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Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of *Caenorhabditis elegans*

(cytoplasmic localization/asymmetric segregation/germ plasm)

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ABSTRACT **By using fluorescent antibody staining, we have followed cytoplasmic granules unique to germ-line cells throughout the life cycle of *Caenorhabditis elegans*. These elements, designated P granules, are segregated exclusively to germ-line precursor cells during early embryogenesis. Prior to mitosis at each of the early cleavages that produce a somatic and germ-line daughter cell, the granules become localized in the region of cytoplasm destined for the germ-line daughter. After the 16-cell stage, the granules appear to be associated with the nuclear envelope. P granules persist in the germ cells throughout the larval and adult stages. The P granules are similar in number, size, and distribution to germ-line-specific structures identified as "germinal plasm" by electron microscopy in *C. elegans* embryos.**

Asymmetric segregation of cytoplasmic components is observed during the early cleavages of many invertebrate embryos (1). Although such cytoplasmic localization is often assumed to be developmentally important, little is known about the nature and function of the segregated components or about the mechanism and control of partitioning. Some of the clearest examples of cytoplasmic localization involve segregation of distinct cytoplasmic structures into germ-line cells. Germ-line-specific structures have been observed by electron microscopy in a variety of organisms (2). These structures have been referred to as nuage in the context of gametogenesis and as germinal plasm, polar plasm, or polar granules in the context of embryogenesis. However, the morphological similarity between nuage and germinal plasm suggests that they represent a common component present in germ-line cells throughout the life cycle. The ubiquity of such germ-line elements has led to suggestions that they serve a role in determination, identification, and differentiation of germ cells (2). Moreover, the transplantation experiments of Illmensee and Mahowald (3) have shown that, in *Drosophila*, cytoplasm containing polar granules acts to determine germ-line cells during embryogenesis. Characterization of germ-line components and their segregation is important for understanding how the fates of these cells, and embryonic cells in general, are specified during early development. Also of interest are questions of the origin of germinal plasm and its continuity during the life cycle.

By using fluorescent antibodies, we have visualized particulate cytoplasmic components specific to germ-line cells of the nematode *Caenorhabditis elegans*. We describe here the segregation of these granules in the germ cell lineage during embryogenesis and their persistence throughout the life cycle in germ cells of juvenile and adult animals.

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MATERIALS AND METHODS

Sera. Fluorescein isothiocyanate-conjugated IgG preparations from rabbit anti-mouse IgG serum (F-RAM) were obtained from Miles (Elkhart, IN), Cappel Laboratories (Cochranville, PA), and U.S. Biochemicals (Cleveland, OH). Experiments in this paper showing P-granule staining were carried out with Miles F-RAM, lot S933.

Immunofluorescence Staining. *C. elegans* was grown on agar plates with *Escherichia coli* as a food source (4). Embryos were obtained by cutting open gravid adult worms in M9 salt solution (4) or by digesting adult worms with 1% NaOCl in 0.5 M NaOH (5). The embryos were transferred to a drop of M9 salt solution on a polylysine-coated slide (6), covered with a silanized coverslip, made permeable by freezing the slide on dry ice and then popping off the coverslip, fixed in absolute methanol at 4°C for 20 min, and air dried. Larvae were picked from plates and fixed as described for embryos. Adult gonads and gametes were obtained by cutting open adult worms in M9 salt solution on polylysine-coated slides, frozen as described above, fixed in acetone at −20°C for 20 min, and air dried. Fixed preparations were incubated with undiluted nonimmune rabbit serum for 1 hr at 25°C and then overnight with F-RAM (1:40) in phosphate-buffered saline (150 mM NaCl/3 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4/1 mM $MgCl_2$) at 4°C. The slides were washed for 90 min with three changes of the same buffer at 10°C, treated with diamidinophenylindole (DAPI) hydrochloride (0.5 μg/ml; Boehringer-Mannheim) in phosphate-buffered saline, rinsed with H_2O, and mounted in Gelutol (Monsanto) mounting fluid.

Microscopy. A Zeiss photomicroscope equipped with Nomarski and epifluorescence optics was used for observation and photography. Each embryo preparation was first photographed with 440- to 490-nm epi-illumination to visualize the immunofluorescence and then simultaneously with visible transmitted light and 365-nm epi-illumination to visualize the embryo and the DAPI-stained chromosomes. For larval and gonad preparations, the Nomarski, DAPI, and immunofluorescence images were photographed separately.

RESULTS

Cleavage of the fertilized *C. elegans* egg (P_0) includes four successive asymmetric divisions (Fig. 1), each generating a larger somatic precursor cell and a smaller P cell (7). The resultant P4 cell is the precursor of the germ line. At the 100-cell stage, P4 divides to produce Z2 and Z3, which proliferate no further until

Abbreviations: F-RAM, fluorescein isothiocyanate-conjugated IgG preparation from rabbit anti-mouse IgG serum; DAPI, diamidinophenylindole; PZA, antibody that stains P granules in P and Z cells.

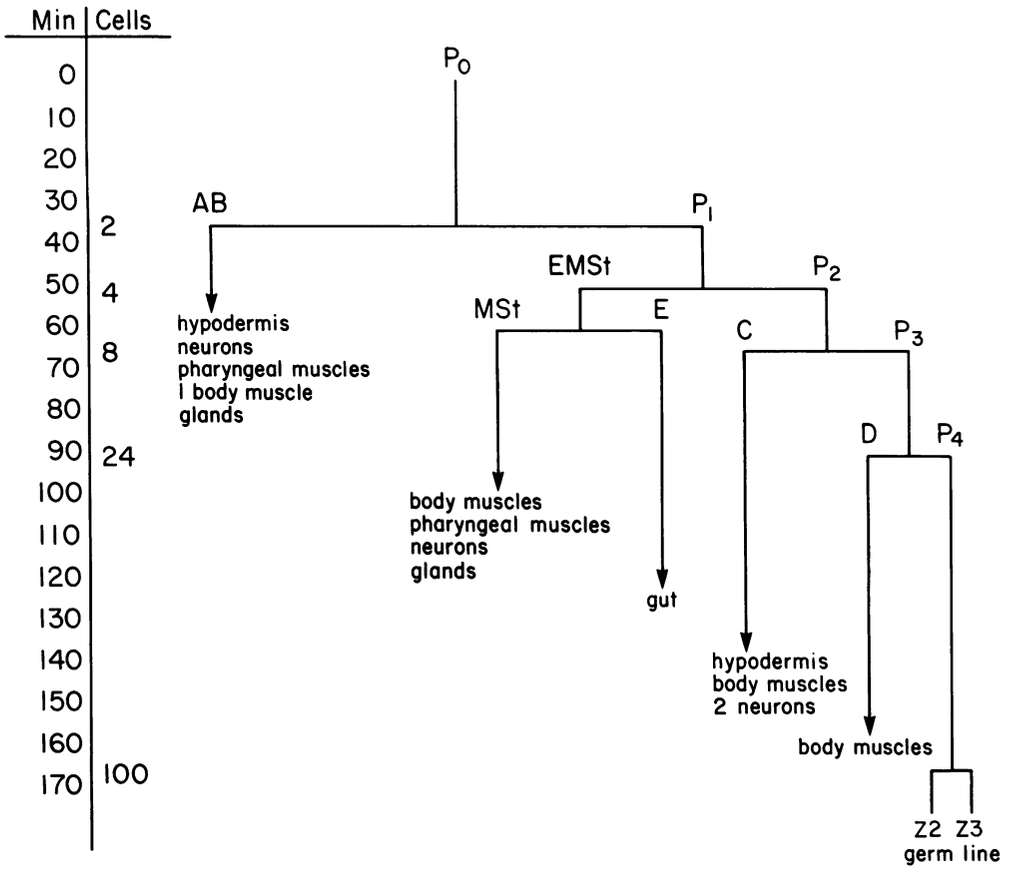


FIG. 1. Lineage relationships, timing of cleavages, and progeny-cell fates in early *C. elegans* embryos at 25°C. All cleavages shown occur approximately in an anterior–posterior direction; anterior is represented to the left and posterior is to the right. The lineage representation and cell nomenclature are adapted from Deppe *et al.* (7); progeny-cell fates have been determined by Krieg *et al.* (8) and by J. Sulston and E. Schierenberg (personal communication).

midway through the first larval stage, when they resume division to produce the germ-line precursor cells of the developing gonad (9, 10).

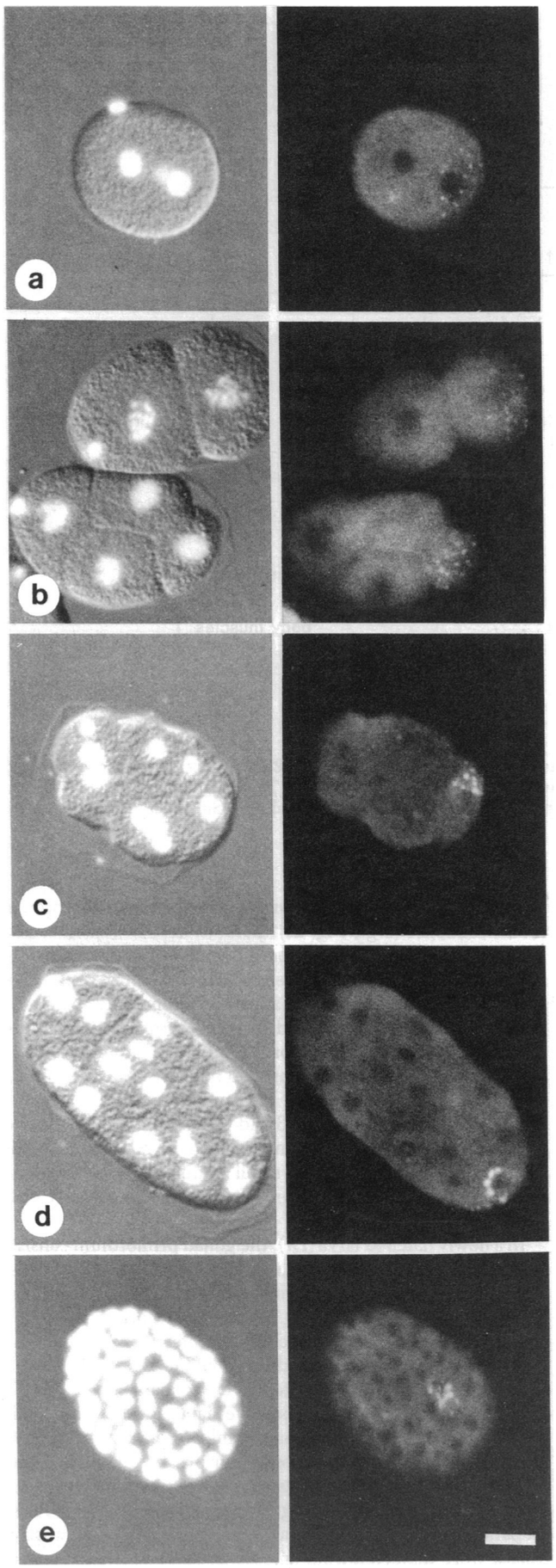
F-RAM is routinely used in our laboratory as a secondary antibody for detecting binding of mouse monoclonal antibodies to *C. elegans* tissue. In the course of such experiments, an F-RAM preparation obtained from Miles was found to react with fixed embryos directly, specifically staining cytoplasmic granules in the germ-line cells of embryos that had not been incubated with primary mouse antibody. We have termed these structures P granules, and we refer to the antibody that stains them in P and Z cells as anti-PZ antibody (PZA). The staining is observed reproducibly in preparations fixed with methanol or acetone as described in *Materials and Methods* but is not seen in aldehyde-fixed preparations.

The staining of P granules at progressive stages of embryogenesis is shown in Fig. 2. The granules are detectable in the uncleaved zygote as prelocalized particles at the posterior pole of the embryo (Fig. 2*a*). After the first cleavage, they are detected only in the P1 cell (Fig. 2*b*). In subsequent divisions, they are progressively segregated into the P2, P3, and P4 cells (Fig. 2 *b–d*). When P4 divides, the P granules are distributed into both Z2 and Z3, which remain the only cells stained in embryos from the 100-cell stage (Fig. 2*e*) to hatching of the first-stage larva (Fig. 3*a*).

A striking feature of segregating P granules in the early embryo is their prelocalization in the P-cell cytoplasm. Prior to each of the first three cleavages, the granules move into the region of cytoplasm destined for the next P-cell daughter. This prelocalization is observed during prophase as the chromosomes condense (e.g., Fig. 2*c*).

During the early cleavage period, the P granules change in size and distribution. In one-cell to four-cell embryos, they are numerous and small, located apparently randomly in the cytoplasm during interphase and near the cortex during cell division. However, in older embryos, the small granules seem to have coalesced; in each cell, three to five large granules are located around the nucleus. In the last two asymmetric P-cell divisions, which give rise to P3 and C cells and then to P4 and D cells, small granules are sometimes observed in the somatic cells C and D. However, these small granules apparently do not persist, because they are never detected in the progeny of C or D cells.

In a newly hatched larva (L1), the gonad primordium consists of four cells: Z2 and Z3, which give rise to all the germ cells, and Z1 and Z4, which are derived from the MSt lineage and give rise to the somatic gonad (10). In the gonad primordium of a young L1, PZA stains perinuclear granules in Z2 and Z3; no staining is detectable in Z1 and Z4 (Fig. 3*a*). During L1 development, staining is confined to the descendants of Z2 and Z3 (Fig. 3 *b* and *c*). The staining becomes fainter as the germ cells proliferate, suggesting that the granules originally present in Z2 and Z3 are distributed among their progeny cells. In later-stage larvae and adults, nongonadal as well as gonadal tissues show staining, as if new antigens recognized by the F-RAM preparation arise during larval development. Consequently, gonads were dissected from animals at these stages for observation of P-granule staining.



In adult hermaphrodites, each half of the symmetrical gonad is a U-shaped structure consisting of an ovary in the distal arm and an oviduct in the proximal arm, connected through a spermatheca to the common uterus. The progression of germ cell differentiation shows a characteristic spatial organization (10). Germ cells divide mitotically in the distal tip of the ovary and enter meiosis in the more proximal region of the distal arm. Oocytes form at the bend of the gonad, mature in the proximal arm, and subsequently are fertilized in the spermatheca. In the distal arm of the adult hermaphrodite gonad, granules around the germ-line nuclei stain intensely (Fig. 4*a*). These granules remain associated with the nuclei as the cells enter meiosis. However, as the oocytes mature, the granules disperse (Fig. 4*b*). PZA stains mature oocytes diffusely; small particles randomly distributed in the cytoplasm are sometimes observed.

In the gonads of adult male worms, stained perinuclear granules are also observed. Mature sperm obtained from males show cytoplasmic staining; however, because of the small volume of cytoplasm in the sperm, localization of the stain is difficult to discern (Fig. 4 *c* and *d*).

In addition to staining P granules, the F-RAM preparation containing PZA also stains centrosomes under some conditions. In embryos previously incubated as in Fig. 2 with an unlabeled nonimmune rabbit serum known (data not shown) to contain anticentrosome antibodies (11), only P granules are stained. However, in embryos previously incubated with unlabeled nonimmune goat serum, both centrosomes and P granules are stained. Therefore, either P granules and centrosomes are antigenically related or, more likely, they are recognized by different antibodies in the F-RAM preparation.

To test the species specificity of PZA, *Drosophila melanogaster* preblastoderm embryos, mouse oocytes, and mouse cleavage-stage embryos were subjected to methanol fixation, treated with PZA-containing F-RAM antibody, and examined for staining essentially as described for *C. elegans* embryos. Under these conditions, background staining of *Drosophila* embryos was fairly high; however, no specific staining of the posterior polar plasm or pole cells was observed. M. Watanabe and A. Mahowald (personal communication) have obtained similar results with the same F-RAM preparation. Mouse embryos and oocytes also gave negative results. Therefore, unless fixation conditions required for staining are different in these species, PZA apparently does not crossreact with their germ-line components.

DISCUSSION

The pattern of asymmetric cleavages, cell positions, and cell fates in early *C. elegans* embryos is invariant (7, 8). Cleavage-arrest and cell-ablation experiments have shown that the gut precursor cells in two-cell, four-cell, and eight-cell embryos require neither cell division nor the presence of normal cell neighbors to subsequently express an intestinal differentiation marker (12). Therefore, early determinative events in *C. elegans* can be specified by cell-autonomous internally segregating determinants that become localized in specific cells well before

FIG. 2. PZA staining of P granules in *C. elegans* embryos at various stages. (*Left*) Nomarski/DAPI image. (*Right*) Immunofluorescence image. Orientations of embryos are anterior left, posterior right. (*a*) Precleavage zygote. (*b*) Two-cell and four-cell embryos. (*c*) Six-cell embryo in the process of EMSt division, before division of the P2 cell. The EMSt nucleus is in late anaphase or telophase; the P2 nucleus is in early prophase. Note the prelocalization of P granules in the ventral region of the P2 cell. (*d*) Embryo of ≈15 cells, showing perinuclear granules in the P3 cell. (*e*) Embryo of >100 cells shortly after P4 division into Z2 and Z3, both of which are stained. Bar = 10 μm.

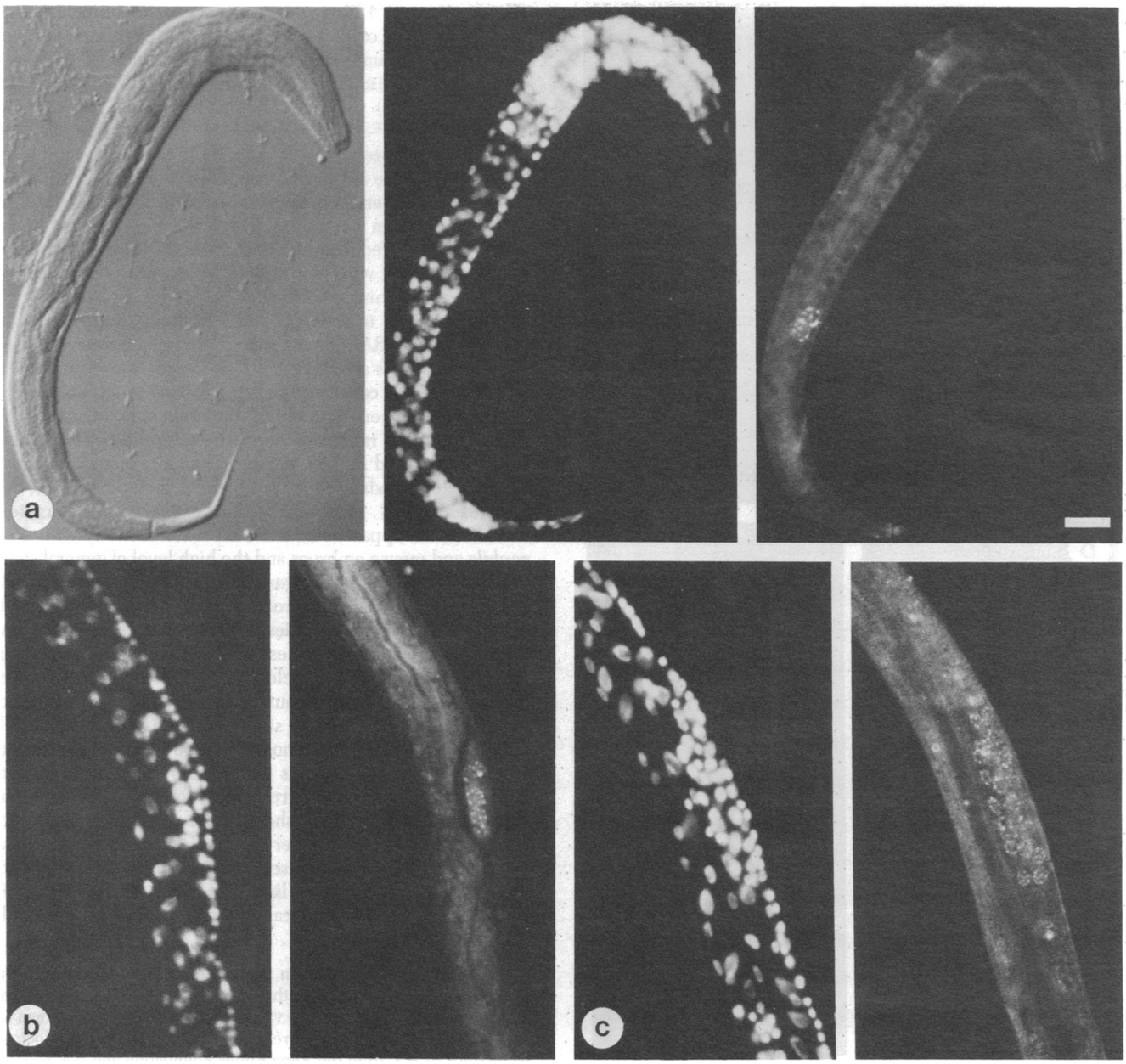


FIG. 3. PZA staining of germ cells in *C. elegans* larvae. (*a*) Nomarski, DAPI, and immunofluorescence images (*Left*, *Middle*, and *Right*) of a newly hatched L1, showing staining of only the Z2 and Z3 cells in the four-cell gonad primordium (evident in the Nomarski image). (*b*) DAPI (*Left*) and immunofluorescence (*Right*) images of a later-stage L1, showing staining of the proliferating germ cells; no staining is detected in the somatic gonad cells. (*c*) DAPI (*Left*) and immunofluorescence (*Right*) images of an L1-L2. Bar = 10 μm.

the differentiative events that they determine. The asymmetries in developmental potential that arise during early cleavages presumably reflect asymmetries at the molecular level that are somehow generated in the cytoplasm or nuclei of early blastomeres. The prelocalization and segregation of P granules, as observed by PZA staining, provide graphic documentation of such an asymmetry.

Cytoplasmic structures that have morphological characteristics of germinal plasm have been observed in cells of the P lineage by transmission electron microscopy of sectioned *C. elegans* embryos (ref. 8; N. Wolf and D. Hirsh, personal communication). These structures, like P granules, are restricted to the P cells during early cleavage stages. Moreover, their number, size, and distribution change during the early cleavage period. In the same manner and at the same time as observed for P granules, numerous small cytoplasmic structures appear to aggregate into larger perinuclear structures. These correlations suggest that PZA reacts with a component of the germinal plasm.

The P granules show continuity from one generation of germ cells to the next, based on their staining throughout the life cycle. The staining becomes fainter during germ cell proliferation in L1 and L2 larvae; presumably P granules must be replenished during the later larval stages or during gametogenesis in adults to regenerate the level of staining observed in adult gonads. Conceivably the P granules could be self-replicating cytoplasmically inherited components. However, in studies of hybrid pole cells, Mahowald *et al.* (13) have shown that the germ-line-specific polar granules in *Drosophila* are not cytoplasmically inherited but depend for their formation on expression of the nuclear genome during oogenesis.

The components of F-RAM that react with P granules are

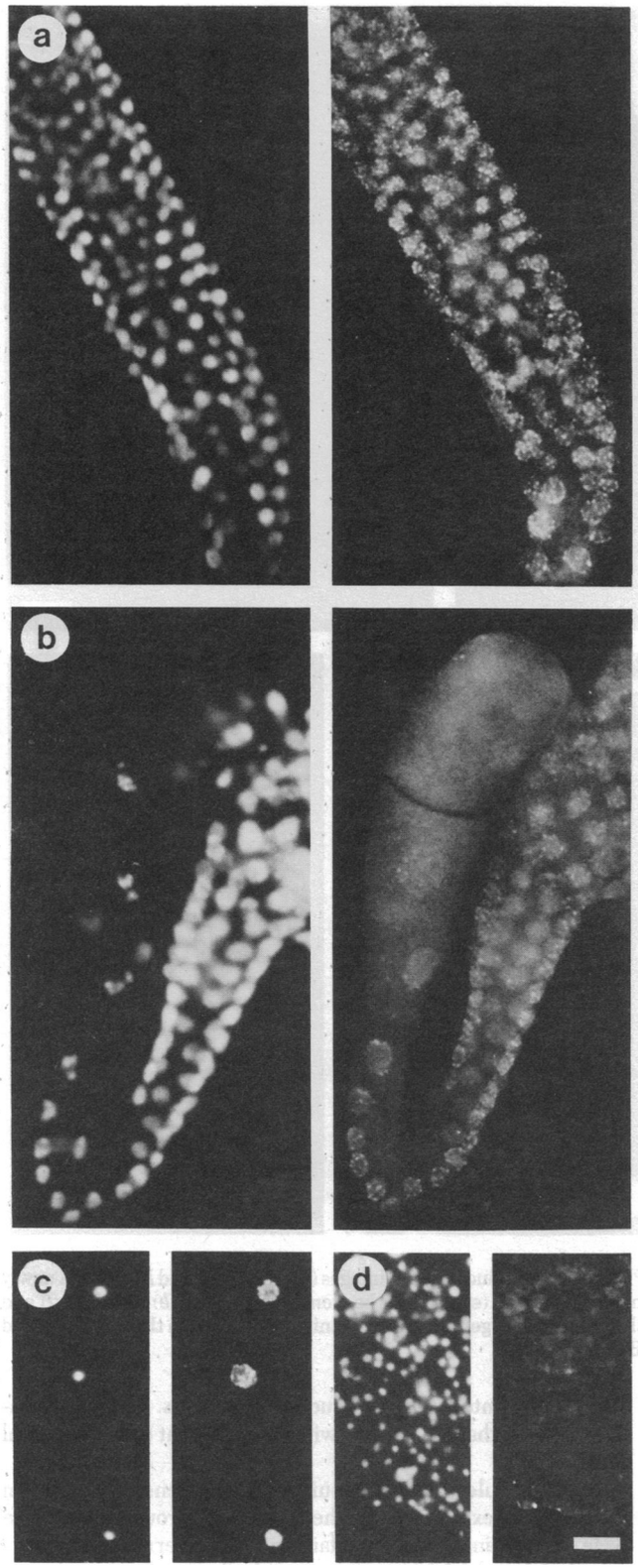


FIG. 4. PZA staining of adult gonads and gametes. (*a*) DAPI (*Left*) and immunofluorescence (*Right*) images of the distal arm of an adult hermaphrodite gonad. (*b*) DAPI (*Left*) and immunofluorescence (*Right*) images showing pachytene nuclei (right side of each) and maturing oocytes (left side) in the proximal portion of an adult hermaphrodite gonad. The perinuclear granules apparently disperse during oocyte maturation. (*c*) Mature sperm from an adult male stained with PZA. (*d*) Mature sperm treated as in *c* with a F-RAM preparation from U.S. Biochemicals that does not stain P granules. Bar = 10 μm.

almost certainly fluorescein isothiocyanate-conjugated IgG molecules, based on their retention by a Staphylococcal protein A-Sepharose column and on the amplification of P-granule staining by fluoresceinated goat anti-rabbit IgG antibodies (unpublished results). However, staining of P granules is not a common feature of F-RAM preparations. This staining has been observed with three different lots of fluorochrome-conjugated rabbit anti-mouse antibody from Miles. However, two lots of F-RAM from other companies and a fourth lot of F-RAM from Miles do not stain P granules. The Miles F-RAM used in this study was prepared from the pooled sera of 15 rabbits that had been immunized with mouse IgG (Miles, personal communication). The staining we observe cannot be explained by homology between mouse IgG and P-granule antigen, because several lots of F-RAM do not stain P granules. Some other possible explanations are (*i*) PZA may be a rabbit autoantibody to an evolutionarily conserved or crossreacting rabbit antigen; (*ii*) PZA may have been elicited by a contaminant in the mouse IgG injected into the rabbits as immunogen; and (*iii*) one or more of the immunized rabbits may have had a nematode infection that elicited production of antibodies, including PZA, that crossreact with *C. elegans*. The third explanation is consistent with both the apparent lack of PZA crossreactivity with *Drosophila* and mouse embryos and the high level of general staining of larval and adult *C. elegans* preparations by Miles F-RAM.

PZA provides a novel approach to studying germ cell-specific elements. In previous studies of nuage and germ plasm, the behavior and continuity of these structures during development have been inferred from electron microscopic observation, which is not only arduous but also relies entirely on morphological similarity for relating structures seen at different developmental stages. An immunologic probe allows correlation of macromolecular components identified by binding of cognate antibodies with the ultrastructural information obtained by electron microscopy. PZA should be a valuable reagent with which to investigate whether P granules are in fact germinal plasm, how P granules are segregated, and whether they are determinative for the germ line. The antibody also should aid in isolation and biochemical characterization of P-granule components.

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