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Implication of a 5′ coding sequence in targeting maternal mRNA to the *Drosophila* oocyte

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Abstract

Early accumulation of maternal mRNA in one of the cells of the cluster of 16 cystocytes is a critical event in the determination of the *Drosophila* oocyte. A number of developmentally important mRNAs have been shown to accumulate in the early oocyte. We report here the early expression of the *yemanuclein-alpha* (*yem-alpha*) transcript, its accumulation in the germarial oocyte and its dynamic localization in the growing oocyte. We have investigated the mechanisms involved in these processes. Microtubules are likely to be involved in both transport and localization as was shown for other maternal transcripts which behave similarly. However, unlike all the cases reported so far, transport and localization are not dependent on 3′UTR sequences. We show that the 5′ coding sequence is necessary for the early accumulation of *yem-alpha* RNA in the oocyte and for its localization pattern during oogenesis. © 1997 Elsevier Science Ireland Ltd.

Keywords: Drosophila; Oocyte; *Yemanuclein-alpha*; RNA transport; RNA localization; Microtubules

1. Introduction

While the organization of oocytes in follicles (germ cells surrounded by somatic cells) is widespread in the animal kingdom, the existence of polarized follicles forming linear arrays of growing oocytes in the ovariole (functional unit of the ovary) is specific for insect oogenesis.

In spite of this common theme, three types of ovaries can be described in insects depending essentially on morphological criteria, i.e. the panoistic ovary, the telotrophic meroistic ovary and the polytrophic meroistic ovary (for reviews see King, 1970; Mahowald, 1972; Spradling, 1993).

The meroistic polytrophic ovary of *Drosophila* is a syncitium of germ cells (15 nurse cells and the oocyte) surrounded by a monolayer of follicle cells. The germarium is subdivided into three regions. Region 1 of the germarium contains the stem cells and the cystoblasts which result from an asymmetric division of the stem cells into a stem cell and a cystoblast. The cystoblast then undergoes a series of four synchronous divisions with incomplete cytokinesis; as a consequence, a cluster of 16 interconnected cystocytes is formed. The cytoplasmic junctions are called ring canals. Two of the 16 cells, called proocytes, have four ring canals; in late region 2 of the germarium, the determined oocyte comes to lie at the posterior of the cyst while the other proocyte eventually has the same fate as the other 14 nurse cells.

Two key questions have been addressed in studies on oogenesis. One deals with the oocyte determination while the other deals with the origin of the macromolecules present in the oocyte. In the case of the panoistic ovaries these two issues are the simplest. The determination of the oocyte in the germarium is a consequence of a classical asymmetric division (on/off switch mechanism). The absence of nurse cells also makes simpler the question of transcript origin. The oocyte nucleus is transcriptionally active; like in amphibian oocytes, lampbrush chromosomes can be observed in orthopteran oocytes for example, which is a sign of active transcription in the oocyte nucleus (for a review see Mahowald, 1972; unpublished data). In the meroistic ovary, the nurse cells provide most if not all of the

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nutrients and macromolecules. Which mechanisms allow the transport of macromolecules from their site of synthesis into the oocyte is an important issue. In the case of telotrophic ovaries, transport and oocyte determination are obviously very distinct mechanisms, as an asymmetric cell division gives rise to a nurse cell and an oocyte. The situation of the polytrophic ovary is very complex as both events, RNA transport and oocyte determination, seem to be linked. Apparently in *Drosophila* the determination of the oocyte does not result simply from a classical on/off mechanism of asymmetric division. However, one should mention some observations which would be in favour of a lineage-based determination (Yue and Spradling, 1992; Lin et al., 1994; Lin and Spradling, 1995). However, a number of arguments are against such a mechanism being sufficient to decide which of the two proocytes is the presumptive oocyte. Meiotic markers such as synaptonemal complexes are still present in more than one cell of the cyst up to late region 2b of the germarium (Carpenter, 1994; Lin and Spradling, 1995). Moreover, mutations which affect genes such as *Bicaudal D* (*Bic-D*) and *egalitarian* (*egl*) result in a reversion of the fate of the presumptive oocyte as no oocyte forms in ovaries of homozygous females bearing these mutations (Schüpbach and Wieschaus, 1991). Apparently these two mutations abolish the directional transport of macromolecules towards the presumptive oocyte by affecting the first steps of microtubule organization (Ran et al., 1994). Recent data indicate that egl and Bic-D proteins act as a complex (Mach and Lehmann, 1997). In conclusion, accumulation of oocyte-specific molecules such as RNA is important in oocyte determination. Various elements of the oocyte cytoskeleton play an important role in this process (Cooley and Theurkauf, 1994; Glotzer and Ephrussi, 1996; Rongo and Lehmann, 1996). From the analysis of this process on different models we expect a better understanding of the mechanisms which underlie it. Some of them must be basic and common to numerous RNAs while others must be more specific to a given RNA.

The *yem-alpha* gene used as a model in the present study was isolated in a molecular screen for differentially expressed maternal genes (Aït-Ahmed et al., 1987). We have shown that it encodes an oocyte-specific nuclear protein with DNA binding properties (Aït-Ahmed et al., 1992). It appears in late germarial region 1 where it is found in the nucleus of the proocytes, whereas in late region 2 it seems to be restricted to the nucleus of the oocyte. Preliminary data are in favour of its role in meiosis (unpublished data).

In the present work we show that *yem-alpha* RNA is an early marker of the oocyte. It has a dynamic localization pattern in the growing oocyte. We have investigated the mechanisms which underlie its early accumulation and localization in the oocyte. Both processes are apparently dependent on the microtubule network. We report that the 5′ coding sequence and not the 3′UTR is involved in this microtubule-dependent transport and distribution of the RNA.

2. Results

2.1. Expression pattern and localization of yem-alpha RNA

We have previously reported the expression pattern of *yem-alpha* transcript as determined by in situ hybridization to ovary sections using radiolabelled probes (Aït-Ahmed et al., 1987; Thomas-Cavallin and Aït-Ahmed, 1988). While we could observe the transient accumulation of *yem-alpha* RNA at the anterior end of the oocyte from stages 8 to 10B of oogenesis, we could not clearly determine the early expression pattern of *yem-alpha* transcript because of the lack of sensitivity in those experiments. For the present work we have carried out the in situ hybridization experiments on whole mounts with digoxygenin-labelled probes. In these conditions *yem-alpha* transcripts were readily detected in region 2 of the germarium (Fig. 1). In the growing pre-vitellogenic egg chamber, the transcripts were concentrated at the posterior pole of the oocyte. After the oocyte nucleus migration towards the anterodorsal position at stage 8, the transcripts were then found to be mainly

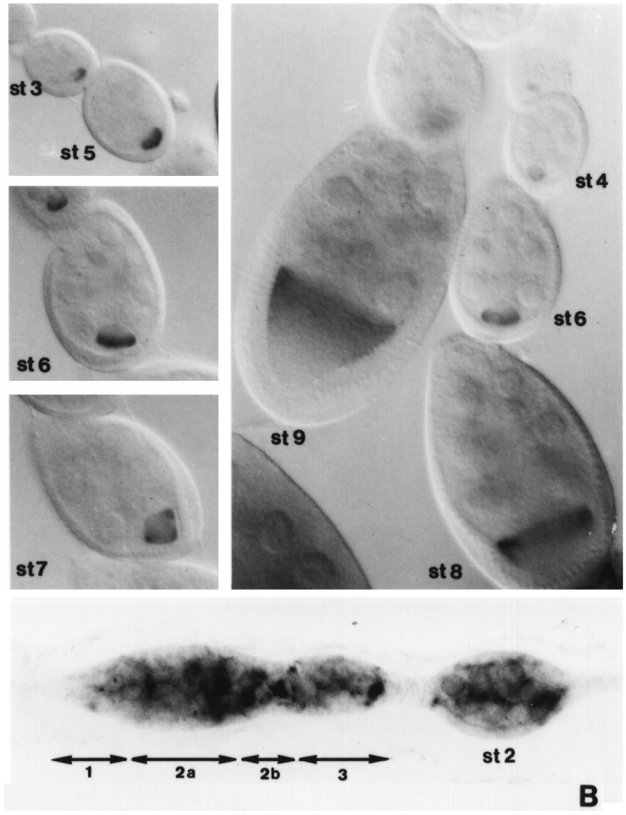

Fig. 1. Distribution of *yem-alpha* RNA in wildtype ovaries. In situ hybridization to whole-mount ovaries with a digoxygenin-labelled *yem-alpha* cDNA probe. (A) Egg chambers at various developmental stages showing the dynamic localization pattern of *yem-alpha* RNA in the growing oocyte. (B) A higher magnification of the germarium; *yem-alpha* RNA is hardly detected in region 1 of the germarium and by germarial region 2 it is readily detected in one cell, the presumptive oocyte. Our reference for egg chambers staging is King (1970).

concentrated at the anterior pole of the oocyte. Interestingly, in the stage 7 egg chamber shown in Fig. 1, transcripts could be transiently observed both at the anterior and posterior ends of the oocyte. From stage 10B on, *yem-alpha* RNA was then uniformly distributed in the egg chamber. This dynamic transport and localization process as evidenced by in situ hybridization has been reported for a number of other maternal transcripts (for reviews see Macdonald, 1992; Ding and Lipshitz, 1993; Micklem, 1995; St Johnston, 1995).

2.2. yem-alpha mRNA transport and localization require an intact microtubule network

It was suspected a while ago that microtubules were involved in RNA transport from the nurse cells into the oocyte of meroistic ovaries, either through the nutritive cord in the telotrophic ovary or through the ring canals in the polytrophic ovary (Mahowald, 1972). In order to address the question of the role of the microtubule network in *yem-alpha* RNA transport and localization, we carried out in situ hybridization experiments on mutant ovaries affected in microtubules, either in very early oogenic stages (*egl* and *Bic-D*) or in post-vitellogenic stages (*capu* and *spir*).

Both *egl* and *Bic-D* mutations result in cysts in which no oocyte forms (Steward and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1991). As shown in Fig. 2 for $Bic\text{-}D^{R26}$, a hypomorphic allele, the mutant egg chambers start to degenerate around stage 7 (stage as determined by egg chamber size). Disruption of the early directional transport of transcripts has been considered as a cause for these defects (Theurkauf et al., 1993). It is noteworthy that the effect of *Bic-D* on the early transport of various transcripts is differential (Suter and Steward, 1991; Ran et al., 1994). As shown in Fig. 2, the hypomorphic allele $Bic\text{-}D^{R26}$ did not prevent a significant amount of *yem-alpha* transcript from transiently accumulating in a single cell of the germarial cyst, while the same mutant never shows any sign of oocyte differentiation. This allele affects neither *Bic-D* nor *osk* or *orb* RNAs while all the other transcripts which are normally targeted to the oocyte fail to accumulate in $Bic\text{-}D^{R26}$ oocytes (Suter and Steward, 1991; Ran et al., 1994). The transport and localization of *yem-alpha* transcript are dramatically affected in $Bic\text{-}D^{PA66}$ mutant ovaries. Like *orb* RNA, *yem-alpha* RNA was found to be uniformly distributed throughout the germ cell cluster in the germarium of $Bic\text{-}D^{PA66}$ mutants (Fig. 2), while *osk* RNA is affected only in *Bic-D* null mutants (Ran et al., 1994). The differential behaviour of *yem-alpha* RNA in the two oocyte-deficient *Bic-D* mutants argues against the uniform localization observed in PA66 being due to the absence of the oocyte target.

In *egl* mutants, *yem-alpha* transcript failed to localize to a single cell of the germarial cysts. Interestingly, *yem-alpha* transcript was no longer detected in a stage 2 *egl* egg chamber while egg chambers degenerated much later (Fig. 2).

The implication of microtubules in the dynamic localization pattern of *yem-alpha* RNA has been analyzed using the two mutations, *capu* and *spir*. Both mutations are known to affect anteroposterior and dorso-ventral polarity of the egg (Manseau and Schüpbach, 1989). These observations led to

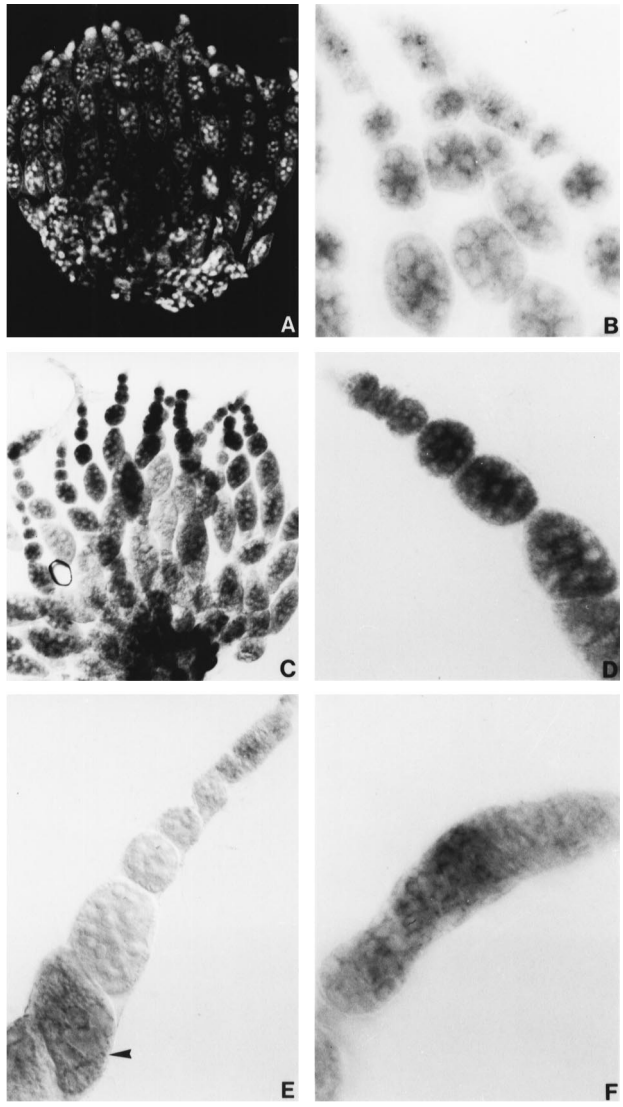

Fig. 2. Distribution of *yem-alpha* RNA in the $Bic\text{-}D^{R26}$, $Bic\text{-}D^{PA66}$ and egl^{RC12} oocyte differentiation mutants. (A) Hoechst staining of an R26 ovary showing that this hypomorphic allele has lost its ability to differentiate a normal oocyte. The egg chambers degenerate by stage 7. (B) In situ hybridization to an R26 mutant ovary. One cystocyte, the presumptive oocyte, still accumulates *yem-alpha* RNA in the germarium; the distribution is uniform later. (C,D) In situ hybridization to a PA66 mutant ovary. Note that the RNA is absent from stage 4 on, while degeneration of the egg chambers starts around stage 7 (C). No accumulation of *yem-alpha* RNA can be observed any more, not even in the germarial oocyte as can be observed at a higher magnification (D). (E,F) egl^{RC12} egg chambers hybridized with a *yem-alpha* cDNA probe; *egl* and *Bic-D* egg chambers are phenotypically very similar yet *yem-alpha* RNA reveals molecular differences (E). The RNA, which is uniformly distributed in the egg chambers from the very first oogenic stages, disappears in post germarial oocytes; it is hardly detected in a stage 2 egg chamber (F). The staining observed in older egg chambers (arrowhead) is due to a non-specific uptake of the probe by pycnotic cells.

the hypothesis that *capu* and *spir* may be implicated in a general mechanism such as cytoskeleton organization in the oocyte. These mutations have actually been shown to cause a premature cytoplasmic streaming in the stage 8 oocyte; this phenomenon which disrupts the polarized microtubules normally occurs at stage 10B (Theurkauf, 1994).

Using an antibody directed against the yemanuclein as a specific marker for the oocyte nucleus, we could show that these mutations also result in a failure of the oocyte nucleus to localize properly at its anterodorsal position (Fig. 3, a stage 9 egg chamber). It seems likely that the oocyte nucleus first localizes properly but fails to maintain its anterodorsal position; a transient anterior localization of the oocyte nucleus could be observed in a stage 8 mutant egg chamber. As can be seen in the stage 5 mutant egg chamber, the early RNA accumulation in the oocyte was maintained while the anterior localization of *yem-alpha* transcripts was largely affected in *capu* and *spir* oocytes (Fig. 3); the residual localization is probably due to the fact that the alleles used in this work are not null alleles. Our results are in good agreement with the known action (although indirect) of *capu* and *spir* mutations on microtubules (Theurkauf, 1994).

None of the other mutations known to affect the localization of specific transcripts had any effect on the *yem-alpha* RNA localization process. *yem-alpha* RNA distribution was unchanged in *exuperantia*, *swallow*, *staufen* and *orb* mutant egg chambers (data not shown).

To further support the data obtained with the mutations which resulted in the microtubule network disruption, we analyzed the effect of the microtubule depolymerizing drug colchicine on *yem-alpha* RNA transport and distribution in the oocyte. It is clear from our results that the RNA does not accumulate any more in the early oocyte. Moreover, its distribution becomes uniform in the growing oocyte (Fig. 4).

2.3. Sequences responsible for yem-alpha RNA transport and localization

In order to identify the RNA sequences which are involved in *yem-alpha* RNA transport and localization, we used transgenic flies which carry various chimeric constructs (reported in Fig. 5). In a preliminary experiment, we first identified the minimal sequences which allow a *lacZ* transgenic RNA to be expressed and localized similarly to the endogenous *yem-alpha* transcript. To construct the E transgene, *lacZ* was fused in frame to a *yem-alpha* 5′ fragment which contains the putative promoter, the 5′UTR and part of the coding region. The whole *yem-alpha* 5′ sequences span 2354 bp. Downstream from the *lacZ* gene, we fused a fragment which bears the entire *yem-alpha* 3′UTR (a 584 bp *Bam*H1-*Eco*R1 DNA fragment). The ovaries from E transgenic flies were submitted to in situ hybridization with a digoxygenin-labelled *lacZ* probe; the data shown in Fig. 6 clearly indicate that the sequences which are necessary and sufficient for a proper *yem-alpha* expression

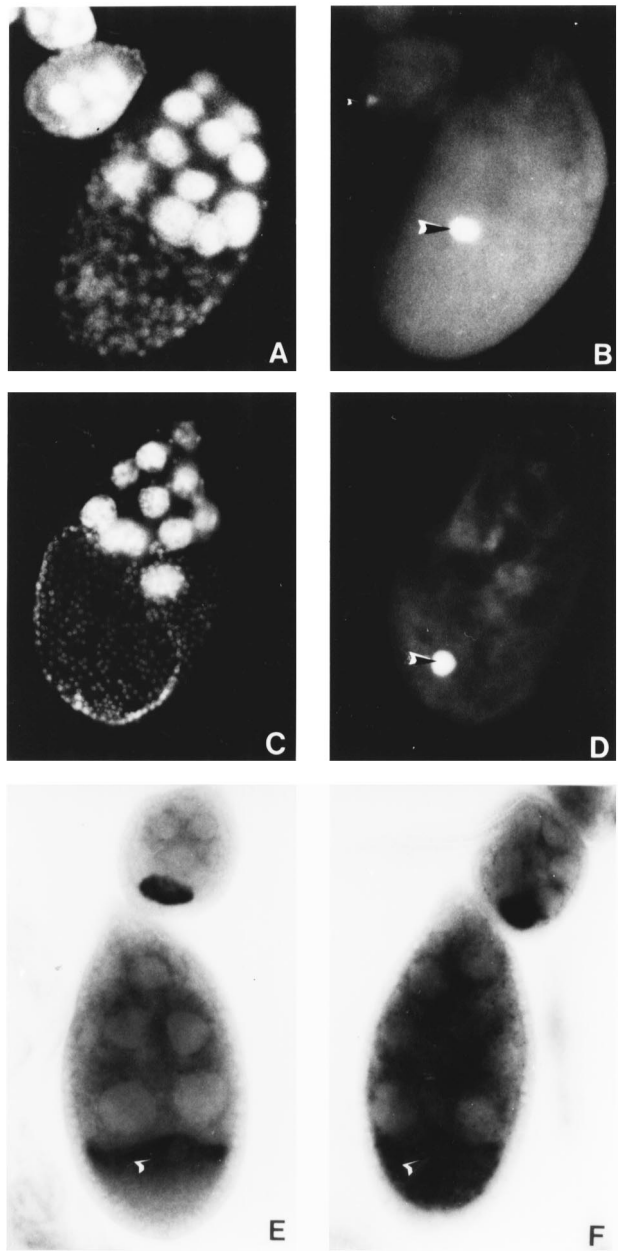

Fig. 3. Distribution of *yem-alpha* RNA in *cappuccino* and *spire* mutants. *Capu* and *spir* mutations affect microtubule repolarization in the stage 8 egg chamber resulting in defects in both anteroposterior and dorsoventral polarity. (A) Hoechst staining of a wildtype late stage 9 egg chamber. (B) Immunostaining of the same egg chamber with an anti-yemanuclein-alpha antibody revealing specifically the oocyte nucleus (arrowhead); in the wildtype, the oocyte nucleus has its characteristic anterodorsal position. (C) Hoechst staining of a $spir^{RP48}$ stage 9 egg chamber. (D) Immunostaining of the same mutant egg chamber with the anti-yemanuclein antibody; the oocyte nucleus seems to float away in a disorganized ooplasm. (E,F) In situ hybridization with a digoxygenin-labelled *yem-alpha* cDNA probe, to whole-mount mutant ovaries. (E) Wildtype stage 5 and stage 8 egg chambers. (F) $capu^{RK12}$ egg chambers, same stages as in (E). Interestingly, the oocyte nucleus which is visible here without any staining is still anterior in a stage 8 $capu^{RK12}$ egg chamber, while most *yem-alpha* transcripts are not any longer localized. On the other hand, transcript distribution in the wild type (E) and the mutant (F) stage 5 egg chambers is very similar.

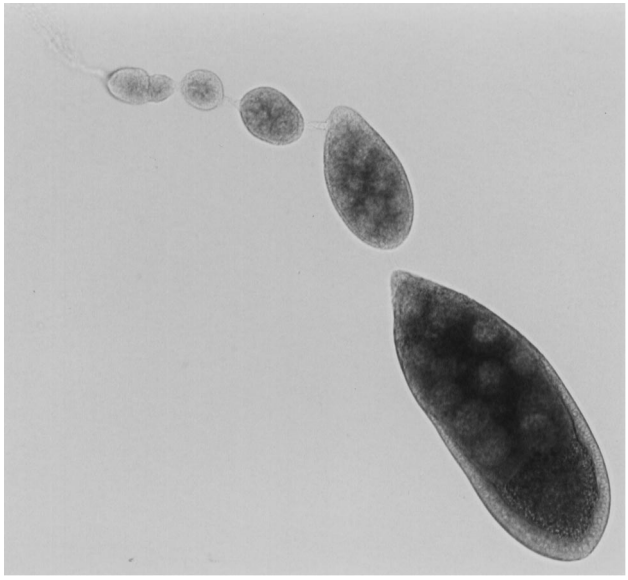

Fig. 4. Distribution of *yem-alpha* RNA in ovaries from colchicine fed flies. Wildtype flies were starved for 8 h and then fed with yeast paste containing 50 μg/ml colchicine for 20 h. RNA staining was carried out as described in Section 4. These data should be compared with those obtained on ovaries from non-treated wildtype flies as shown in Fig. 1.

during oogenesis were present on this construct, including the post-transcriptional control sequences.

All the maternal transcripts identified so far which display a differential localization in the oocyte bear the control sequences in their 3′UTR. Moreover, the sequences responsible for their localization seem to be closely linked to the transport sequences (Ferrandon et al., 1994; Serano and Cohen, 1995a). The aim of this study was to identify those sequences on *yem-alpha* transcript. In order to address this question, we made a second construct in which we replaced the *yem-alpha* 3′UTR with a sequence containing SV40 polyadenylation signals (Thummel et al., 1988). The D transgenic flies were submitted to in situ hybridization. Surprisingly, similar results were obtained for D and E transgenic ovaries. The *lacZ* transcript accumulates in the oocyte according to the same pattern as the endogenous *yem-alpha* transcript. The *yem-alpha* 3′UTR seems to be dispensable with respect to the transport/localization process of the transgenic RNA. In order to test a possible effect of *yem-alpha* 3′UTR on transcriptional activity, we fused this sequence upstream from the promoter in the D construct to generate the DAE construct. The in situ hybridization pattern as revealed with a *lacZ* probe was unchanged (data not shown). Therefore, the role of *yem-alpha* 3′UTR is still unclear.

Obviously, the sequences responsible for the correct transport and localization of the transgenic RNA were still present on the D construct. They should be found either on *yem-alpha* 5′UTR or in its 5′ coding sequences present on the chimeric transcript, unless the early accumulation of the RNA in the early oocyte is due to the activity of the *yem-alpha* promoter in the oocyte nucleus. However, we first had to rule out any implication of the SV40 3′ sequences used in our constructs in the accumulation and localization of the D transgenic transcript. This was achieved with the Delta B construct which bears *yem-alpha* promoter sequences fused to lacZ-SV40 sequences. The chimeric transcript expressed from this transgene fails to concentrate and localize properly within the oocyte (data not shown). These data rule out any implication of SV40 sequences in the process we have been analyzing. Moreover, it is clear from these results that the accumulation of the *yem-alpha* transcript in the early oocyte cannot be accounted for by transcription from *yem-alpha* promoter in the oocyte nucleus.

In order to identify the *yem-alpha* transcribed sequences involved in its RNA transport and distribution, we constructed the Delta A′ transgene in which the entire *yem-alpha* coding region was deleted (Fig. 5). In situ hybridization data on *lacZ* transcript localization are reported in Fig. 6. A dramatic change occurred in the localization pattern of the *lacZ* chimeric transcript expressed from Delta A′ transgene. The RNA was uniformly distributed in the entire Delta A′ egg chamber, both in nurse cells and oocyte. Even at early stages, high levels of *lacZ* transcripts could be detected in nurse cells. In conclusion, these *yem-alpha* sequences (spanning a region from the ATG initiation site to the StyI restriction site, called ORF 5′) are necessary to drive proper transport and localization of a chimeric transgene in the *Drosophila* oocyte. Although such a role for an RNA coding region is completely novel, once more both transport and localization seem to be tightly linked, presumably because the same mechanism is involved (Serano and Cohen, 1995a).

An attractive hypothesis would be that the yemanuclein nascent peptides play a role in this ORF mediated transport. In order to test this model we constructed a transgene (DM) similar to D in every point except that the ATG initiation codon of *yem-alpha* ORF was mutated to TTG. We first verified by histochemical staining of the DM ovaries for β-galactosidase activity that no alternative ATG codon was used to initiate protein synthesis (data not shown). Obviously, the same in situ hybridization pattern as in D was observed. These experimental data rule out a role of the nascent peptides in *yem-alpha* RNA transport and localization.

3. Discussion

3.1. The role of RNA localization

In the present work, we have analyzed the transport and localization of *yem-alpha* RNA. We have shown that *yem-alpha* RNA concentrates in a single cell in late germarial region 2. Like a number of other maternal transcripts, *yem-alpha* RNA then displays a dynamic localization pattern in the growing oocyte. It is first localized to the posterior pole

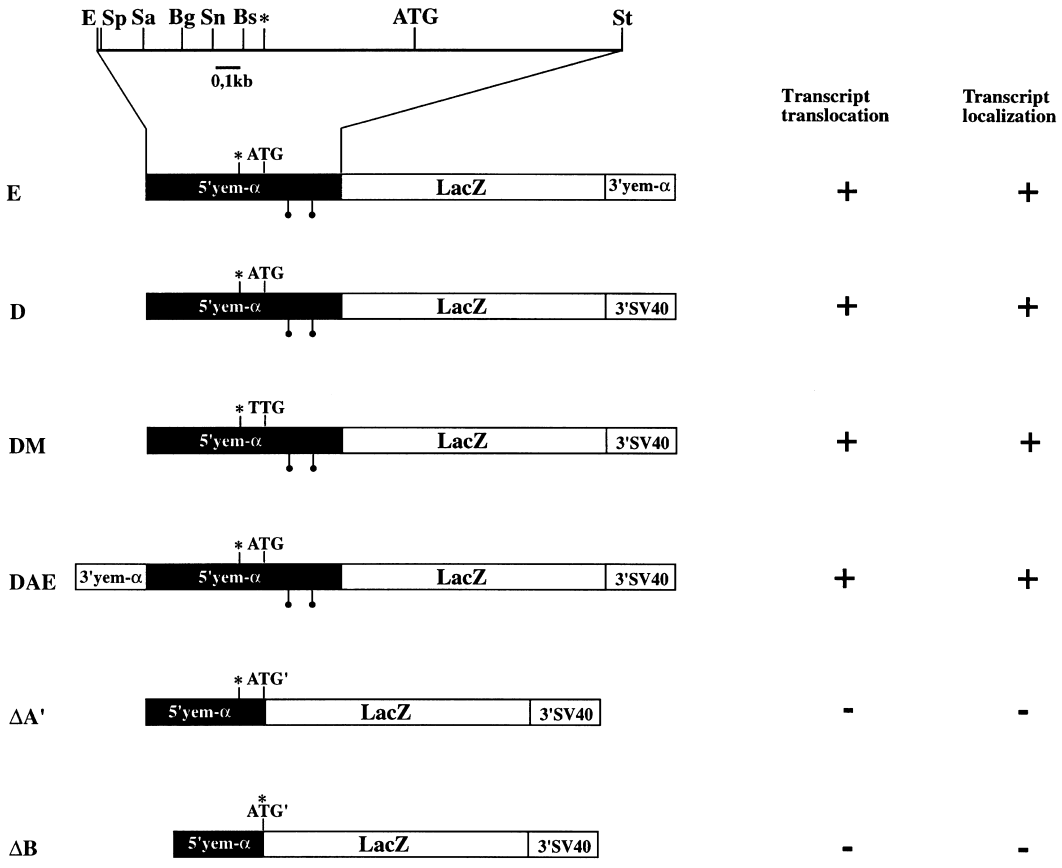

Fig. 5. Structure of the various constructs used in the transformation experiments and summary of the transport and localization patterns of the corresponding *lacZ* chimeric transcripts. (+) and (–) indicate respectively wildtype and abnormal translocation and localization pattern of the reporter transcripts expressed from the various constructs. All transgenes (E, D, DM, DAE, Delta A′ and Delta B) are under the control of *yem-alpha* promoter. All constructs except the E construct bear SV40 3′ polyadenylation signals. The E construct bears the *yem-alpha* control sequences both 5′ and 3′ to the *lacZ* gene. The black part of the drawings represents *yem-alpha* 5′ genomic sequences. The symbols represent the introns and the star indicates *yem-alpha* transcription start. ATG, *yem-alpha* translational start; ATG′, *lacZ* translational start. Restriction sites: E, EcoRI; Sp, SphI; Sa, SacII; Bg, BglII; Sn, SnaBI; Bs, BssHII; St, StyI.

of the oocyte up to stage 7. By stage 8, when the oocyte nucleus migrates to its anterodorsal position, *yem-alpha* transcript relocalizes to the anterior end of the oocyte. This anterior localization is transient for most localized RNAs as their distribution is even from stage 10B on (Macdonald, 1992; Ding and Lipshitz, 1993; Micklem, 1995; St Johnston, 1995). Other RNAs (usually determinants) remain anchored to their position: *bicoid* to the anterior margin of the oocyte, *gurken* to the antero-dorsal corner and *oskar* and *cyclin B* to the posterior end of the oocyte (St Johnston et al., 1989; Ephrussi et al., 1991; Kim-ha et al., 1991; Dalby and Glover, 1993; Neuman-Silberberg and Schüpbach, 1993). Obviously *K10, yem-alpha, Bic-D, orb* or *egl* mRNAs cannot be considered as molecular determinants (Prost et al., 1988; Suter et al., 1989; Aït-Ahmed et al., 1992; Christerson and McKearin, 1994; Lantz et al., 1994; Serano and Cohen, 1995b; Mach and Lehmann, 1997). It is possible that this localization is simply a consequence of microtubule rearrangement in the oocyte. Nevertheless, we cannot rule out a role of this microtubule-dependent localization in preventing RNA from degradation or in ensuring its efficient translation.

3.2. Microtubule-dependent movement of RNA within the oocyte

The dynamic localization pattern and the early transport into the germarial oocyte led us to the hypothesis that microtubules could be involved in *yem-alpha* RNA transport and localization. Indeed, it is well established that important changes occur in the cytoskeletal organization of the egg chamber from the very first oogenic stages. Microtubules always organize from a microtubule organizing centre (MTOC). The first signs of microtubule organization can be observed in a subset of the cysts present in region 2a of the germarium (Theurkauf et al., 1993). It is also at that time that intracyst transport of organelles is initiated (Mahowald and Strassheim, 1970). Within region 2b, the MTOC is clearly localized to one cell at the expected position of the oocyte. Microtubules appear to originate from this MTOC and pass through the ring canals allowing the directional transport (Theurkauf et al., 1993). Microtubule inhibitors (colchicine) and mutations such as *egl* and *Bic-D* disrupt the microtubule-dependent transport and as a result no oocyte differentiates in colchicine treated or in *egl* and

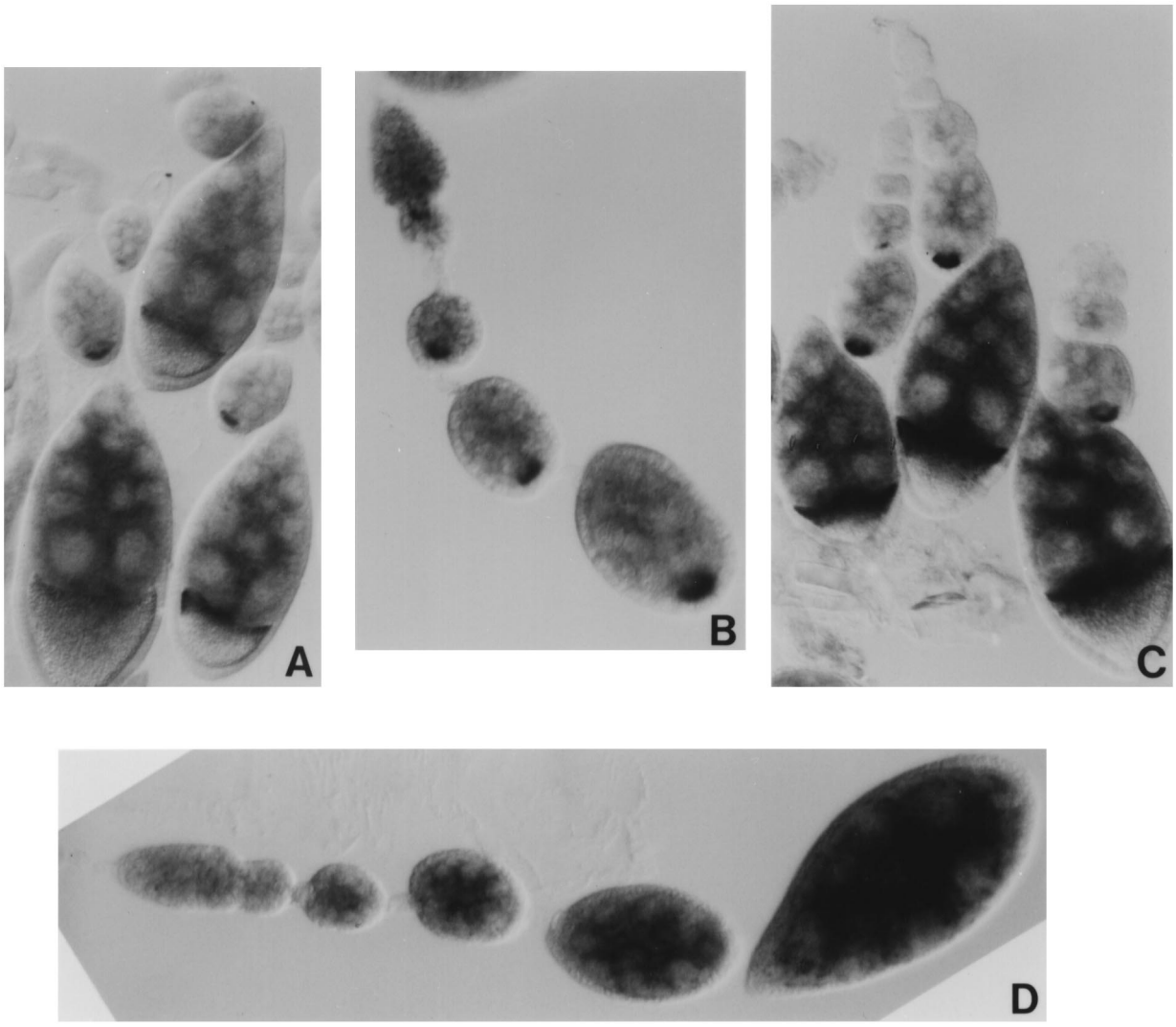

Fig. 6. Distribution of *lacZ* transcripts in E (A), D (B,C) and Delta A′ (D) transgenic ovaries. In situ hybridization to whole-mount ovaries was performed with a digoxygenin-labelled *lacZ* DNA probe. For transgene E (A), the distribution of *lacZ* transcripts is similar to the endogenous *yem-alpha* transcript distribution. The substitution of the *yem-alpha* 3′ UTR with SV40 3′ sequences does not affect *lacZ* RNA expressed from the D construct, either in pre-vitellogenic egg chambers (B), or in post-vitellogenic egg chambers (C). Early transport and dynamic localization within the oocyte are dramatically affected in the Delta A′ ovariole (D). Delta A′ is derived from the D construct by deleting the *yem-alpha* coding region present on E and D constructs.

Bic D mutant egg chambers (Theurkauf et al., 1993; Schüpbach and Wieschaus, 1991).

Both mutations result in a degeneration of the egg chambers from stage 7 on, but *yem-alpha* transcripts disappear earlier (stage 2) in *egl* mutant egg chambers. This may reflect other differences between these mutations at the molecular level, although the resulting phenotypes are very similar.

For *Bic-D* a differential effect has been shown on RNA translocation which depends on the allele and the transcript considered. Apparently *osk* RNA still localizes to one cell in *Bic-D*^{PA66} egg chambers, while this mutation prevents the accumulation of all other transcripts including *yem-alpha* RNA. *Bic-D*^{R26} and *Bic-D*^{PA66} alleles differ in that no Bicaudal protein accumulates in PA66 mutant oocytes while a post-translational mechanism apparently allows the accumulation of the protein in R26 mutants. It is noteworthy that *Bic-D* transcripts no longer accumulate in R26 mutant egg chambers (Suter and Steward, 1991; Ran et al., 1994).

One often refers to this microtubule-dependent RNA transport as a default pathway; if this was the case, any RNA sequence, given that it is expressed early, should accumulate in the oocyte, such as the maternal transcripts listed above. Our data clearly show that this is not the case; the *lacZ* transcript without an appropriate transport and localization sequence is transported into the oocyte as early as the germarial region yet it does not display the characteristic accumulation pattern unless specific sequences are present on the transgenic transcript.

In conclusion, the microtubule-dependent transport can-

not be considered as a default pathway for maternal transcripts. To support this idea, the K10 transcript, although it has such sequences, does not accumulate in the oocyte before stage 2 (Cheung et al., 1992; our observations). This means that the presence of specific sequences is necessary but not sufficient for the early accumulation of transcripts in a single germarial cystocyte and that this phenomenon is highly regulated.

To our knowledge, all the transcripts which accumulate in a single cell at early oogenic stages undergo a series of movements which are characteristic of the microtubule reorganization during the oocyte maturation process (Theurkauf et al., 1992). It seems that once the RNA is associated with microtubules, it then follows the microtubules fate. This is probably the reason why one cannot dissociate the mechanisms which allow the early translocation process from the transient anterior localization of the transcripts.

At stage 10B, a cytoplasmic streaming occurs which disrupts the microtubule organization. Mutations such as *capu* and *spir* affect the microtubules by inducing a premature streaming of the ooplasm at stage 8 (Theurkauf, 1994). As a consequence, the asymmetrical distribution of RNA was affected in all the cases reported so far but one, *bcd* RNA, which remained anchored to the anterior cortex of *capu* and *spir* oocytes. However, the action of microtubules on *bcd* RNA transport and localization has been shown (Pokrywka and Stephenson, 1991). As has been recently found for *osk* RNA localization, other cytoskeletal components may account for RNA transport and/or localization (Erdelyi et al., 1995; Glotzer et al., 1997).

The biological significance of the microtubule-dependent transient asymmetric localization of transcripts remains elusive. An attractive hypothesis would be that some of these RNAs, if not all, are associated with polysomes.

3.3. The yem-alpha 5′ coding sequence is necessary for RNA transport and localization

In all the cases reported so far, the *cis*-acting sequences involved in RNA transport and localization are found in the 3′ untranslated region of the RNA (Macdonald, 1992; Ding and Lipshitz, 1993; Wilhelm and Vale, 1993; Micklem, 1995; St Johnston, 1995). In a number of cases, such as *bicoid* and *K10*, it has been shown that RNA secondary structures present on the 3′UTR account for its action (Ferrandon et al., 1994; Serano and Cohen, 1995a). Our finding that the *yem-alpha* 3′UTR is involved neither in its transport nor in its localization is surprising. More surprising is the fact that the coding sequence is necessary for this process. As it is clear that *yem-alpha* RNA is recruited on polysomes as soon as it is present in the cystocyte cytoplasm (the yemanuclein protein being detected as early as late germarial region 1; our unpublished data), we hypothesized a model which could account for the early transport. In this model *yem-alpha* transcript would be transported into the oocyte as a polysomal complex through the nascent peptides. However, experimental data did not support this model which leaves us with the necessity to determine whether ORF 5′ is sufficient and to precisely map which part of the 5′ coding sequence is involved in the transport and how the interaction of the RNA coding sequence with *trans*-acting factors is compatible with its translation.

4. Experimental procedures

4.1. Fly stocks

The wildtype flies used in this study were Oregon R. For microinjection experiments, a yw^{1118} stock was used. The mutants *egalitarian* (*egl*), *Bicaudal-D* (*Bic-D*), *cappuccino* (*capu*) and *spire* (*spir*) were previously described (Mohler and Wieschaus, 1986; Steward and Nüsslein-Volhard, 1986; Manseau and Schüpbach, 1989; Suter et al., 1989; Schüpbach and Wieschaus, 1991; Suter and Steward, 1991). The alleles $Bic\text{-}D^{R26}$ and $Bic\text{-}D^{PA66}$ were used in our experiments as hemizygotes in combination with Df (2L) TW119, egl^{RC12} allele was used in combination with Df (2R) 54B and $capu^{RK12}$ and $spir^{RP48}$ were used as homozygotes.

4.2. Transformation constructs

In all the transformation constructs we used the pCasper vector which contains the *white* gene as a selectable marker (Thummel et al., 1988). For E, D and DAE, the *lacZ* gene from pPMC plasmid (Pharmacia) was fused in frame to a *yem-alpha* 5′ genomic fragment which spans the promoter region, the 5′UTR and part of the coding sequence (up to the StyI site, nucleotide 2354). The numbering of the sequence was as previously reported (Aït-Ahmed et al., 1992).

In a first step, a SmaI-PstI fragment containing the *lacZ* gene was subcloned into pBluescript SK^+ vector (SK^+-lacZ). A SmaI-StyI blunted fragment containing the *yem-alpha* 5′ sequences was cloned into the SmaI site of the SK^+-*lacZ* construct, which resulted in an in-frame fusion of the *yem-alpha* and *lacZ* ORFs. This intermediate construct called 5′yZ/ED was then inserted using PstI flanking sites into a pCasper 4 vector which contained either the *yem-alpha* 3′UTR (construct E3) or a sequence containing the SV40 polyadenylation signals for constructs D and DAE (construct D3). For the DAE construct the *yem-alpha* 3′UTR was also fused upstream of the promoter region.

To generate the Delta A and Delta B constructs, we used clones obtained by exonuclease III nested deletions according to Aït-Ahmed et al. (1992). To construct Delta B, a fragment containing the first 716 nucleotides was subcloned into the EcoRI-KpnI site of the pCasper AUG-βGal vector. The removal of the *yem-alpha* translated DNA made it necessary to have a translational start site provided by this vector (Thummel et al., 1988).

The Delta A′ construct was derived from Delta A. Eleven nucleotides upstream from the ATG translational start were missing from the 5′UTR in Delta A. It was important to make a construct which contained the entire 5′UTR. To do so, the *yem-alpha* sequences were removed from Delta A by cutting at SphI and KpnI sites and replaced by a PCR fragment which spanned nucleotide 13 to nucleotide 1393 and which contained the *yem-alpha* 5′ control sequences and the whole 5′UTR. The oligonucleotides used were ROS 10 (13–29) and OA8 (1379–1393).

The DM construct is a mutated version of D in which the ATG which initiates the *yem-alpha* ORF is mutated to TTG in order to prevent its translation. The mutated ORF was obtained by recombinant PCR by using two sets of primers (ROS 10 and OA40 on the one hand and OA41 and P2 on the other hand).

The OA40 (5′ 1402-CCCCTTTGACAATTCCGGCGG-1382) and OA41 (5′ 1382-CCGCCGGAATTGTCAAAGG-1402) primers which are the internal primers are complementary and bear the point mutation which results in the replacement of the ATG initiator codon by a TTG (underlined codon). ROS 10 (see above) and P2 (2563–2583) are the external primers. The template was a *yem-alpha* genomic EcoRI fragment (Aït-Ahmed et al., 1992).

The Appligene thermocycler (Crocodile III) and the Boehringer Expand High Fidelity enzyme were used in the amplification assay. The various constructs were checked by sequencing.

4.3. P element mediated transformation

A *yw* fly stock was used as a recipient to construct all the transgenic flies. The test DNA was injected at a 100 ng/μl concentration along with the pUC hs (P Delta 2–3) helper DNA (300 ng/μl) as a source of transposase (Rio and Rubin, 1985).

4.4. β-Galactosidase histochemical staining

Newly hatched flies were reared for 3 days on fresh yeast. The ovaries were hand dissected and placed in ice-cold PBS. They were fixed for 10 min at 4°C in a 0.25% glutaraldehyde solution in PBS. After rinsing twice in PBS, the X-Gal staining was carried out at 37°C in a classical staining solution containing X-Gal at a 0.5 mg/ml final concentration (Sanes et al., 1986). To account for effects of chromosomal location on gene activity, at least two transformed lines were systematically examined for each construct.

4.5. Whole-mount in situ hybridization

The ovaries were hand dissected as described above. The in situ hybridization to whole-mount ovaries was carried out essentially as described in Tautz and Pfeifle (1989). The probes were made either from gel-purified cloned DNA or PCR fragments. The digoxygenin-labelling of the probes was achieved by nick translation using the Boehringer nick translation kit.

4.6. DNA staining and immunochemical detection on whole-mount ovaries

For DNA staining, hand-dissected ovaries were treated with bis benzimide (Hoechst 33258, Sigma) by dipping the specimens for 30 s in a 2 μg/ml Hoechst solution in PBS. After several washes in PBS the samples were mounted in Permafluor (Immunotech).

The immunochemical staining was performed using the AS2 polyclonal antibodies from rabbit, described in Aït-Ahmed et al. (1992). In order to prevent a loss of the yemanuclein antigenic response, we avoided any kind of specimen fixation. The following procedure was used. Ovaries from etherized females were incubated overnight at 4°C with the primary antibody immediately after a blocking step in 1X PBS, 0.1% Triton and 5% low-fat dried milk. All the subsequent steps were carried out in 1X PBS, 0.1% Triton and 0.5% low-fat dried milk. The secondary antibody was a donkey anti-rabbit FITC-affiniPure F(ab′)2 fragment from Jackson ImmunoResearch.

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References

Aït-Ahmed, O., Thomas-Cavallin, M., Rosset, R., 1987. Isolation and characterization of a region of the *Drosophila* genome which contains a cluster of differentially expressed maternal genes (*yema* gene region). Dev. Biol. 122, 153–162.

Aït-Ahmed, O., Bellon, B., Capri, M., Joblet, C., Thomas-Delaage, M., 1992. The yemanuclein-alpha: a new *Drosophila* DNA binding protein specific for the oocyte nucleus. Mech. Dev. 37, 69–80.

Carpenter, A.T., 1994. *Egalitarian* and the choice of cell fates in *Drosophila melanogaster* oogenesis (discussion). Ciba Found. Symp. 182, 223–254.

Cheung, H.K., Serano, T.L., Cohen, R.S., 1992. Evidence for a highly selective RNA transport system and its role in establishing the dorsoventral axis of the *Drosophila* egg. Development 114, 653–661.

Christerson, L.B., McKearin, D.M., 1994. *orb* is required for anteroposterior and dorsoventral patterning during *Drosophila* oogenesis. Genes Dev. 8, 614–628.

Cooley, L., Theurkauf, W.E., 1994. Cytoskeletal functions during *Drosophila* oogenesis. Science 266, 590–596.

Dalby, B., Glover, D.M., 1993. Discrete sequence elements control posterior pole accumulation and translational repression of maternal *cyclin* B RNA in *Drosophila*. EMBO J. 12, 1219–1227.

Ding, D., Lipshitz, H.D., 1993. Localized RNAs and their functions. BioEssays 15, 651–658.

Ephrussi, A., Dickinson, L.K., Lehmann, R., 1991. *Oskar* organizes the germ plasm and directs localization of the posterior determinant *nanos*. Cell 66, 37–50.

Erdelyi, M., Michon, A.M., Guichet, A., Glotzer, J.B., Ephrussi, A., 1995. Requirement for *Drosophila* cytoplasmic tropomyosin in *oskar* mRNA localization. Nature 377, 524–527.

Ferrandon, D., Elphick, L., Nüsslein-Volhard, C., St Johnston, D., 1994. Staufen protein associates with the 3′UTR of *bicoid* mRNA to form particles that move in a microtubule-dependent manner. Cell 79, 1221–1232.

Glotzer, J.B., Ephrussi, A., 1996. mRNA localization and the cytoskeleton. Semin. Cell. Dev. Biol. 7, 357–365.

Glotzer, J.B., Saffrich, R., Glotzer, M., Ephrussi, A., 1997. Cytoplasmic flows localize injected *oskar* RNA in *Drosophila* oocytes. Curr. Biol. 7, 326–337.

Kim-ha, J., Smith, J.L., Macdonald, P.M., 1991. *oskar* mRNA is localized to the posterior pole of the *Drosophila* oocyte. Cell 66, 23–35.

King, R.C., 1970. Ovarian Development in *Drosophila melanogaster*, Academic Press, New York.

Lantz, V., Chang, J.S., Horabin, J.I., Bopp, D., Schedl, P., 1994. The *Drosophila* orb RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. Genes Dev. 8, 598–613.

Lin, H., Spradling, A.C., 1995. Fusome asymmetry and oocyte determination in *Drosophila*. Dev. Genet. 16, 6–12.

Lin, H., Yue, L., Spradling, A.C., 1994. The *Drosophila* fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. Development 120, 947–956.

Macdonald, P.M., 1992. The means to the ends: localization of maternal messenger RNAs. Semin. Dev. Biol. 3, 413–424.

Mach, J.M., Lehmann, R., 1997. An Egalitarian-Bicaudal D complex is essential for oocyte specification and axis determination in *Drosophila*. Genes Dev. 11, 423–435.

Mahowald, A.P., 1972. Oogenesis. In Counce, S.J. and Waddington, C.H. (eds.), Developmental Systems: Insects, Academic Press, New York, pp. 1–47.

Mahowald, A.P., Strassheim, J.M., 1970. Intercellular migration of centrioles of *Drosophila* germarium. J. Cell Biol. 45, 306–320.

Manseau, L.J., Schüpbach, T., 1989. *cappuccino* and *spire*: two unique maternal-effect loci required for both the anteroposterior and dorsoventral patterns of the *Drosophila* embryo. Genes Dev. 3, 1437–1452.

Micklem, D.R., 1995. mRNA localization during development. Development 172, 377–395.

Mohler, J., Wieschaus, E.F., 1986. Dominant maternal-effect mutations of *Drosophila melanogaster* causing the production of double-abdomen embryos. Genetics 112, 803–822.

Neuman-Silberberg, F.S., Schüpbach, T., 1993. The *Drosophila* dorsoventral patterning gene *gurken* produces a dorsally localized RNA and encodes a TGF alpha-like protein. Cell 75, 165–174.

Pokrywka, N.J., Stephenson, E.C., 1991. Microtubules mediate the localization of *bicoid* RNA during *Drosophila* oogenesis. Development 113, 55–66.

Prost, E., Deryckere, F., Roos, C., Haenlin, M., Pantesco, V., Mohier, E., 1988. Role of the oocyte nucleus in determination of the dorsoventral polarity of *Drosophila* as revealed by molecular analysis of the *K10* gene. Genes Dev. 2, 891–900.

Ran, B., Bopp, R., Suter, B., 1994. Null alleles reveal novel requirements for *Bic-D* during *Drosophila* oogenesis and zygotic development. Development 120, 1233–1242.

Rio, D.C., Rubin, G.M., 1985. Transformation of cultured *Drosophila melanogaster* cells with a dominant selectable marker. Mol. Cell. Biol. 5, 1833–1838.

Rongo, C., Lehmann, R., 1996. Regulated synthesis, transport and assembly of the *Drosophila* germ plasm. Trends Genet. 12, 102–109.

Sanes, J.R., Rubenstein, J.L.R., Nicolas, J.F., 1986. Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. EMBO J. 5, 3133–3142.

Schüpbach, T., Wieschaus, E., 1991. Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. Genetics 129, 1119–1136.

Serano, T.L., Cohen, R., 1995. A small predicted stem-loop structure mediates oocyte localization of *Drosophila K10* mRNA. Development 121, 3809–3818.

Serano, T.L., Cohen, R.S., 1995. Gratuitous mRNA localization in the *Drosophila* oocyte. Development 121, 3013–3021.

Spradling, A.C., 1993. Developmental genetics of oogenesis. In: Bate, M., Martinez Arias, A. (Eds.), The Development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 1–70.

Steward, R., Nüsslein-Volhard, C., 1986. The genetics of the dorsal-Bicaudal-D region of *Drosophila melanogaster*. Genetics 113, 665–678.

St Johnston, D., 1995. The intracellular localization of messenger RNAs. Cell 81, 161–170.

St Johnston, D., Driever, W., Berleth, T., Richstein, S., Nüsslein-Volhard, C., 1989. Multiple steps in the localization of *bicoid* RNA to the anterior pole of the *Drosophila* oocyte. Development 107 (Suppl.), 13–19.

Suter, B., Steward, R., 1991. Requirement for phosphorylation and localization of the Bicaudal-D protein in *Drosophila* oocyte differentiation. Cell 67, 917–926.

Suter, B., Romberg, L.M., Steward, R., 1989. *Bicaudal-D*, a *Drosophila* gene involved in developmental asymmetry: localized transcript accumulation in ovaries and sequence similarity to myosin heavy chain tail domains. Genes Dev. 3, 1957–1968.

Tautz, D., Pfeifle, C., 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma 98, 81–85.

Theurkauf, W.E., 1994. Premature microtubule-dependent cytoplasmic streaming in *cappuccino* and *spire* mutant oocytes. Science 265, 2093–2096.

Theurkauf, W.E., Smiley, S., Wong, M.L., Alberts, B.M., 1992. Reorganization of the cytoskeleton during *Drosophila* oogenesis: implications for axis specification and intercellular transport. Development 115, 923–936.

Theurkauf, W.E., Alberts, B.M., Nung Jan, Y., Jongens, T.A., 1993. A central role for microtubules in the differentiation of *Drosophila* oocytes. Development 118, 1169–1180.

Thomas-Cavallin, M., Aït-Ahmed, O., 1988. The random primer labeling technique applied to in situ hybridization on tissue sections. J. Histochem. Cytochem. 36, 1335–1340.

Thummel, C.S., Boulet, A.M., Lipshitz, H.D., 1988. Vectors for *Drosophila* P-element-mediated transformation and tissue culture transfection. Gene 74, 445–456.

Wilhelm, J.E., Vale, R.D., 1993. RNA on the move: the mRNA localization pathway. J. Cell Biol. 123, 269–274.

Yue, L., Spradling, A.C., 1992. *Hu-li tai shao*, a gene required for ring canal formation during *Drosophila* oogenesis encodes a homolog of adducin. Genes Dev. 6, 2443–2454.