[REVIEW]

# Molecular Analysis of Germ Line Formation in *Drosophila* Embryos

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Germ-line precursor cells are formed as pole cells in *Drosophila* embryos at the stage of preblastoderm. Pole cells are defined as those including polar plasm besides as those with determined fate to develop to germ line. The term polar plasm refers to posteriorly localized cytoplasm in oocytes and early embryos with a prominent morphological marker, polar granules (Mahowald, 1962), and with dual function, abdominal segmentation and germ-line determination (for review see Lehmann and Rongo, 1993). It has been disclosed that *nanos* protein starts the genetic process to ensue abdominal segmentation, and that other posterior class genes have respective roles to localize *nanos* RNA in the polar plasm (Ephrussi *et al.*, 1991). In contrast, no molecule has been specified as the germ cell determinant. We have demonstrated that the mechanism underlying germ line formation is not such simple that a single molecule species regulates everything from segregation to differentiation of germ line. In this article we review recent understanding of polar plasm functions to support pole cell formation. Furthermore, we present our on-going attempts to reveal genes that are involved in differentiation of pole cells as germ cells.

**Polar Plasm, a Cytoplasmic System to Initiate Germ Line Formation**

*a) Pole cell forming factor*

Since microinjection techniques became available in *Drosophila* embryos, the polar plasm has been transplanted into ectopic periplasm or u.v.-inactivated polar plasm (Illmensee and Mahowald, 1974; Okada *et al.*, 1974). These transplantation experiments have revealed that polar plasm has an autonomous ability to generate germ-line precursor cells *in situ*.

Relying on a bioassay system, in which the activity of a material is assessed by injecting it into u.v.-sterilized embryos and see if they form pole cells, we attempted to isolate cytoplasmic factors active in pole cell formation. Firstly, we collected an active subcellular fraction from total homogenate of early cleavage embryos (Ueda and Okada, 1982). Secondly, we found that polyadenylated RNA represents the pole cell forming activity of the subcellular fraction (Togashi *et al.*, 1986). Finally, the cDNA to the poly(A)RNA with the activity was cloned (Kobayashi and Okada, 1989).

RNA transcribed *in vitro* from the cloned cDNA was clearly able to restore pole cell forming ability to u.v.-irradiated embryos, when injected. The pole cells induced with the injection of the RNA is apparently normal as far as the morphology is concerned. However, they have never differentiated as germ cells even if the embryos with RNA-induced pole cells were allowed to develop to adulthood.

Taken together, RNA encoded in the cDNA we cloned represents part of the polar plasm function, and a genuine germ cell determinant is also present in polar plasm, although no molecular information has yet been

accumulated to specify the determinant. Furthermore, our experiments to inject a mixture of the RNA and u.v.-irradiated polar plasm into the anterior revealed that ploe cell segregation requires a u.v.-resistant factor localized in polar plasm, besides the RNA whose function we have disclosed.

*b) Mitochondrial large ribosomal RNA as a pole cell forming factor*

Nucleotide sequencing revealed that the cDNA encoding a pole cell forming factor was completely identical to the mitochondrial large ribosomal RNA (mtlrRNA) gene (Kobayashi and Okada, 1989, 1990). The implication of a gene in the mitochondrial genome in pole cell formation was so unexpected that we were very careful to accept our own results as evidencing a fact. The first question was if there was any possibility that a gene with sequences identical to mitochondrial large rRNA is present in chromosomal genome. Restriction map analyses of total DNA extracted from embryos probing with mtlrRNA showed that no sequence identical to mtlrRNA was detected in chromosomal DNA. In addition, *in situ* hybridization of mtlrRNA to salivary chromosomes gave no signal. Thus we concluded that only mitochondrial genome included sequences idêntical to the cDNA encoding RNA capable of inducing pole cells.

The second question is that mtlrRNA might not be a pole cell forming factor, but only could have rescued u.v.-damaged mitochondria. To answer this question, we assessed mitochondrial respiratory activity. Mitochondria in the u.v.-irradiated polar plasm take up Rhodamin-123. Besides, the mitochondria follow the same developmental change in Rhodamin-123-incorporation rate as in normal embryos (Akiyama and Okada, 1992). These attest that the mitochondria are not affected by u.v. in respiratory activities.

The third question is how mtlrRNA, transcribed in mitochondria to compose ribosomes, contributes to an event outside mitochondria. We have had a notion, since early stages of this work, that mtlrRNA is transported out of mitochondria, because a remarkable pole cell forming activity is detectable from a post-mitochondrial fraction of a homogenate of early cleavage embryos (Togashi *et al.*, 1986). This is the very point that has to be determined *in situ* in the embryos.

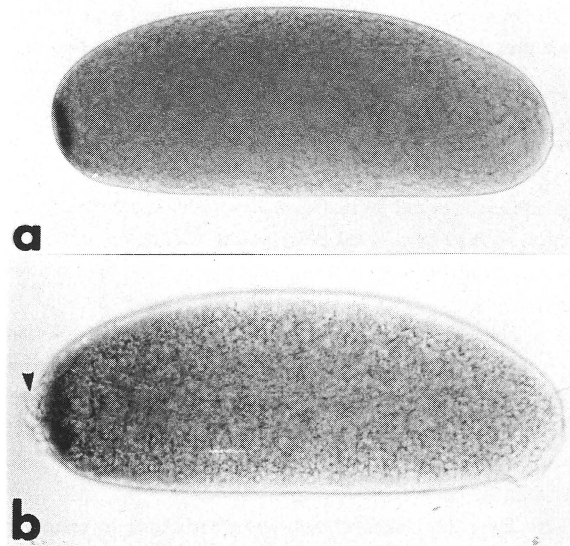


Fig. 1 Micrographs showing the distribution of extramitrochondrial mtlrRNA in *Drosophila* embryos at the cleavage stage (a), and blastoderm stage (b). Fixed embryos were hybridized *in situ* with mtlrRNA cDNA, then visualized with digoxigenin-alkaline phosphatase-labelled antidigoxigenin method. Anterior to the left. Arrowhead points to pole cells.

*c) MtlrRNA localized in polar granules*

Whole mount *in situ* hybridization techniques were adopted to demonstrate distribution of mtlrRNA in embryos. Since the procedure we followed for fixing embryos and hybridizing mtlrRNA probes onto embryos allows no probe to penetrate mitochondria, hybridization signals notify only extra-mitochondrial mtlrRNA (Amikura *et al.*, 1993).

Hybridization signals in cleavage embryos were clearly restricted to polar plasm. However, as polar plasm flowing into the pole buds, the signals are left behind and remains in the cortex beneath pole cells (Fig. 1). When gastrulation sets forth, the signal is no longer discernible. Furthermore, electron-microscope-level *in situ* hybridization verified in cleavage embryos that silver grains representing hybridization signals were associated with polar granules but no other part of cytoplasm carried the signals (Kobayashi *et al.*, 1993a) (Fig. 2). When pole cells have started to form, silver grains were not in association with polar granules but distributed sporadically in cytoplasm (Amikura, unpublished).

Our findings provide a foundation to the observation by Mahowald (1971b): polar granules are associated with mitochondria in late oogenesis, but separated after fertilization; and polar granules include RNA as well as protein in oocytes and cleavage embryos, but only protein in preblastoderms. Our hypothesis is that mtlrRNA represents the RNA that Mahowald demonstrated cytochemically in polar granules, and that mtlrRNA is transferred from mitochondria to polar granules while they are associated, and later in the preblastoderm stage mtlrRNA is released from polar granules out into cytosoles for contribution to pole cell formation.

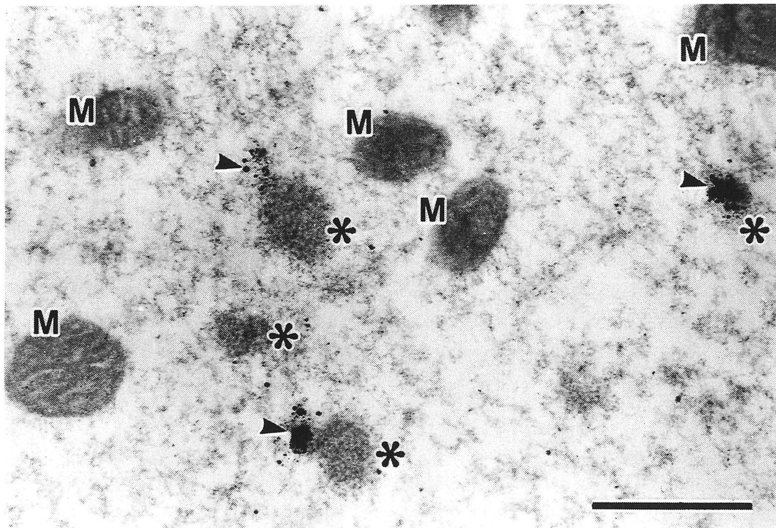


Fig. 2 Electron micrograph of a cleavage embryo sectioned through polar plasm, where mtlrRNA in polar granules is visualized with an *in situ* hybridization method. Asterisks mark polar granules, and arrow heads point to silver grains representing mtlrRNA cDNA signal. Bar = 1$\mu$m. M: mitochondrion.

To verify the hypothesis, there are questions we have to answer. To what extent is a mitochondrion associated with a polar granule in a condition that allows the transfer of mtlrRNA? It will be worthwhile to examine extra-mitochondrial mtlrRNA in polar plasm in *Drosophila hydei* that was notified as having no stage when mitochondria were in association with polar granules (Mahowald, 1971a). Is mtlrRNA able to drive itself through the mitochondrial membranes, or does it need any helper molecule? What would happen to other

rRNA species that lost their partners and were left in mitochondria? What molecules in polar granules does mtlrRNA bind to? What does signal mtlrRNA to leave polar granules? Finally, what a role does mtlrRNA have in pole cell formation?

*d) Implication of posterior class gene products*

According to Ding and Lipshitz (1993) embryos produced by females with mutation in either one of posterior class genes (except *nanos* and *pumilio*) showed no mtlrRNA accumulation in polar plasm. Posterior class genes were first defined for their common mutant phenotypes: failure in pole cell and abdomen formation (for review see St Johnston and Nüsslein-Volhard, 1992). Ephrussi and Lehmann (1992) elegantly demonstrated that localization of *oskar* RNA to an ectopic site is sufficient to trigger germ line formation as well as *nanos* RNA localization that consequently directs abdomen formation. *vasa* and *tudor* proteins, which are components of polar granules, cooperate with *oskar* protein in both germ line and abdominal segment formation. Based on accumulated data from genetic and molecular basis analyses, genetic interactions have been illustrated among posterior class genes and their products (Ephrussi and Lehmann, 1992). We propose that mtlrRNA also comes into this scheme (Kobayashi *et al.*, 1994), as Lehmann and Rongo (1993) also noted (Fig. 3). It is interesting from evolutionary point of view that different molecules, ones for germ-line determination and others for abdomen formation depend on a common transportation and localization system.

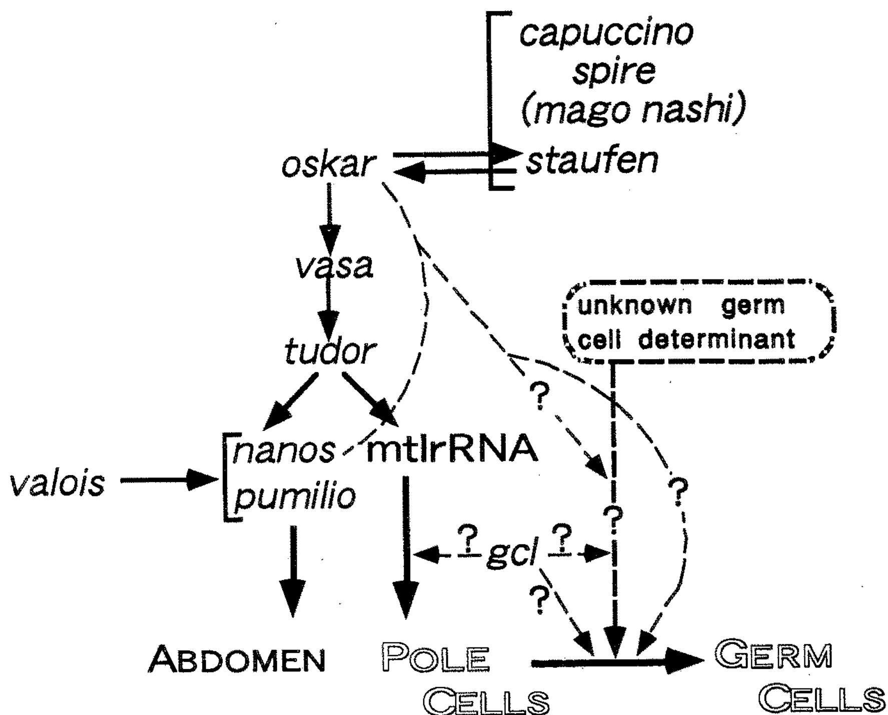


Fig. 3 The diagram illustrating genetic interactions laying foundation for germ plasm assembly, and abdomen/germ line formation. Modified from Lehmann and Rongo (1993). Arrows not necessarily represent direct regulation. A broken line with a guestion mark denotes a hypothetical assumption. *gcl* : *germ cell less*.

Among the posterior class genes, *tudor* can be most important, when mtlrRNA transportation is concerned. *tudor* protein is the only posterior gene products localized in mitochondria (Bardsley *et al.*, 1993). Taken this and others together, we are inclined to speculate that *tudor* protein binds to and carries mtlrRNA to penetrate mitochondrial membranes by way of channels.

**Genes Expressed in Germ Line**

*a) Two types of pole cells*

In addition to morphological markers, pole cells have a remarkable molecular-level property. Germ-line cells in adult flies were reported to be capable of splicing all three introns from P-element transcripts, in contrast to somatic cells that can splice the first and second introns, but not the third intron (Laski *et al.*, 1989). We extended those experiments to embryos and demonstrated that pole cells are also capable of splicing the third P-element intron. However, we noted that all pole cells are not capable. Furthermore, we noticed a strong tendency that pole cells with the splicing ability did, but those without the ability did not differentiate as germ cells in gonads (Kobayashi *et al.*, 1993b).

Since the P element is a transposon that is an extrinsic gene of *Drosophila melanogaster*, a splicing system facilitated for the regulation of some intrinsic genes must have successfully been allocated to this extrinsic gene when it transfected to *Drosophila*. It is postulated that these intrinsic genes are regulated by this splicing system for their specific expression in germ line. On this premise we have been attempting to isolate genes that are expressed in germ-line cells in developmental stages. Our strategy is to find genes including a sequence identical to the 20-bp sequence that assigns germ-line specific splicing of the P-element third intron (Laski and Rubin, 1989). We will hopefully select prospective genes from some candidates we have cloned (Kitamura, unpublished).

On the other hand, we have demonstrated that the P-element third intron is spliced in some somatic tissues at particular embryonic stages (Kitamura *et al.*, 1993). This implies the presence of a developmentally regulated splicing system that acts on transcripts from a variety of genes expressed not only in germ line but also in somatic line during embryogenesis.

*b) A gene responsible for spermatocytes proceeding to spermatids*

Our attempt to find every gene involved in differentiation of pole cells into eggs or sperms, has revealed a gene that works only for male germ cells to start meiosis. This gene was found and cloned from a P-element insertional mutant. The phenotype was abortive spermatogenesis. Germ cells do not develop beyond spermatocytes. DNA fragments on the flank of the inserted P element have been cloned and partially sequenced (Endo, unpublished).

**Conclusion**

Germ-line segregation occurs as pole cells form in preblastoderm embryos. The segregation results in restriction of polar plasm into pole cells, and secluding pole cells from the influence of somatic cells. Consequently this installs genes in pole cells under the control of germ plasm in a closed system, at least until pole cells start migration through the midgut rudiment to be exposed to signals from surrounding somatic cells. Considering that penetration by a centrosome (not a nucleus) is sufficient for polar plasm to initiate pole cell formation (Raff and Glover, 1989), together with above mentioned our observations, mtlrRNA may participate in cytoplasmic reorganization (probably through reorganization of cytoskeleton) to play a role in segregation of polar plasm. Subsequently, molecules accumulated in polar plasm activate zygotic genes, which drives pole cells to differentiate as germ cells. This story includes some speculations. Our efforts are, hopefully, to substantiate the speculations to complete the story.

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