Induction of pole cells in sterilized *Drosophila* embryos by injection of subcellular fraction from eggs

cytoplasmic determinants/germ cells/insect embryogenesis/microinjection into eggs

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**ABSTRACT** A subcellular fraction isolated from a homogenate of young *Drosophila* embryos was shown to be capable of inducing pole cells when injected into UV-sterilized *Drosophila* embryos. Most of the pole cell-inducing activity was recovered from the precipitate after centrifugation at 27,000 × g. The activity remained in this precipitate (called F-3 fraction hereafter) even after membranous structures were removed from it through centrifugation on a sucrose density gradient. Dialysis, lyophilization, and heating at 80°C for 10 min did not inactivate the F-3 fraction. The pole cells, which were produced when the F-3 fraction was injected at the posterior pole of UV-sterilized embryos, did not develop into germ cells. Furthermore, the F-3 fraction was unable to induce pole cells when injected into the anterior region of the egg. These results can be explained by assuming that (i) pole cell formation and germ cell determination are controlled by different factors, (ii) pole cell formation requires at least two factors, which are normally localized in the posterior-pole cytoplasm, one of which is sensitive and one resistant to the UV dosage we used, and (iii) the subcellular fraction we obtained contains the UV-sensitive factor but not the UV-resistant factor.

In *Drosophila*, a specific cytoplasm is localized at the posterior pole of oocytes and young embryos. During early embryogenesis, this cytoplasm becomes included in pole cells, which have been shown to make primordial germ cells (1, 2). Experiments in which this cytoplasm is inactivated with UV light indicate that the posterior-pole cytoplasm plays an important role in the formation of pole cells and the determination of germ cells (3–6). The hindrance of pole cell formation and germ cell formation caused by UV-irradiation at the posterior pole can be cured by transplantation of normal posterior-pole cytoplasm, but not anterior-pole cytoplasm, into this egg region (7, 8). Furthermore, posterior-pole cytoplasm induces ectopic pole cells when injected elsewhere in the periplasm (8–10) and the ectopically induced pole cells are able to develop into germ cells when transplanted into the appropriate region of a host embryo (9, 10).

Clearly, the accumulated evidence suggests the existence of substances localized in the posterior region of oocytes and embryos of *Drosophila* that control the determination of a specific cell type, the germ cell. As a first step towards the isolation and identification of the functional substances, we have examined the pole cell-inducing activity of subcellular fractions of *Drosophila* eggs. The technique of microinjection into *Drosophila* embryos (8, 11) was used to assay the subcellular fractions for pole cell-inducing activity.

**MATERIALS AND METHODS**

*Drosophila melanogaster* Strains. Eggs from a wild-type stock, Oregon R, were used for the fractionation of egg cytoplasm. As recipients of the subcellular fractions in the bioassay, embryos of a *muh e*¹¹ stock were used (8).

Microinjection Bioassay. The apparatus and procedure for test material microinjection were basically the same as described (8, 11). Eggs were collected on agar plates during a period of 40 min from a *muh e*¹¹ stock reared at 25°C, and the egg-bearing agar plates were immediately placed in a room at 18°C. The following procedure was then carried out. First, the eggs were dechorionated and placed on a glass slide parallel to each other. These eggs were exposed to a rectangular UV beam at the dose of 1.3 × 10⁴ erg/mm², with the posterior poles facing a Matsushita germicidal lamp, which emitted mainly UV light at 253 nm, at a distance of 40 mm. The rectangular UV beam was directed through a window, 3 mm × 40 mm, which opened onto the UV lamp whose surface was covered with black paper. Each irradiated egg was injected with 0.2 nl (approximately 1.5% of the egg volume) of the test material. Prior to the injection, each embryo contained less than 32 nuclei. For each series of the bioassay, some of the irradiated eggs on the same glass slide were separated from the experimental group and kept as an un injected control group.

After the injection, the recipients were covered with a drop of paraffin oil and incubated in a moist chamber at 25°C. After the recipients developed to the blastoderm stage, they were examined under a compound microscope for the presence of pole cells. When pole cells could not be identified because of injury, the recipients were examined again at the gastrula stage, when pole cells were possibly present in the proctodaeal pit. More than 95% of the control eggs, UV-irradiated but not injected, formed no pole cells. However, no more than 5% of the control eggs formed normal or abnormal pole cells, which were usually smaller than normal pole cells and less than five per embryo. Similar abnormal pole cells were rarely observed in embryos irradiated and injected with subcellular fractions. However, it was not always possible in a routine bioassay to exclude embryos with abnormal pole cells from embryos with normal pole cells on the basis of cell size and shape. Thus, in the bioassay, in order to judge more precisely as to whether pole cells were present, a cellular blastoderm embryo was recorded as having pole cells only when more than five pole cells could be found. Actually, in most of the experimental and control embryos that formed pole cells, more than 10 pole cells per embryo were observed at the blastoderm stage. The pole cell-inducing activity of the assay material was expressed as the “restoration rate,” i.e., the percentage of recipients that formed normal pole cells.

**Fractionation of Egg Cytoplasm.** Eggs age 30 min ± 30 min after being laid were collected. After the eggs were washed with distilled water to remove any yeast, they were frozen imme-
diately and stored at −20°C until used. Ten grams of frozen eggs were thawed in 10 volumes of ice-cold homogenizing medium (0.25 M sucrose/3 mM CaCl₂/10 mM Tricine, pH 6.9) and gently homogenized in a Potter homogenizer with a Teflon pestle. In the first step of the fractionation, differential centrifugation of the egg homogenate was carried out sequentially at 1,000, 7,000, and 12,000 × g for 10 min each and finally at 27,000 × g for 60 min. The pellet formed at 1,000 × g was discarded, and the remaining pellets were collected separately and designated P-1, P-2, and P-3.

For further purification, the P-3 fraction was centrifuged on a stepwise sucrose density gradient. A suspension of the P-3 fraction in the homogenizing medium was layered at the top of a gradient consisting of four 2-ml layers of 1.4, 1.6, 1.8, and 2.0 M sucrose solution containing 3 mM CaCl₂ and 10 mM Tricine (pH 6.9) and centrifuged at 35,000 × g for 30 min. After the centrifugation, the four layers of increasing sucrose concentration were recovered and designated F-1, F-2, F-3, and F-4. Each fraction was dialyzed overnight against distilled water and lyophilized. From 10 g of frozen eggs, about 15, 20, 4, and 7 mg of lyophilized F-1, F-2, F-3, and F-4 fractions were obtained, respectively. All procedures were carried out at 0–4°C.

Unless otherwise mentioned, the lyophilized fractions were suspended at 100 mg/ml in a standard buffered Drosophila Ringer’s solution [balanced saline solution (12) but without bovine serum albumin], and 0.2 ml of the suspension was injected into the recipients for bioassay (20 ng of the lyophilized fraction per embryo).

Electron Microscopy. Embryos were fixed by the method of Zalokar and Erk (13) and embedded in epoxy resin by Spurr’s method (14). In fixing the subcellular fractions, prefixation in heptane/glutaraldehyde was omitted. Thin sections were observed with a JEOL 100C electron microscope.

### RESULTS

Pole Cell-Inducing Activity of Cytoplasm from Frozen Embryos. Dechorionated eggs from a 1-hr egg collection period were placed on double-stick Scotch tape and stored in a −20°C freezer. A week later, 0.2 nl of the posterior-pole cytoplasm was withdrawn from each of these eggs immediately after the eggs were thawed and injected into a UV-irradiated recipient. This posterior-pole cytoplasm exhibited a restoration rate of 28% (Table 1). Fresh (not frozen) posterior-pole cytoplasm showed a restoration rate of 42%. Freezing and thawing apparently reduced the pole cell-inducing activity of the posterior-pole cytoplasm but left enough activity to be detectable in the bioassay.

Functional Subcellular Fractions. Each of the P-1, P-2, and P-3 fractions was assayed for activity in inducing pole cell formation. The highest activity was found in the P-3 fraction (Table 2). When only the homogenizing medium was injected into the UV-irradiated eggs, the restoration rate was as low as that of the pole cell-forming embryos in the un.injected control group (Table 2, control 1).

The P-3 fraction was further purified by centrifugation on a sucrose density gradient. Of the F-1 to F-4 fractions, the F-3 fraction showed the highest activity (Table 3).

A portion of the F-3 fraction was taken before lyophilization and fixed for electron microscopy. The electron micrographs showed that the F-3 fraction was almost free of mitochondria and other membranous structures (Fig. 1). Small electron-dense particles (15–20 nm in diameter) occupied most of the fraction. They were distributed sporadically in the electron micrographs, constituting groups with diameters of 0.2–0.5 μm.

In the next series of experiments, the germ cell-forming ability of the pole cells induced in recipients by injection of the F-

### Table 1. Transplantation of posterior-pole cytoplasm from normal or frozen embryos into UV-irradiated eggs

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Injected material</th>
<th>No. of eggs treated</th>
<th>Blastoderm-stage embryos</th>
<th>Significance†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. developed</td>
<td>No. with pole cells*</td>
</tr>
<tr>
<td>1</td>
<td>Normal polar plasm</td>
<td>215</td>
<td>163</td>
<td>68 (42)</td>
</tr>
<tr>
<td>C-1†</td>
<td>None</td>
<td>164</td>
<td>141</td>
<td>3 (2)</td>
</tr>
<tr>
<td>2</td>
<td>Polar plasm from frozen embryos</td>
<td>198</td>
<td>87</td>
<td>24 (28)</td>
</tr>
<tr>
<td>C-2†</td>
<td>None</td>
<td>201</td>
<td>146</td>
<td>2 (1)</td>
</tr>
</tbody>
</table>

* Restoration rate; percentage of blastoderm-stage embryos developed with pole cells is in parentheses.
† Probability was calculated by Fisher’s exact probability test (15) against control 2.
‡ C-1, control 1. Eggs were UV-irradiated but were not injected.
§ C-2, control 2. Eggs were UV-irradiated and transplanted with homogenizing medium.

### Table 2. Pole cell-inducing activity of subcellular fractions obtained from egg homogenates by differential centrifugation

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Injected material</th>
<th>No. of eggs treated</th>
<th>Blastoderm-stage embryos</th>
<th>Significance†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. developed</td>
<td>No. with pole cells*</td>
</tr>
<tr>
<td>1</td>
<td>P-1</td>
<td>51</td>
<td>28</td>
<td>3 (11)</td>
</tr>
<tr>
<td>2</td>
<td>P-2</td>
<td>140</td>
<td>76</td>
<td>8 (11)</td>
</tr>
<tr>
<td>3</td>
<td>P-3</td>
<td>188</td>
<td>92</td>
<td>28 (30)</td>
</tr>
<tr>
<td>C-1†</td>
<td>None</td>
<td>329</td>
<td>244</td>
<td>9 (4)</td>
</tr>
<tr>
<td>C-2†</td>
<td>Homogenizing medium</td>
<td>64</td>
<td>24</td>
<td>1 (4)</td>
</tr>
</tbody>
</table>

* Restoration rate; percentage of blastoderm-stage embryos developed with pole cells is in parentheses.
† Probability was calculated by Fisher’s exact probability test (15) against control 2.
Table 3. Pole cell-inducing activity of subcellular fractions obtained by centrifuging the F-3 fraction on a stepwise sucrose density gradient

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Injected material</th>
<th>No. of eggs treated</th>
<th>No. of blastoderm-stage embryos developed</th>
<th>No. of embryos with pole cells*</th>
<th>Significance†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F-1</td>
<td>112</td>
<td>43</td>
<td>9 (81)</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>2</td>
<td>F-2</td>
<td>104</td>
<td>39</td>
<td>13 (33)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>3</td>
<td>F-3</td>
<td>101</td>
<td>40</td>
<td>22 (55)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>4</td>
<td>F-4</td>
<td>124</td>
<td>32</td>
<td>12 (38)</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>C-1†</td>
<td>Saline</td>
<td>173</td>
<td>92</td>
<td>1 (1)</td>
<td>—</td>
</tr>
<tr>
<td>C-2‡</td>
<td>None</td>
<td>121</td>
<td>49</td>
<td>3 (6)</td>
<td>—</td>
</tr>
</tbody>
</table>

* Restoration rate; percentage of blastoderm-stage embryos developed with pole cells is in parentheses.
† Probability was calculated by χ²-test against control 2.
‡ C-1, control 1. Eggs were UV-irradiated but were not injected.
§ C-2, control 2. Eggs were UV-irradiated and injected with saline that is used for suspending fractions for the assay.

3 fraction was tested. When embryos, which were irradiated and injected with F-3 fraction, developed to the blastoderm stage, those which formed a conspicuous polar cap (an accumulation of pole cells) were picked out and allowed to develop to adulthood. Nine adults (six females and three males) developed from 92 embryos that had formed pole cells. None of the nine adults contained germ cells in their gonads when they were dissected after being fed for 3 days (Table 4). As a control, posterior-pole cytoplasm from normal embryos was injected into the UV-irradiated embryos, and four out of nine adults that emerged from 42 blastoderm embryos with pole cells were fertile. A statistical test showed that the results from the experiment and the control are different with more than 95% confidence.

To rule out a possibility that we are dealing with a general type of "cellularization rescue" by F-3 fraction, we transplanted cytoplasm from the anterior pole into the posterior pole of UV-irradiated eggs. None of the 61 blastoderm embryos developed from these eggs formed pole cells.

Quantitative Evaluation of the Bioassay. The lyophilized F-3 and F-4 fractions were separately suspended in saline solution at a concentration of 200 mg/ml, and each suspension was serially diluted twice; 0.2 ml of each diluted suspension was then injected into an UV-irradiated embryo. The amount of the lyophilized F-3 and F-4 fraction injected into each egg and the restoration rate were both reciprocally plotted (Fig. 2). The graphs allow us to estimate that a unit mass of F-3 fraction contains about 3 times as much activity as that of F-4 fraction. Because 4 mg of the lyophilized F-3 fraction was harvested from 8 × 10⁶ eggs weighing 10 g, 20 ng of the fraction was found to be equivalent to the fraction from four embryos.

Heat Treatment of the F-3 Fraction. The F-3 fraction was dialyzed against distilled water overnight to remove sucrose, heated for 10 min at 80°C, and then lyophilized. The heat-treated and lyophilized F-3 fraction was injected into UV-irradiated eggs (20 ng per embryo). The heat-treated F-3 fraction still retained some pole cell-inducing activity [a restoration rate of 22% (Table 5)]. Because the extrapolation of the line in Fig. 2 to the level of 22% shows that an injection of 1.7 ng of the lyophilized intact F-3 fraction per UV-irradiated embryo gives this restoration rate value, the heat treatment was considered to reduce the pole cell-inducing activity of the F-3 fraction to about 1/10 of what it was originally.

Electron Microscopy. A normal pole cell at the cellular blastoderm stage is shown in Fig. 3A. Polar granules and nuclear bodies are characteristic of pole cells. The polar granules at this stage were approximately 1 μm in diameter.

The embryos irradiated with UV at the posterior pole never formed any typical pole cells, although they did form an otherwise normal cellular blastoderm. An electron micrograph (Fig. 3B) shows that polar granules and nuclear bodies are present in morphologically normal somatic cells occupying the posterior pole of the embryo. The polar granules in these cells, however, were much smaller (ca. 0.3 μm in diameter) than those found in normal pole cells at the blastoderm stage. Nuclear bodies were also smaller than normal, but they were found in every cell that included the above-mentioned small polar granules.

In addition to restoring pole cell formation, injection of the F-3 fraction (20 ng per egg) seemed to have affected the morphology of the polar granules in the UV-irradiated embryos. The pole cells that were formed in embryos that had been irradiated with UV and injected with the F-3 fraction were morphologi-
cally almost identical to the normal ones, except that in most cases two types of polar granules were included in these pole cells (Fig. 3C). Granules of the first type were similar to those observed in the posteriormost cell of the blastoderm developed from the UV-irradiated embryos, and those of the second type were doughnut-shaped and morphologically similar to, but a little larger than, normal polar granules in the pole cells at the blastoderm stage (ca. 1.5 \( \mu \)m in diameter). The nuclear bodies found in such pole cells seemed morphologically normal, although they were a little smaller than the normal ones.

**Injection of Materials into the Anterior Region of Embryos.**

The F-3 fraction was tested for its ability to cause anterior-pole cytoplasm to form pole cells. The F-3 fraction was injected into 84 embryos (0.2 ng per embryo), and 32 embryos developed to the blastoderm stage. Observation of the 32 embryos under a compound microscope showed that no pole cells were formed in the anterior region where the F-3 fraction had been injected. Electron micrographs of thin sections from 13 of these blastoderm embryos were examined. Normal blastoderm cells were formed, but neither pole cells nor polar granules were recognized at the portion where the F-3 fraction had been injected.

It was confirmed that ectopic pole cells were detectable in living blastoderm embryos under a compound microscope. Ectopic pole cells were perceived in 8 out of 18 living blastoderm embryos that had been injected in their anterior region with polar plasm from normal embryos. Observation of 1-\( \mu \)m-thick sections cut from these eight embryos verified the presence of ectopic pole cells in these embryos.

**Table 5.** Effect of heat treatment on the pole cell-inducing activity of the F-3 fraction

<table>
<thead>
<tr>
<th>Blastoderm-stage embryos</th>
<th>No. of eggs treated</th>
<th>No. developed</th>
<th>No. with pole cells*</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp.</td>
<td>258</td>
<td>119</td>
<td>26 (22)</td>
<td>( P &lt; 0.01 )</td>
</tr>
<tr>
<td>Control\†</td>
<td>108</td>
<td>71</td>
<td>2 (3)</td>
<td></td>
</tr>
</tbody>
</table>

* Restoration rate: percentage of blastoderm-stage embryos developed with pole cells is in parentheses.

† Probability was calculated by the \( \chi^2 \)-test.

‡ Eggs were irradiated but were not injected.

**Fig. 2.** The restoration rates obtained by the injection of various amounts of the F-3 (○) and the F-4 (△) fractions in the bioassay. Abscissa, amount of injected material (dry weight; 40, 20, 10, and 5 ng indicated) into one egg (reciprocal plot); volume of injection per egg was always 0.2 nl. Ordinate, restoration rate; % blastoderm embryos which formed pole cells (reciprocal plot). Each point represents the mean value of two or three experiments, and the vertical bars show the SEM.

**Fig. 3.** Electron micrographs of thin sections through pole cells or somatic cells at the posterior pole of embryos. (A) A normal pole cell in a blastoderm embryo. (B) A posteriormost cell of an embryo that was previously irradiated with UV and allowed to develop up to the blastoderm stage. (C) A pole cell formed in an embryo that was previously irradiated with UV, followed by the injection of the F-3 fraction. Two types of polar granules are seen. (Inset) A nuclear body seen in another section of the same cell at the same magnification. N, nucleus; NB, nuclear body; PG, polar granule. (Bars = 1 \( \mu \)m.)

**DISCUSSION**

Most of the pole cell-inducing activity could be recovered from the subcellular fraction pellet at 27,000 \( \times \) g. This shows that the pole cell-inducing factor may possibly be associated with certain subcellular organelles. Mitochondria and other mem-
branous structures may be excluded from the candidate organ-
elles because electron micrographs showed that the F-3 frac-
tion, which had the highest pole cell-inducing activity, contained
almost no membranous structures. The F-3 fraction consisted
almost entirely of particles resembling ribosomes, but it is un-
likely that the ribosomes themselves are responsible for the in-
duction of the pole cells. The posterior pole cytoplasm may
possibly contain certain specific agents, such as mRNA, that
play an important role in pole cell formation and that sediment
with ribosomes. Another possibility may be that some of
the particles were derived from the polar granules. The polar
granules at the stage used are known to contain small granules
similar in size to ribosomes (16), although these granules do not
bind indium as do ribosomes (17). The fractionation procedure
may have caused the polar granules to dissociate into small gran-
ules and other components. Only the small granules may have
been recovered in the F-3 fraction.

Because we obtained the F-3 fraction from whole egg homog-
enates, there is no direct evidence to show that the F-3 frac-
tion included a specific polar plasm component. However, elec-
tron microscopy showed that UV-irradiation brought about
morphological changes in polar granules and nuclear bodies and
that the injection of the F-3 fraction restored morphologically
the normal polar granules and nuclear bodies to the irradiated
embryos. Furthermore, the UV of the dose and wavelength
specified above did not inhibit the irradiated regions of embryos
from forming normal somatic cells either in the posterior region
(this work) or in other regions (unpublished data); only pole cell
formation was prevented. The ability of the UV-irradiated eggs
to form pole cells was restored only by injecting them with the
polar plasm or with the subcellular fractions we obtained. No
other means are presently known for restoring the pole cell to
UV-irradiated embryos. These results strongly suggest the pos-
sibility that the pole cell-forming activity of the F-3 fraction is
a function of the polar plasm.

Our experiments provide some indications for the inde-
pendence of pole cell formation and germ cell determination in
Drosophila. The posterior-pole cytoplasm has been consid-
ered to contain the agents that are required for both pole cell
formation and germ cell determination (3, 6, 8, 9). Our control
experiments verified this. However, no germ cells were de-
tectable in nine flies that emerged from UV-irradiated embryos
that had received F-3 fraction and apparently formed pole cells.
The number of flies we obtained was rather small. However,
a statistical test allowed us to consider it probable that the pole
cells induced by the F-3 fraction in the UV-irradiated recipients
were incapable of developing to germ cells. These results sug-
gest that pole cell formation and germ cell determination in
Drosophila are controlled by different factors, and that the fac-
tors essential for germ cell determination were lost during the
fractionation procedure or sedimented in a fraction or fractions
other than F-3. It has been shown that in some species of insects
that normally form pole cells, pole cell formation and germ cell
formation are not necessarily coupled (18–20).

Our results suggest that at least two factors are involved in
pole cell formation. Injection of the F-3 fraction restored pole
cell-forming ability to the UV-irradiated posterior-pole cyto-
plasm. However, the F-3 fraction failed to induce any ectopic pole
cells when injected into the anterior region of the embryo.
We showed by duplicating earlier work (9, 10) that intact pos-
terior-pole cytoplasm can induce ectopic pole cells in the an-
terior region. The data may be explained if we speculate that
(i) the posterior-pole cytoplasm of Drosophila eggs contains two
specific factors, both of which are necessary for pole cell for-
modation (one is UV sensitive and the other is UV resistant at the
dose and wavelength we applied); (ii) UV-irradiation at the pos-
terior pole impairs the UV-sensitive factor; (iii) the F-3 fraction
contains only the UV-sensitive factor; and (iv) anterior pole cy-
toplasm contains neither of these factors. The idea that more
than one specific factor is involved in pole cell formation was
also proposed by Jazdowska-Zagrodzinska (21).

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