A test for uv-caused cytoplasmic deficiencies preventing RNA-induced pole cells from developing to germ cells in *Drosophila*

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Abstract

RNA-induced pole cells are round cells segregated from the posterior of *Drosophila* embryos that were uv-irradiated posteriorly at the early cleavage stage then injected with poly (A)$^+$ RNA or mitochondrial large rRNA at the irradiated site. The RNA-induced pole cells are morphologically very similar to normal pole cells. They migrate to gonads where they are morphologically identical to primordial germ cells (PGC). However, those PGCs deriving from RNA-induced pole cells never develop to germ cells. A germ cell determinant proper was presumed to explain the fate of RNA-induced pole cells. No evidence has been presented for this hypothesis. In this paper we tested an alternative hypothesis that uv-damage to mitochondria prevents RNA-induced pole cells to develop to germ cells. The results were against the hypothesis. RNA-induced pole cells include respiratory active mitochondria. Furthermore, coinjection of poly (A)$^+$RNA and cytoplasm from mid-ventral region of cleavage embryos did not develop to germ cells, nevertheless intact mitochondria were provided by transplantation of ventral cytoplasm.

Introduction

Pole cells, germ line precursors in *Drosophila* embryos, are segregated from the somatic line at the posterior pole of early syncytial blastoderms. The posterior pole cytoplasm or polar plasm of oocytes and early cleavage embryos has been well documented for its including cytoplasmic factors essential for embryogenesis, one for abdominal pattern formation and one for germ line determination. Uv-irradiation of early cleavage embryos at polar plasm results in sterile flies (Geigy, 1931) because of failure in pole cell formation. The pole cell forming activity is restored if polar plasm is transplanted into uv-irradiated embryos from unirradiated embryos. Restoration activity is detected exclusively from polar plasm, no other part of the cytoplasm could accomplish this reversal of sterility (Okada *et al.*, 1974; Warn, 1975).

The pole cell-inducing activity was recovered from a 27,000 g precipitate (designated P3 fraction) from the homogenate of early cleavage embryos (Ueda and Okada, 1982) and from poly (A)$^+$RNA extracted from the P3 fraction (Togashi *et al.*, 1986). It has finally been shown that the molecule restoring pole cell formation to uv-irradiated embryos is mitochondrial large rRNA (mtlrRNA) (Kobayashi and Okada, 1989). MtlrRNA has been located outside mitochondria intimately associating with polar granules (Kobayashi *et al.*, 1993). Furthermore, the pole cells induced in uv-irradiated embryos by the poly (A)$^+$RNA or mtlrRNA were demonstrated never to develop to germ cells (Togashi *et al.*, 1986; Kobayashi and Okada, 1989), in spite of their least morphological difference from normal pole cells (Yamazaki and Okada, 1989).

As for what causes inability of RNA-induced pole cells to develop to germ cells, a hypothesis has been proposed (Togashi *et al.*, 1986; Kobayashi and Okada, 1989). Two uv-sensitive cytoplasmic factors are present in the polar plasm, one responsible for pole cell formation, and one for the determination of pole cells to germ cells. MtlrRNA represents the factor for pole cell formation. Injection of poly (A)$^+$RNA or mtlrRNA provided uv-irradiated embryos with pole cell forming factors but not with germ cell determining factors. Thus pole cells are restored to uv-irradiated embryos, but those pole cells are unable to differentiate as germ cells. The hypothesis is supported by *agametic* mutation, in which pole cells are formed but degraded in the following development (Engstrom *et al.*, 1982). However, no direct evidence has been produced for the germ cell deter-

minant as a polar plasm component. An alternative hypothesis can be possible. Uv damages mitochondria in polar plasm to disable them for proliferation as well as respiration after they are incorporated into pole cells.

We have already shown that uv does not impair respiratory activities of mitochondria in cleavage embryos (Akiyama and Okada, 1992). In the present work we exerted rhodamine 123 staining to RNA-induced pole cells and found that mitochondria in these pole cells are as active as those in normal pole cells. We further report that poly (A)$^+$RNA-induced pole cells are still unable to develop to germ cells, even when unirradiated mitochondria are provided by coinjection with cytoplasm from the mid-ventral region of cleavage embryos. We used, in place of mtlrRNA, poly (A)$^+$RNA extracted from early cleavage embryos in the present work, because we confirmed that mtlrRNA, which is polyadenylated, is only component of the poly (A)$^+$RNA that has pole cell forming activities.

Materials and Methods

Drosophila melanogaster *strains*

Eggs from a wild-type Oregon R were used for the source of poly (A)$^+$RNA, and as donors for the ventral cytoplasm or polar plasm to be transplanted. Eggs from the mutant *mwh* e^{11} were uv-irradiated posteriorly and used as recipients for cytoplasm and RNA.

Poly (A)$^+$RNA preparation from embryos

The procedures for collection of embryos, fractionation of the homogenate of the embryos and preparation of poly (A)$^+$RNA were performed according to Kobayashi and Okada (1989). The stage of embryos at the egg collection for RNA extraction was 20±20 min after egg laying (AEL).

Uv-irradiation and microinjection

The procedure for microinjection was the same as described (Kobayashi and Okada, 1989). Embryos aged 30±20 min AEL were uv-irradiated (280 nm, 200 J/m^2) posteriorly. Poly (A)$^+$RNA, 5 μg/ml, was dissolved in an injection buffer (0.1 mM sodium phosphate pH 6.8, 5 mM KCl), and 0.2 nl of the suspension was injected into a recipient embryo (*mwh* e^{11}). Poly (A)$^+$RNA, 20 μg/ml, was mixed in a drop of silicon oil (Sinetsu Kagaku, FL100-450CS) with threefold volume of cytoplasm taken from mid-ventral periplasm, and 0.2 nl of the mixture was injected into a recipient embryo (*mwh* e^{11}). When the embryos had developed to the blastoderm stage, they were observed for the presence of pole cells. All the embryos were allowed to develop to the mature adults and they were observed for the presence of germ cells.

Vital staining of pole cells with rhodamine 123

Pole cells were taken into a glass needle from embryos in process of cellularization. These pole cells were expelled from the needle into a drop of dye solution (10 μg/ml of rhodamine 123 and 1 μg/ml of Hoechst 33342 in a basic medium described by Limbourg and Zalokar, 1973) on a 0.1%-poly-L-lysine coated glass slide. After 10 min of incubation, the pole cells were rinsed in a dye-free medium for 10 min, and were examined under an Olympus epifluorescence microscope. For applying an inhibitor for mitochondrial activities, pole cells were incubated in a dye solution containing 50 μg/ml valinomycin (Johnson *et al.*, 1981).

Results

Vital staining of pole cells with rhodamine 123

In embryos subjected to transplantation, pole cells are readily exposed because of a puncture caused by microinjection. This prevents us to use n-octane for permeabilize vitelline membrane. Thus we adopted the method that collecting pole cells from embryos into a fine glass capilary to be double stained with rhodamine 123 and Hoechst 33342 (Fig. 1). Pole cell nuclei were stained with Hoechst 33342. The granular cytoplasmic structures stained with rhodamine 123 were observed in normal pole cells (Fig. 1C). The exposure of pole cells to valinomycin inhibited the rhodamine staining (Fig. 1F). Rhodamine 123 accumulates only in active mitochondria (Johnson *et al.*, 1980), and valinomycin inhibits the mitochondrial activities. These results indi-

cate that rhodamine 123-stained structures in pole cells are mitochondria. Pole cells induced in uv-irradiated embryos by transplantation of polar plasm or poly(A)$^+$RNA exhibited rhodamine stained mitochondria, whose size, number and distribution pattern were practically the same as those in normal pole cells (Fig. 1C, I, L). Thus we conclude that poly(A)$^+$RNA-induced pole cells have mitochondria with normal respiratory activities.

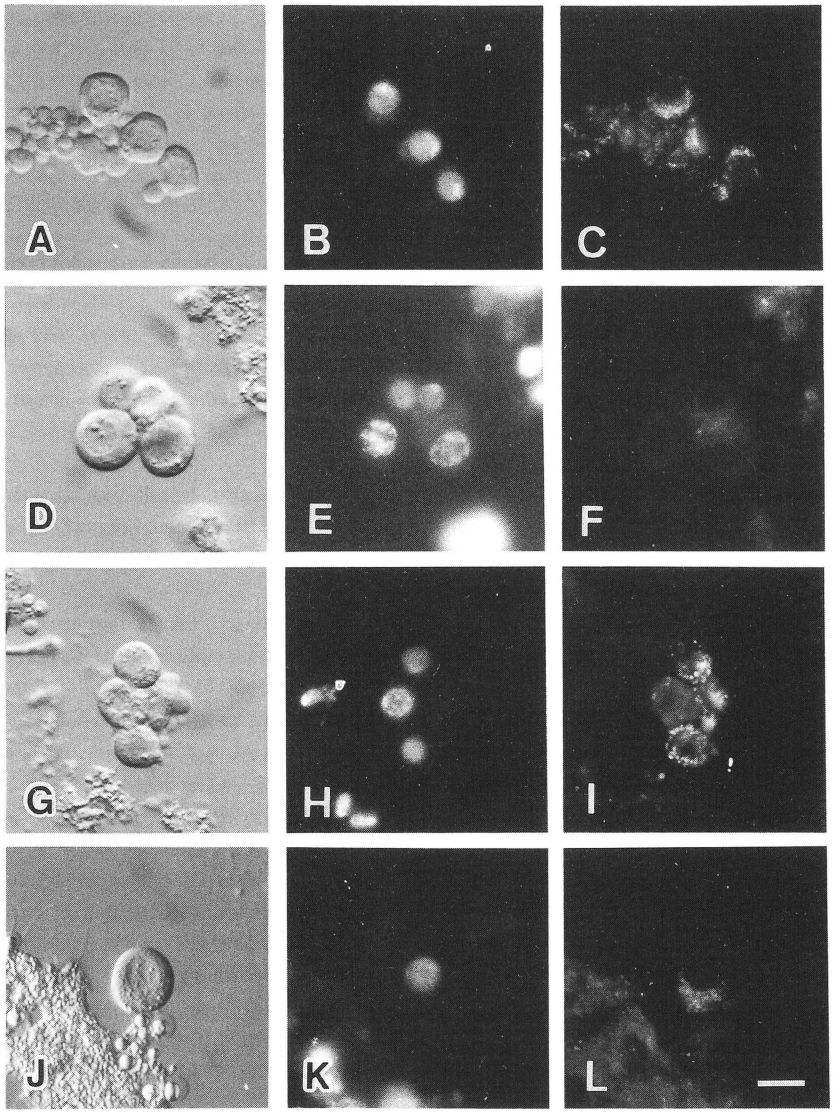

Fig. 1 Photomicrograms of live pole cells collected from cellular blastoderms with a glass capillary. Sets of horizontal 3 micrographs show the same pole cells observed with different optics. A, B and C, normal pole cells; D, E and F, normal pole cells treated with valinomycin; G, H and I, pole cells induced in a uv-irradiated embryo by polar plasm transplantation; J, K and L, pole cells induced in a uv-irradiated embryo by poly(A)$^+$RNA injection. A, D, G and J, photomicrographs taken with Nomarski optics; B, E, H and K, exhibiting fluorescence from Hoechst 33342; C, F, I and L, exhibiting fluorescence from rhodamine 123. Bar = 10 μm.

Table 1 Germ cell forming activity of polar plasm.

Injected material	No. of eggs treated	No. of embryos developing to blastoderms		No. of adults examined	
		Total	With pole cells	Fertile	Sterile
Polar plasm	121	63	38(60.3%)[a]	3	3[b]
None	158	88	9(10.2%)	0	9

[a] Statistical significance, $P < 0.01$. Probability was calculated by the χ^2-test against the non-transplantation control.

[b] Statistical significance, $P < 0.05$. Probability was calculated by the Fisher's exact probability test against the control.

Germ line formation by coinjection of poly(A)$^+$RNA fraction and cytoplasm from mid-ventral periplasm

When normal pole plasm was injected into the posterior of uv-irradiated embryos, 38 out of 63 embryos formed pole cells (Table 1). Six flies emerged from these 63 embryos and three (one female and two males) among the six flies had mature germ cells (Table 1).

Ventral cytoplasm, when solely transplanted, did not exhibit significant pole cell forming activities (Table 2). When poly(A)$^+$RNA and ventral cytoplasm were coinjected, 79 of 217 formed pole cells (Table 2). The difference in the rate of embryos formed pole cells between experimentals and controls, in which embryos were irradiated but not transplanted, was statistically significant.

Thirty-four flies emerged from 217 embryos. Although 15 flies among the 34 flies derived from blastoderms with pole cells, 33 of 34 flies had completely germ-cell free gonads. Only one fly had fertile gonads. In the controls, 18 of 185 uv-irradiated embryos formed pole cells, and 37 flies emerged. Of the 37 flies 6 derived from blastoderms with pole cells. However, none of these 37 flies had germ cells (Table 2).

Table 2 Germ cell forming activity of a mixture of ventral cytoplasm and poly(A)$^+$RNA.

Injected material	No. of eggs treated	No. of embryos developing to blastoderms		No. of adults examined	
		Total	With pole cells	Fertile	Sterile
Ventral cytoplasm	68	48	7(14.6%)[a]	ND	ND
None	72	38	6(15.8%)	ND	ND
Ventral cytoplasm with poly(A)$^+$RNA	315	217	79(36.4%)[b]	1	33[c]
None	289	185	18(9.7%)	0	37

[a] Statistical significance, $P > 0.05$. Probability was calculated by the χ^2-test against the non-transplantation control.

[b] Statistical significance, $P < 0.01$. Probability was calculated by the χ^2-test against the control.

[c] Statistical significance, $P > 0.05$. Probability was calculated by the Fisher's exact probability test against the control.

Discussion

Poly (A) $^{+}$RNA-induced pole cells have been shown to migrate to gonads to become cells morphologically very similar to primordial germ cells, but they never develop to germ cells (Togashi *et al.*, 1986). To explain this, a hypothesis was proposed: the function of poly (A) $^{+}$RNA or mtlrRNA is confined to pole cell formation; and an additional cytoplasmic factor (s), which is also a component of polar plasm, is required for pole cells to differentiate as germ cells. However, no direct evidence for this hypothesis has so far been presented. An alternative hypothesis is that RNA-induced pole cells have latent ability to differentiate as germ cells, but their cytoplasm has been uv-irradiated at earlier stages causing mitochondria so impaired that can not support RNA-induced pole cells to survive cell divisions and differentiation after they reach gonads. In the present work we tested the second hypothesis by examining two main properties of mitochondria in RNA-induced pole cells: whether they keep respiratory activities, and whether transplantation of functional mitochondria can rescue RNA-induced pole cells from abortive development.

Uv-irradiation does not reduce mitochondrial activities in pole cells

We have previously reported that mitochondrial respiratory activities in early cleavage embryos are higher in the posterior pole region than in the other somatic regions. To the later cleavage stages the elevated activities in the posterior decline to reach the level of the somatic regions. Uv-irradiation at the early cleavage stage did not affect the higher activity in the posterior. After polar plasm was incorporated into pole cells mitochondria acquire higher activities again in normal development (Akiyama and Okada, 1992). In the present work we showed that RNA-induced pole cells exhibit mitochondrial activities as high as those in normal pole cells.

We have not checked mitochondrial activities in primordial germ cells in normal development. However, it is unlikely that mitochondrial activities of RNA-induced pole cells become different from normal pole cells after they reach gonads, considering that no uv effect has ever been detected in younger embryos so far examined.

Cytoplasm with intact mitochondria can not rescue RNA-induced pole cells from inability of developing to germ cells

In this work we have demonstrated that the poly (A) $^{+}$RNA induces pole cells even when it was coinjected with cytoplasm from mid-ventral periplasm. The region of around 50% egg length (posterior pole as 0%) is known to be lowest in coordinate gene products, such as *Bicoid, Oskar, Vasa* and *Nanos* (see review for Bate and Martinez-Arias, 1993). Consequently, cytoplasm from mid-ventral region is likely to be neutral for germ cell determination as well as abdomen formation.

Coinjection of poly (A) $^{+}$RNA and ventral cytoplasm into uv-irradiated embryos produced RNA-induced pole cells. Since ventral cytoplasm has been confirmed to include as many mitochondria as polar plasm (Akiyama and Okada, 1992), RNA-induced pole cells from coinjection are provided very likely with unirradiated mitochondria. These mitochondria from ventral cytoplasm are probably keeping their ability of proliferation after transplantation. This view is supported by the fact that polar plasm transplanted to an ectopic site supports pole cell formation, their division, and development to germ cells (Okada *et al.*, 1974; Illmensee and Mahowald, 1974). The results of the present experiment strongly suggest that RNA-induced pole cells are not capable of developing to germ cells even when intact mitochondria are provided. Thus we can discard the second hypothesis that loss of mitochondrial respiration and/or proliferation activities causes failure of RNA-induced pole cells in development beyond primordial germ cells.

We have thus returned to the hypothesis to presume the germ cell determinant proper. No genetic studies have so far revealed the gene (s) encoding germ cell determinant. One of the posterior group genes may be responsible for expression of germ cell determinant genes in pole cells. To analyze multiple roles of most of posterior group genes, a strategy to separate pole cell formation and germ cell determination is needed for further survey of germ line determining genes.

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