DEVELOPMENTAL BIOLOGY **180,** 119–130 (1996)
ARTICLE NO. 0289

Elaboration of the Messenger Transport Organizer

Cortex of *Xenopus* Oocytes

Malgorzata Kloc, Carolyn Larabell,* and Laurence D. Etkin[1]

*Department of Molecular Genetics, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030; and *Life Sciences Division, Lawrence Berkeley Laboratory, University of California, Berkeley, California*

Previous studies demonstrated that there were two pathways, the messenger transport organizer (METRO) or early and the Vg1 or late, which function during stages 1 to 3 of oogenesis for the localization of RNAs at the vegetal cortex of *Xenopus* oocytes. In the present study we analyzed the properties of the METRO pathway, which localizes Xlsirt, Xcat2, and Xwnt11 RNAs to a specific region of the vegetal cortex during stage 1 of oogenesis. A combination of methodologies involving both fixed material and living oocytes was used to analyze RNA localization. We show that in early diplotene pre-stage 1 oocytes (25–50 μm in diameter) both endogenous and injected exogenous METRO RNAs translocated to multiple mitochondrial aggregates (pre-mitochondrial clouds) that surround the germinal vesicle (GV). However, by early stage 1 (diplotene oocytes, 50–200 μm), all three of the RNAs discriminated between the different clouds and translocated exclusively within the METRO of a single mitochondrial cloud. Therefore, in stage 1 diplotene oocytes there is a unique mechanism causing a change in the intrinsic property of the mitochondrial clouds which designates one of them as the RNA transport vehicle. During translocation through the cytoplasm Xlsirt and Xcat2 RNAs were detected associated with cytoplasmic particles of different morphologies. Additionally, we also found that the translocation of RNAs through the early or METRO pathway, unlike that of the late pathway, occurred in the absence of intact microtubule and actin microfilament cytoskeletal elements. This supports a cytoskeletal-independent model for localization of RNAs through the METRO pathway.

INTRODUCTION

The localization of RNAs is a universal process occurring in a large variety of cell types including somatic cells and germ cells. In both vertebrates and invertebrates RNA localization during oogenesis is critical to the establishment of polarity in the oocyte, the designation of specific cell lineages, and the proper axial patterning of the embryo (Jeffrey, 1988; Melton, 1991; Gurdon, 1992; St. Johnston, 1995). In the frog, *Xenopus laevis*, full-grown stage 6 oocytes possess a primary polarity that includes the unequal distribution of yolk platelets, pigment, and various RNAs which designates an animal/vegetal axis (Dumont, 1972; Gerhart *et al.*, 1989; Rebagliati *et al.*, 1985).

During oogenesis in *Xenopus* there are several morphological features within the oocyte that foreshadow this emerging polarity. One such feature encompasses the mitochondrial aggregates which in pachytene and early diplotene oocytes surrounds the GV. At this time these aggregates possess a prominent region that will become more compact and eventually form the spherical mitochondrial cloud seen in stage 1 oocytes (Al-Mukhtar and Webb, 1971; Hausen and Riebessell, 1991; Heasman *et al.*, 1984; M. Kloc and L. D. Etkin, unpublished observations). For convenience we will refer to the mitochondrial aggregates in oogonia and meiotic oocytes as precloud structures since they, either completely or partially, contribute to the fully elaborated mitochondrial cloud structure typical of stage 1 diplotene oocytes. Another indication of polarity is seen during the zygotene stage when the chromosomes form the so-called bouquet arrangement at one side of the nucleus. All of these structures are predictive of the future vegetal pole.

Numerous RNAs were found localized at either the animal or the vegetal hemisphere in full-grown stage 6 oocytes (Rebagliati *et al.*, 1985; Weeks and Melton, 1987; Kloc *et*

[1] To whom correspondence should be addressed.

0012-1606/96 $18.00

al., 1989, 1991; Reddy *et al.*, 1992; Ku and Melton, 1993; Mosquera *et al.*, 1993). Recently, two major pathways were identified for the localization of RNAs at the vegetal cortex of oocytes (Kloc and Etkin, 1994, 1995; Forristall *et al.*, 1995). One of these involves the localization during stage 1 (Dumont, 1972) of oogenesis through a specialized region of the mitochondrial cloud called the messenger transport organizer (METRO) which was first described for the localization of Xlsirts (Kloc *et al.*, 1993). Later several other RNAs such as Xwnt11 (Kloc and Etkin, 1994, 1995) and Xcat2 (Forristall *et al.*, 1995) also were shown to use this pathway. The second pathway is used by Vg1 which is localized during stages 2–3 of oogenesis (Melton, 1987; Yisraeli and Melton, 1988; Yisraeli *et al.*, 1990; Kloc and Etkin, 1994, 1995; Forristall *et al.*, 1995). Interestingly, two of these RNAs, Xwnt11 and Vg1, manifest properties of molecules involved in axial patterning, while Xcat2 possesses homologies to the *Drosophila* nanos zinc binding domains (Mosquera *et al.*, 1993). Xlsirt RNAs appear to function, in part, in the anchoring of the Vg1 RNA at the vegetal cortex (Kloc and Etkin, 1994).

RNAs localized through the METRO and Vg1-like pathways exhibit different patterns of cortical distribution. The Vg1-like RNAs were detected anchored throughout the entire cortex of the vegetal hemisphere from the vegetal pole to the region of the future marginal zone while METRO-localized RNAs were detected as a discrete disk in the cortex at the apex of the vegetal pole.

Previous studies have focused on the analysis of RNA localization during the later stages of oogenesis from stage 2 through stage 6 when the oocyte already exhibits numerous morphological signs of polarization (Melton, 1987; Yisraeli and Melton, 1988; Yisraeli *et al.*, 1990; Kloc and Etkin, 1994, 1995; Forristall *et al.*, 1995). However, in the present study we focused on the process by which the early or METRO pathway is established during oogenesis prior to and during stage 1 of oogenesis. In so doing we also describe a method to visualize the transport of exogenous RNAs in living oocytes.

MATERIALS AND METHODS

Preparation of Oocytes

Ovaries were surgically removed from anesthetized adult *X. laevis* frogs, young froglets (2.5–4.0 cm), and late metamorphosing and newly metamorphosed froglets (stages 63–66) and were stored in 1× modified Barth's solution. Pieces of 2.5- to 4.0-cm and adult frog ovaries were treated with collagenase for 40 min at 28°C [1 mg/ml collagenase (Sigma)] in calcium-deficient buffer (1× OR). Ovaries from stage 63–66 animals contained germ cells from primordial germ cell stages (18–20 μm) through pre-stage 1 early diplotene oocytes (35–50 μm), while the 2.5- to 4.0-cm frog ovaries contain oocytes through stage 1. These ovaries were dissected into small fragments and used for *in situ* hybridization or for histological analysis.

In Situ Hybridization to Detect Endogenous Transcripts

After extensive washing in 1× Barth's solution, groups of oocytes or pieces of ovary were fixed in MEMFA (0.1 *M* MOPS, 2 m*M* EGTA, 1 m*M* $MgSO_4$, 3.7% formaldehyde) for 1 to 2 hr and transferred to 100% methanol and stored at −20°C. Complementary RNA probes were synthesized from linearized plasmid templates in the presence of digoxigenin-11-UTP (BMB) as in Kloc and Etkin (1994, 1995). All *in situ* hybridization procedures were done in 6- and 24-well culture plates using 50 to 100 oocytes per well with shaking on rotating platform according to Harland (1991) and Kloc and Etkin (1994, 1995). Therefore, observations were based on the analysis of hundreds of oocytes of different stages of oogenesis. The detection procedure and reagents were from a Genius 3 kit (Boehringer). The color reaction was developed for 1 hr in the dark and oocytes were postfixed in MEMFA overnight and stored in 100% methanol. Oocytes were embedded in paraplast and sectioned.

Injection and Detection of Exogenous RNA

Pre-stage 1 or stage 1 oocytes were injected with 100–500 pg of synthetic Xlsirt RNA containing three repeat units labeled *in vitro* with digoxigenin-11-UTP (Harland, 1991). RNA was targeted for the nucleus; however, there was always a significant amount within the cytoplasm due to leakage from the pipette and the nucleus. Injected oocytes were cultured in 50% Liebowitz L-15 media containing 5 mg/ml vitellogenin, 1 m*M* L-glutamine, 15 m*M* Hepes, 1 μg/ml insulin, and antibiotics gentamycin (100 μg/ml), tetracycline (50 μg/ml), and nystatin (50 units/ml) at 18°C (Kloc *et al.*, 1993; Wallace and Misulovin, 1978; Wallace *et al.*, 1981). After 2 days of culture injected oocytes were fixed in MEMFA for 1–2 hr, washed in PBS, treated with proteinase K, and postfixed in MEMFA. They were incubated with antidigoxigenin antibody and stained according to the Genius 3 kit protocol (Boehringer). The oocytes were photographed as whole mounts using a compound microscope and incident lighting.

Confocal Microscopy Analysis of RNA Localization in Living Oocytes

Oocytes of various stages were manually defolliculated or treated with collagenase to remove follicle cells. Fluoroscein- or Texas red-labeled RNAs made *in vitro* by incorporation of fluoroscein (BMB)- or Texas red (Molecular Probes)-derivatized UTP were injected into oocytes. Oocytes were viewed in the MRC 1024 laser scanning confocal microscope (Bio-Rad Microsciences, Cambridge, MA) equipped with an inverted Nikon Diaphot 200 microscope and a 20× Fluor lens (0.75 numerical aperture) or a 60× Plan Apo oil immersion objective lens (1.4 numerical aperture). Optical sections were collected at 0.5- to 1-μm intervals using fluorescein and rhodamine channels of krypton/argon laser (at 10% power) fitted with the appropriate filters. Each image is a single optical section.

Nocodazole and Cytochalasin B Treatment and Confocal Analysis of Microtubules and Microfilaments

Injected oocytes were cultured in L-15 medium in the presence of nocodazole (4–10 μg/ml; Sigma), to depolymerize microtubules,

or 25 μg/ml cytochalasin B (Sigma), to depolymerize actin, in Barth's for 4 hr. Oocytes were fixed overnight in 4% paraformaldehyde and 0.1% glutaraldehyde in a cytoskeleton stabilization buffer (CSB; 100 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* Hepes, 1 m*M* EGTA, 0.1% Triton X-100, pH 7.6). Prior to immunolabeling, oocytes were rinsed in CSB (×3) then incubated in SuperBlock (Pierce Chemical, Rockford, IL) plus 0.1% Triton X-100 (SBT) for 1 hr to block nonspecific protein binding. The nocodazole-treated oocytes were incubated in mouse anti-tubulin IgG (Calbiochem–Novabiochem International, San Diego, CA) for 4 hrs, rinsed in SBT for 20 min (×3), then incubated in fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibodies (Sigma) for 4 hr. Cytochalasin B-treated oocytes were incubated in SBT for 1 hr then in rhodamine-labeled phalloidin (Sigma) for 2 hr. Control oocytes were incubated in the secondary antibodies (without exposure to a primary antibody) after the blocking step. After labeling oocytes were rinsed in CSB for 20 min (×3) and mounted in ProLong Antifade (Molecular Probes, Inc., Eugene, OR) between two coverslips, then analyzed by confocal microscopy.

RESULTS

Endogenous Xlsirt, Xcat2, and Xwnt11 RNAs Are Detected within Several Mitochondrial Aggregates in Pre-Stage 1 Early Diplotene Oocytes (25–50 μm)

When oocytes reach the early diplotene stage (25–50 μm in diameter), prior to stage 1, the mitochondrial aggregates surround the GV (Heasman *et al.*, 1984; Fig. 1a). We refer to these structures as pre-mitochondrial clouds, one of which will eventually form the METRO-containing mitochondrial cloud during stage 1 (>50 μm in diameter) (Kloc and Etkin, 1995). The close association of the pre-mitochondrial clouds to the GV is evident even after removal of the GV–cloud complex from live early diplotene oocytes (Fig. 1b). The number of pre-mitochondrial clouds varies but generally ranges from 2 to 4 per oocyte. The spatial pattern of distribution of the localized RNAs has never been analyzed in these early stage oocytes. Therefore, we investigated the patterns of localization of Xcat2, Xlsirt, Xwnt11, and Vg1 in early diplotene oocytes.

Endogenous Xcat2, Xlsirt, and Xwnt11 were detected in pre-stage 1 diplotene oocytes by *in situ* hybridization localized within all of the precloud structures surrounding the GV (Figs. 1c, 1d, and 1e). In some instances, besides localization in the preclouds, RNAs were detected in the cytoplasm. Vg1 mRNA was also detected in prestage 1 oocytes; however, it was distributed throughout the cytoplasm and was excluded from all of the pre-mitochondrial cloud structures (Fig. 1f). This is consistent with its distribution throughout the cytoplasm later during oogenesis until it begins to translocate to the cortex during late stage 2 and stage 3.

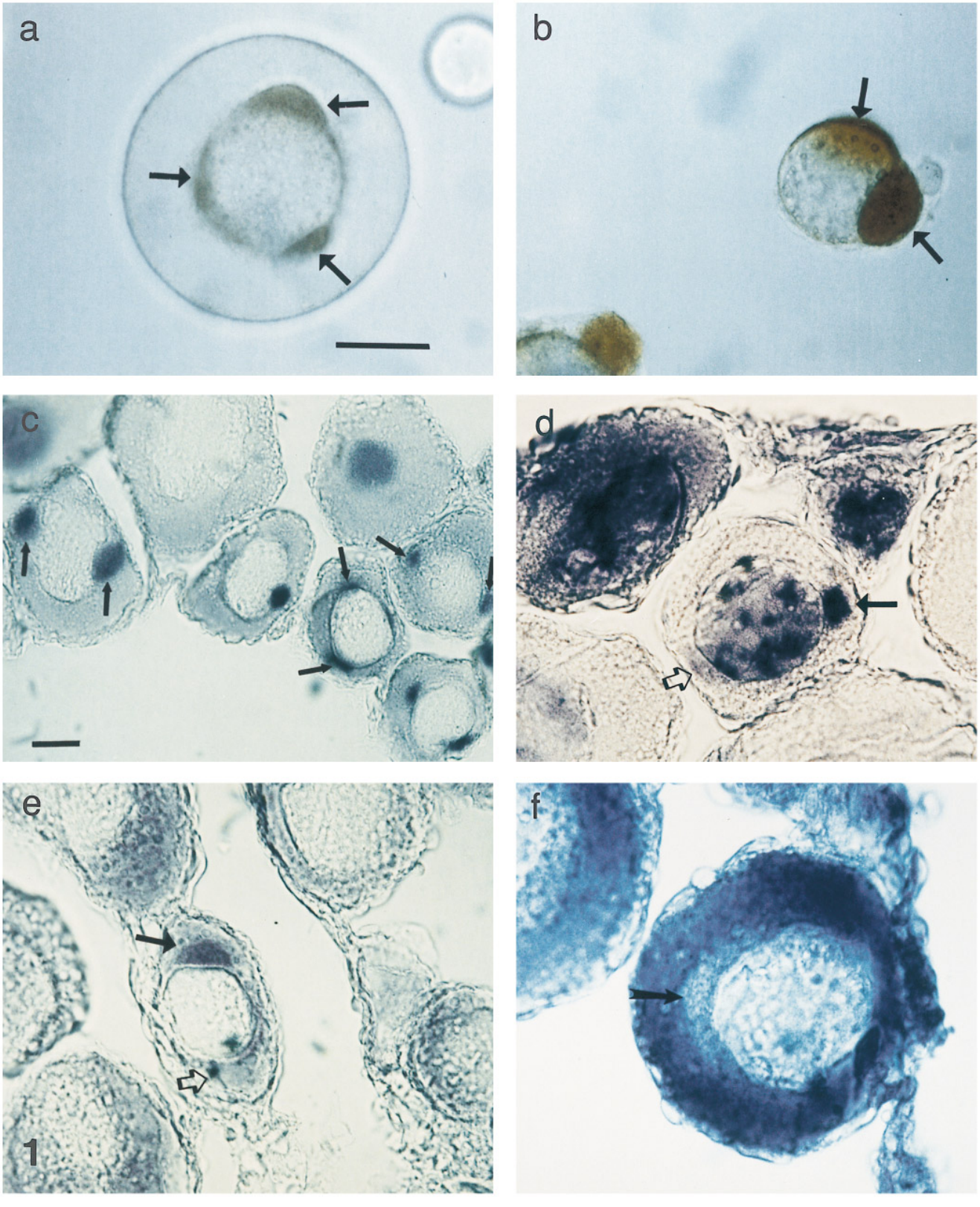
These results suggest that in pre-stage 1 diplotene oocytes, although there may be some quantitative differences among the different RNAs, there is no discrimination by Xlsirt, Xcat2, or Xwnt11 among the numerous precloud structures and that the RNAs are targeted to all mitochondrial aggregates.

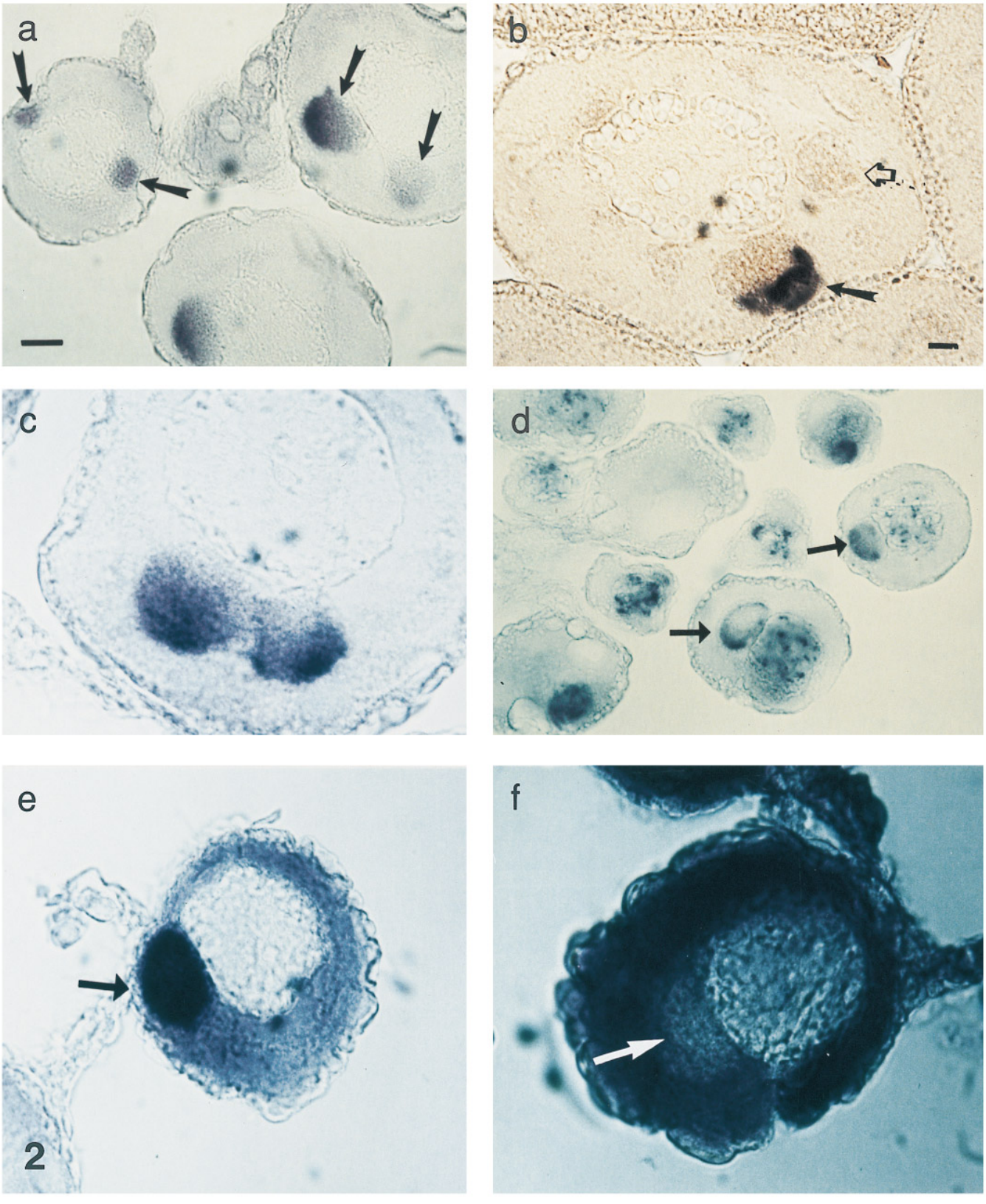
Endogenous RNAs Were Localized to the METRO Region of a Single Mitochondrial Cloud in Diplotene Stage 1 Oocytes

In early stage 1 diplotene oocytes (50–200 μm) the Xcat2 mRNA became increasingly more abundant within one of the mitochondrial clouds (Fig. 2a). Eventually, in the majority of stage 1 oocytes it was detected localized in the METRO region at the tip of the cloud with other visible clouds lacking Xcat2 mRNA (Fig. 2b). This resulted in a distinct molecular polarity of Xcat2 mRNA in the oocyte with its position within the METRO region of the mitochondrial cloud presumably designating the future vegetal pole. Occasionally, several clouds were detected that still possess equivalent amounts of Xcat2 mRNA in early stage 1 oocytes; however, invariably they were located within very close proximity to one another, suggesting that there may be a specific region of the oocyte cytoplasm that main-

FIG. 1. Xlsirt, Xcat2, and Xwnt11 were detected within all of the precloud mitochondrial aggregates in early diplotene oocytes. (a) Unstained living prestage 1 oocytes (25 μm in diameter) showing the distribution of the pre-mitochondrial clouds surrounding the GV (arrows); (b) isolated GV showing pre-mitochondrial cloud tightly juxtaposed to the nuclear membrane; both a and b were photographed as live unfixed material. Oocytes in c–f were embedded in paraffin and sectioned. (c) *In situ* hybridization of early diplotene oocytes (25–50 μm in diameter) using the Xcat2 probe. Arrows point at the pre-mitochondrial clouds showing the accumulation of Xcat2 mRNA; (d) hybridization of Xlsirt probe to early diplotene oocytes (filled arrow points to high level of accumulation in one precloud while open arrow points to low level of accumulation); (e) hybridization of Xwnt11 probe to early diplotene stage oocytes (filled arrow points to high level of accumulation in one precloud while open arrow points to low level of accumulation); (f) hybridization of Vg1 probe to early diplotene stage oocytes (arrow points to unstained pre-mitochondrial cloud). Each bar represents 10 μm.

FIG. 2. Localization of Xcat2, Xlsirt, and Xwnt11 in a single METRO-containing cloud in stage 1 oocytes. (a) Pre-stage 1 oocytes showing the decrease of Xcat2 mRNA in all but one of the pre-mitochondrial clouds (arrows); (b) stage 1 oocyte showing accumulation of Xcat2 mRNA in the METRO region of the dominant cloud (solid arrow) while other clouds do not contain Xcat2 mRNA (open arrow); (c) stage 1 oocyte containing two clouds in close proximity, both of which retain the Xcat2 mRNA; (d) group of early stage 1 oocytes showing localization of Xlsirt to one of the clouds; (e) Xwnt11 mRNA localized to one of the clouds in stage 1 oocyte. Solid arrows point to mitochondrial clouds containing Xlsirt or Xwnt11 RNA; (f) stage 1 oocyte hybridized with Vg1 probe. The arrow points to the METRO-containing mitochondrial cloud that lacks Vg1 mRNA. Each bar represents 25 μm.





tains the Xcat2 mRNA within the METRO region of the clouds (Fig. 2c).

In early stage 1 oocytes Xlsirt and Xwnt11 RNAs, like Xcat2, were only associated with the METRO-containing cloud (Figs. 2d and 2e). Xlsirt transcripts also were detected within the GV as nuclear bodies the nature of which is not known (Fig. 2d). Vg1 mRNA, however, was distributed throughout the cytoplasm and was excluded from the mitochondrial cloud in stage 1 oocytes (Fig. 2f). These results indicate that by early stage 1 there is a designation of one of the precloud structures as the METRO-containing cloud which will transport the Xlsirt, Xwnt11, and Xcat2 RNAs to the vegetal pole cortex.

Injected Xlsirt RNA Possesses the Proper Information to Translocate to All of the Precloud Structures in Pre-Stage 1 Oocytes and to the METRO Region of a Single Cloud in Stage 1 Oocytes

To test for intrinsic differences between the pre-stage 1 precloud structures and the stage 1 METRO-containing clouds we injected digoxigenin-labeled Xlsirt and analyzed the oocytes by fixing them and performing a color reaction to detect the injected RNA. Alternatively, we also analyzed the translocation of the exogenous RNA by confocal laser microscopy in living oocytes. For this type of experiment we injected a mixture of fluoroscein-labeled Xlsirt and Texas red-labeled Xcat2 RNAs and detected each as a characteristic green color for fluoroscein-labeled Xlsirt or red color for Xcat2.

For the first experiment we injected a mixture of fluoroscein-labeled Xlsirt and Texas red-labeled Xcat2 into early diplotene pre-stage 1 oocytes and performed a time course. Figure 3a shows that immediately following injection Xlsirt and Xcat2 RNAs overlap within the cytoplasm exhibiting a yellow color characteristic of the overlapping of the two fluors. However, within 5 hr both RNAs had sorted from one another and were detected within discrete cytoplasmic particles (Fig. 3b). There were two important features of these particles: (1) the two different RNA particles had segregated from one another within the cytoplasm and (2) the morphology of the two types of particles was different. The Xlsirt formed large particles measuring approximately 0.4–1.2 μm, while the Xcat2 particles were uniformly smaller. This suggests that although both RNAs localize through a common pathway they may be associated with unique proteins resulting in different conformations.

Figure 4A shows that after 16–48 hr in living oocytes the injected exogenous Xlsirt and Xcat2 were localized within all of the precloud structures as were the endogenous Xcat2, Xlsirt, and Xwnt11 RNAs. At this level of resolution both RNAs appear in an overlapping pattern, although when passing through the different focal planes in the confocal we observe that the RNAs do occupy different positions within the METRO. This is consistent with our previous observations (Kloc and Etkin, 1995). Figures 5a and 5b also show that the injected Xlsirt RNA was detected within all of the precloud structures in fixed oocytes. In both living and fixed oocytes (Figs. 4A, 5a, and 5b), although the RNAs accumulate in all of the preclouds, it appears that the exogenous RNAs tended to accumulate to a greater extent in one of the clouds, perhaps foreshadowing the differentiation of the METRO-containing cloud. Control uninjected oocytes (Fig. 5c) or oocytes injected with a nonlocalized RNA such as Xnf7 (Reddy *et al.*, 1991) (data not shown) did not show any signal in the precloud structures.

When Xlsirt and Xcat2 RNAs were injected into stage 1 oocytes the exogenous RNAs were directed to the METRO region of only one of the clouds while the other clouds were devoid of detectable Xcat2 or Xlsirt RNA (Fig. 4B). This was also the case when Xlsirt was injected and the oocytes were fixed and analyzed by the color reaction (Fig. 5d). Injected Vg1 mRNA was not localized and was excluded from the METRO-containing cloud in stage 1 oocytes (Fig. 5e). However, consistent with previous observations of endogenous Vg1 (Kloc and Etkin, 1995), exogenous Vg1 injected into early stage 2 oocytes was localized to the vegetal cortex in a region overlapping the already localized Xlsirt (Fig. 6).

Translocation of the Injected Xlsirt RNA to the METRO Is Not Dependent upon Intact β-Tubulin-Containing Microtubules and Actin Microfilaments

Our previous results demonstrated the existence of a novel pathway in diplotene stage 1 oocytes through which Xlsirt, Xcat2, and Xwnt11 were localized to the vegetal cortex using the METRO region of the mitochondrial cloud. In addition, there is a second pathway through which Vg1 was localized to the vegetal cortex in stage 2–3 oocytes. Yisraeli *et al.* (1990) showed that the translocation of Vg1 to the vegetal cortex in stage 3 oocytes was dependent upon intact microtubules. We were interested in determining whether the transport of Xlsirt, Xwnt11, and Xcat2 RNAs to the METRO region of the mitochondrial cloud during stage 1 utilized a microtubule- and/or microfilament-dependent mechanism. Therefore, we treated stage 1 oocytes with nocodazole or cytochalasin B following the injection of Xlsirt RNA.

Figure 7a shows that in stage 1 oocytes injected with 100–500 pg of Xlsirt the RNA accumulated within the METRO region of the mitochondrial cloud. Treatment of injected oocytes with either nocodazole or cytochalasin B had no effect on the localization of these RNAs in the cloud (Figs. 7b and 7c). To control for the effectiveness of the nocodazole and cytochalasin B in treated oocytes, we analyzed for the presence of detectable microtubules by immunostaining with fluorosceinated antibodies against β-tubulin and analyzing the oocytes by laser confocal microscopy. To detect cytoskeletal actin microfilaments we used rhodamine-conjugated phalloidin. The concentration of nocodazole and cytochalasin B used in these experiments resulted in the depolymerization of the microtubules and actin microfilaments as shown by the loss of staining with the tubulin

antibodies and phalloidin in treated oocytes, thus indicating the effectiveness of this treatment (Fig. 8). These results strongly suggest that the transport of the exogenous Xlsirt to the METRO in stage 1 oocytes was independent of the presence of intact β-tubulin-containing microtubules and actin microfilament cytoskeletal networks.

DISCUSSION

The localization of RNAs during oogenesis is crucial to subsequent development of the embryo. Therefore, we investigated the origin of the early or METRO pathway responsible for the localization of RNAs during stage 1 of oogenesis. To analyze the origin of the METRO pathway we performed experiments using a variety of approaches. These included the analysis of the time and spatial distribution of endogenous RNAs and the fate of exogenous RNAs in both fixed and living oocytes. The results show that the morphogenesis of the early transport vehicle, or METRO, involves a series of steps beginning in the early diplotene stages of oogenesis.

The first step is the translocation of these RNAs from their site of synthesis in the GV to the mitochondrial aggregates that surround the GV. As they translocate to these precloud aggregates the RNAs are detected within the cytoplasm as discrete particles that are specific for at least two of the RNAs, Xlsirt and Xcat2. During this phase the RNAs accumulate within all of the precloud structures that surround the GV. The final phase involves the maturation and differentiation of the METRO during early stage 1, resulting in a single cloud being utilized for the transport of Xlsirt, Xcat2, and Xwnt11 to the vegetal cortex. The movement of the RNAs from their site of injection to the mitochondrial aggregate in stage 1 oocytes is independent of intact β-tubulin-containing microtubule or actin microfilament cytoskeletal elements. We believe that the accumulation of injected RNAs into the mature dominant cloud in stage 1 oocytes reflects the movement of the endogenous Xlsirt RNA from its site of synthesis in the GV to the METRO.

Reflective of the changes in the properties of the mitochondrial aggregates, injected Xlsirt and Xcat2 RNAs were targeted to all of the precloud structures in early diplotene pre-stage 1 oocytes; however, they were targeted to the METRO region of only one of the clouds in stage 1 oocytes. On the other hand, injected Vg1 mRNA remained in the cytoplasm in these oocytes and was excluded from all of the clouds in pre-stage 1 and in stage 1 oocytes. The question of how the RNAs target the clouds and the eventual designation of a single cloud in early diplotene oocytes is still unclear. One possibility is that the dominant cloud may inherit the remnants of the original centrosome which was located in the mitochondrial aggregate present in the early meiotic stage oocytes (Coggins, 1973; Tourte *et al.*, 1981; Klymkowski and Karnovsky, 1994). Additionally, one could propose that the dominant cloud acquires a specific gene product or organelle such as germinal granules during its maturation process (Fig. 9 for model).

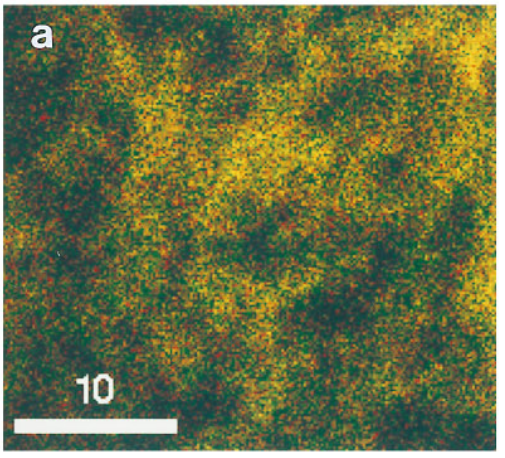
When Vg1 mRNA was injected into stage 2 oocytes, after endogenous Xlsirt, Xcat2, and Xwnt11 were localized as a disk at the vegetal pole, it associated with the vegetal cortex overlapping this disk. This suggests that the observation that endogenous Vg1 associates with the vegetal cortex overlapping the localized Xlsirt during stage 2 (Kloc and Etkin, 1995) also occurs with injected mRNA. We interpret these findings as indicating that the Vg1 mRNA localizes at the vegetal cortex following the pathway established by the cloud when it associates with the vegetal cortex (Kloc and Etkin, 1995). This is further supported by the observation that destruction of the Xlsirt RNA at the vegetal cortex releases the anchored Vg1 and suggests that there is an interaction between the components of these two pathways (Kloc and Etkin, 1994).

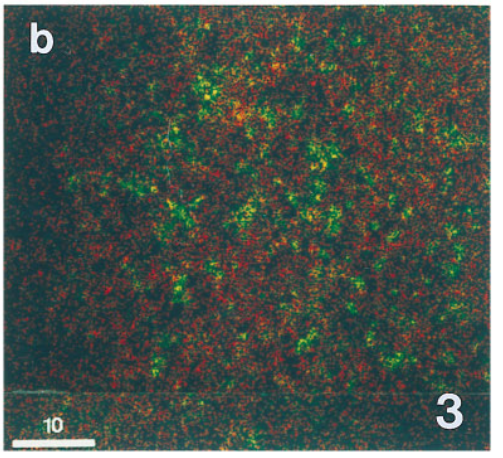
Translocation of Vg1 from the cytoplasm to the vegetal cortex during stage 3 is a two-step process involving a microtubule-dependent translocation step and a microfilament-dependent anchoring step (Yisraeli *et al.*, 1990). In *Drosophila,* microtubules were also implicated in the translocation of localized RNAs to the anterior and posterior pole (Pokrywka and Stephenson, 1991) and from the nurse

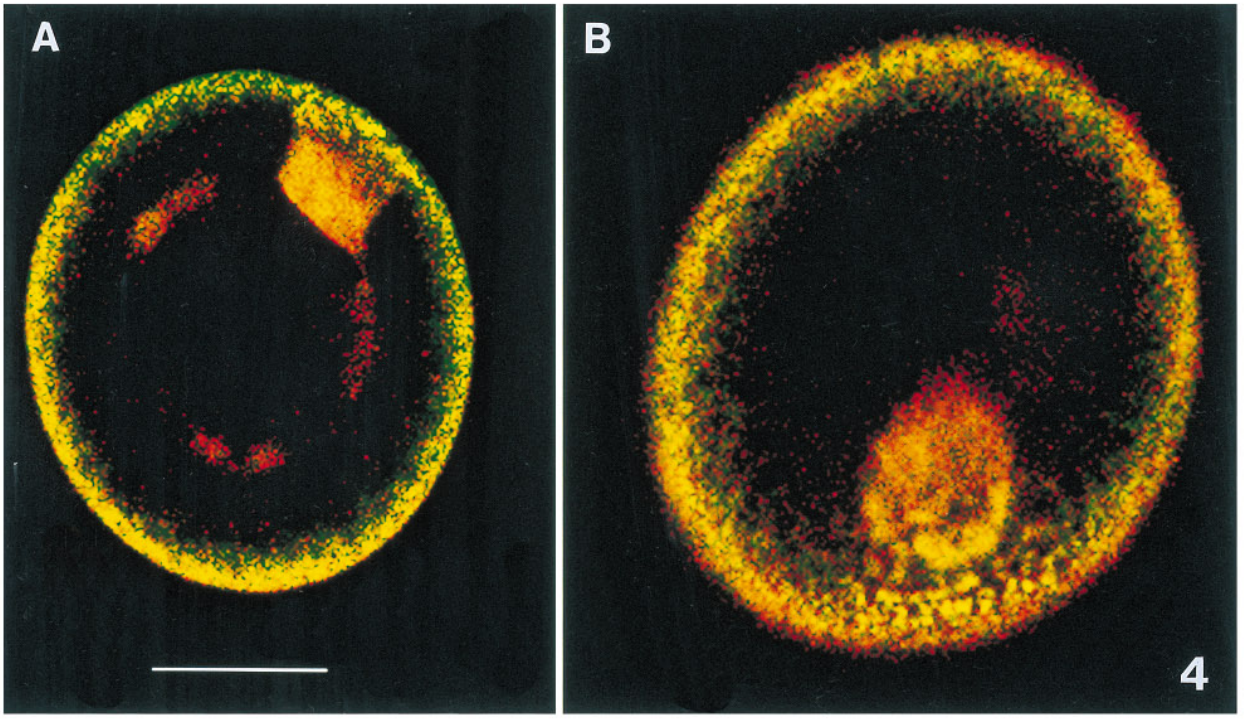
FIG. 3. Exogenous Xlsirt and Xcat2 form distinct cytoplasmic particles when translocating through the cytoplasm. (a) Confocal microscopy image of fluoroscein-labeled Xlsirt (appear green) and Texas red-labeled Xcat2 (appears red) in the cytoplasm within 1 hr following injection into stage 1 oocyte. The area of overlap between the two RNAs appears yellow. (b) Segregation of the two different RNAs into different-type particles 5 hr after injection into stage 1 oocytes.

FIG. 4. Confocal analysis of the localization of exogenous Xlsirt and Xcat2 RNAs within living oocytes shows the RNAs colocalizing in all clouds in pre-stage 1 oocytes but are restricted to a single cloud in stage 1 oocytes. Fluoroscein-labeled Xlsirt (green) and Texas red-labeled Xcat2 RNAs were co-injected into pre-stage 1 early diplotene oocytes (A) or into stage 1 diplotene oocytes (B) and examined after 16 hr for the localization of the RNAs.

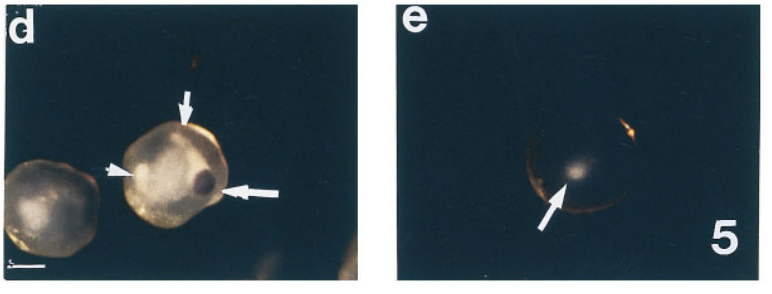
FIG. 5. Exogenous Xlsirt RNA translocates to all preclouds when injected into pre-stage 1 oocytes but accumulates in the METRO region of one cloud in stage 1 oocytes. (a) Pre-stage 1 oocytes that were injected with digoxigenin-labeled Xlsirt RNA and cultured for 2 days. The oocytes were stained and the injected Xlsirt RNA was detected within all of the precloud structures (arrowheads). (b) Another example of a pre-stage 1 oocyte injected with exogenous Xlsirt RNA. (c) Control oocytes that were not injected but went through the staining reaction. (d) Early stage 1 oocyte injected with Xlsirt RNA showing the distribution of the transcripts in the METRO region of one of the cloud. (e) Control early stage 1 oocyte injected with exogenous Vg1 RNA showing the distribution of Vg1 throughout the cytoplasm. The arrow points to the cloud where Vg1 was excluded. Bars represent 25 μm.

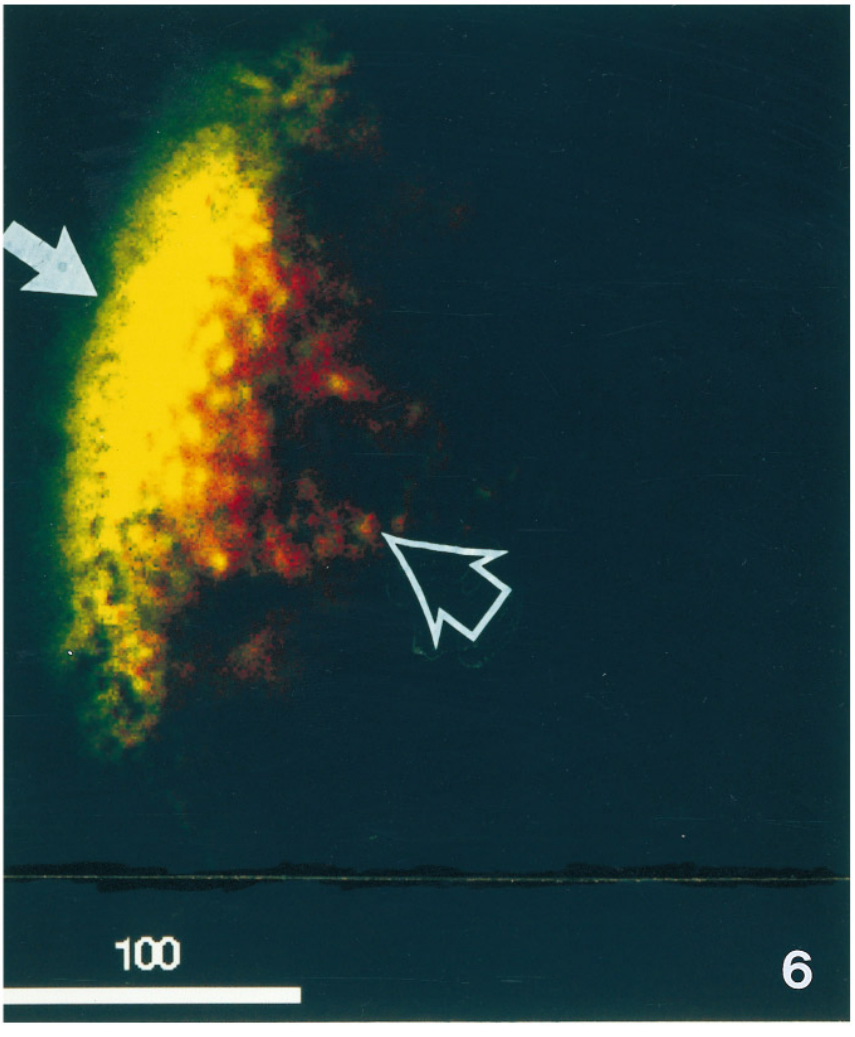

a
10


b
10
3


A
B
4


a
b
c


d
e
5



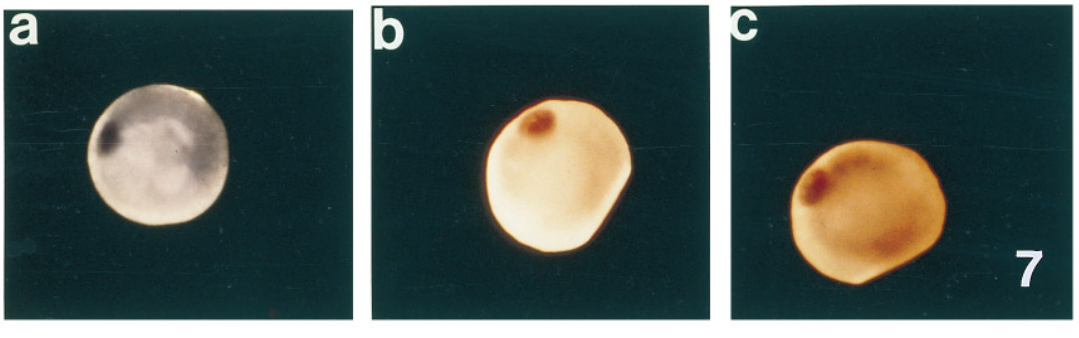


FIG. 6. Co-injected Vg1 and Xlsirt translocate to the vegetal cortex in living stage 2 oocytes. Texas red-labeled Vg1 (open arrow) and fluoroscein-labeled Xlsirt (filled arrow) were co-injected into stage 2 oocytes. The oocytes were cultured for 36 hr and visualized by confocal microscopy. The Xlsirt RNA (green) has already reached the cortex while the Vg1 RNA (red) is localizing at the cortex in the region overlapping the localized Xlsirt (yellow).

FIG. 7. The effect of nocodazole treatment on the localization of injected Xlsirt in the METRO. Oocytes were injected with Xlsirt RNA and cultured for 48 hr (a), in the presence of 5 μg/ml nocodazole (b) or in the presence of 25 μg/ml cytochalasin B (c). The RNA was visualized using the color reaction with antidigoxigenin alkaline phosphatase.

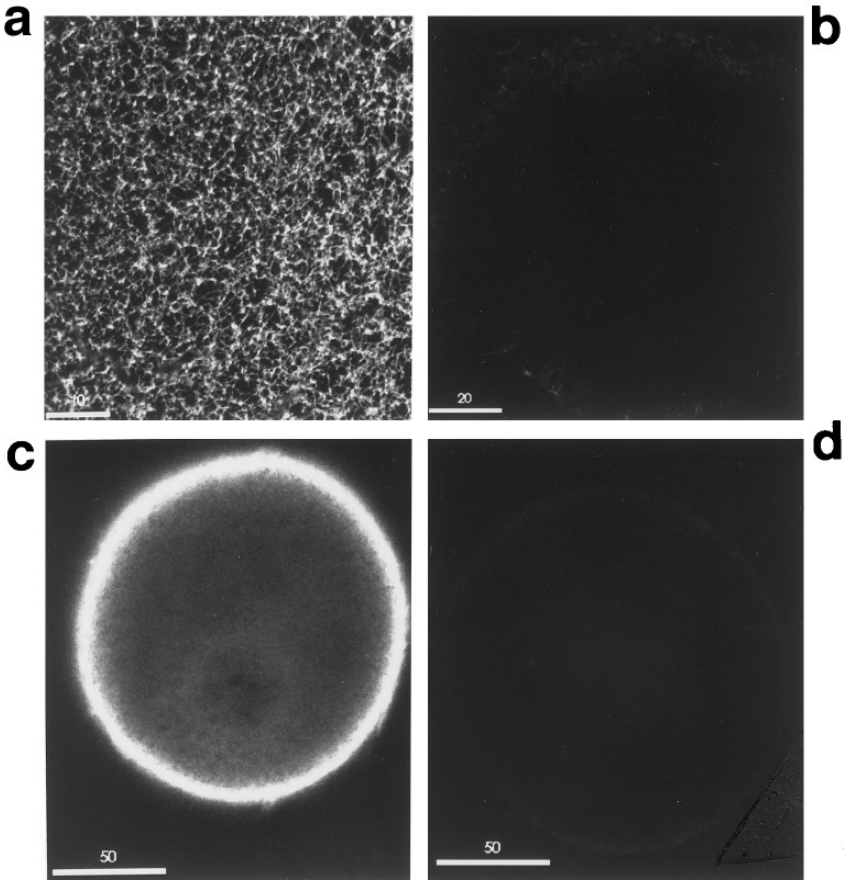


FIG. 8. Effectiveness of nocodazole and cytochalasin B treatment on the integrity of microtubules and microfilaments. Stage 3 oocytes were treated with either nocodazole or cytochalasin B as above and were analyzed for the effects on microtubules (b) or actin microfilaments (d). (a and c) Control untreated oocytes showing the presence of intact microtubules and actin microfilaments, respectively.

cells to the proocyte (Theurkauf, 1994). In the present study we demonstrated that the translocation of Xlsirt to the METRO region of the mitochondrial cloud is not dependent upon intact β-tubulin-containing microtubules or actin microfilaments. This raises the question of how Xlsirt, Xcat2, and Xwnt11 accumulate within the METRO.

There are several potential mechanisms by which these RNAs could become localized within oocytes and eggs (St. Johnston, 1996). One is the differential stability of RNA in different regions of the oocyte. This mechanism may account for the accumulation of hsp83 mRNA at the posterior pole of *Drosophila* (Ding *et al.*, 1993). A second mechanism involves the directed transport along cytoskeletal elements such as microtubules. This is used by Vg1 in *Xenopus* and a variety of RNAs in *Drosophila*. Another mechanism is vectorial transport from one region of the nucleus directly toward a cytoplasmic target (Davis and Ish-Horowicz, 1991; Davis *et al.*, 1993). A fourth method is the anchoring of the RNAs to localized binding sites with subsequent loss of the RNA in other regions of the oocyte (Raff *et al.*, 1990; Jongens *et al.*, 1992). Our data indicate that Xlsirt RNAs do not require intact β-tubulin-containing microtubules or actin microfilaments to accumulate in the METRO region of the cloud. Therefore, either cytoskeletal elements other than β-tubulin-containing microtubules or actin microfilaments are used or one of the other mechanisms is responsible for Xlsirt localization. Other cytoskeletal elements could include γ tubulin or intermediate filament networks. Further analyses will be necessary to answer this question.

Interestingly, at the same time that Xlsirt, Xcat2, and Xwnt11 are accumulating in the METRO region of the cloud, Vg1 is dispersed throughout the cytoplasm. It is unclear at the present time why Vg1 does not localize during this early time period. Two alternatives are that Vg1 localization may require the establishment of the proper microtubule pathway to permit its translocation or that it may be anchored within the cytoplasm and require a specific signal to release it. Kloc and Etkin (1995) proposed that the movement of the mitochondrial cloud containing the Xlsirt, Xcat2, and Xwnt11 RNAs to the vegetal cortex during late stage 1 resulted in the establishment of this cytoskeletal pathway which is subsequently used by Vg1 to localize at

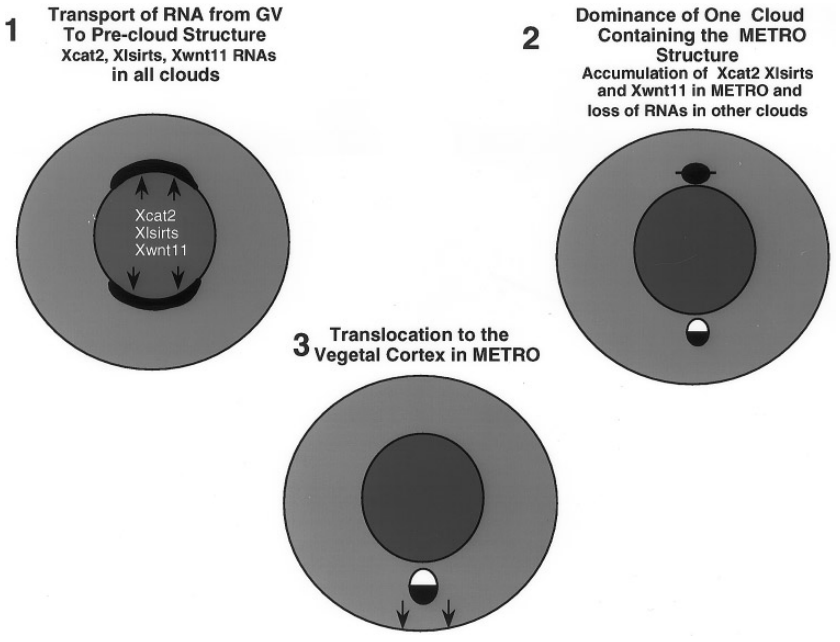


FIG. 9. The organization of the early or METRO pathway in early diplotene oogenesis. In early diplotene oocytes, mitochondrial aggregates (preclouds) surround the GV. Xcat2, Xlsirt, and Xwnt11 RNAs accumulate indiscriminately within all of the precloud structures (1). During the period from pre-stage 1 to stage 1 there is a change in the intrinsic property of one of the clouds that designates it as the messenger transport organizer-containing cloud. Thus, the second step involves the accumulation of RNAs within the METRO structure of one of the clouds. Xcat2, Xwnt11, and Xlsirt transcripts are lost in all of the other clouds. The third step is the transport of the RNAs to the vegetal cortex in the METRO containing cloud. Exogenous Xlsirt RNA injected into pre-stage 1 oocytes was targeted to all of the clouds; however, when injected into older oocytes they localize to the METRO-containing cloud. This suggests that there are changes that occur within the clouds that produce a mature transport vehicle into which the RNAs accumulate. Additionally, it also indicates that the METRO RNAs also possess a *cis*-acting signal directing them to the proper subcellular compartment within the METRO.

the cortex. Alternatively, it is possible that both mechanisms may operate in Vg1 localization. Preliminary data show that a chimeric Vg1 RNA containing the Xlsirt repeat localization signal will associate with the mitochondrial cloud. This result suggests that Vg1 is not anchored in the cytoplasm and its localization may await the establishment of the late pathway (Kloc and Etkin, unpublished observations).

The results of this study demonstrate that the translocation of RNAs through the METRO or early pathway involves several steps which, unlike the translocation of Vg1 in the late pathway, are not dependent upon β-tubulin-containing microtubules or actin microfilament cytoskeletal elements. The first step is the assembly of the individual RNAs into distinct subcellular particles which are transported from their site of synthesis to all of the precloud structures in pre-stage 1 oocytes. The second stage occurs during stage 1. At this time there are changes in the intrinsic properties of the clouds resulting in the formation of a single dominant cloud resulting in a maturation and refinement of the RNA transport machinery. These results demonstrate that the localization of RNAs within the same cell type is a complex process that may involve several different mechanisms functioning in a well-defined temporal order.

ACKNOWLEDGMENTS

We thank the members of the Etkin laboratory for helpful comments and suggestions throughout the course of this work. This work was supported by a grant from the National Institutes of Health.

REFERENCES

Al-Mukhtar, K., and Webb, A. (1971). An ultrastructural study of primordial germ cells, oogonia and early oocytes in *Xenopus laevis. J. Expt. Embryl. Morph.* **26,** 195–217.

Coggins, L. (1973). An ultrastructural and autoradiographic study of early oogenesis in the toad, *Xenopus laevis. J. Cell Sci.* **12,** 71–93.

Davis, I., and Ish-Horowicz, D. (1991). Apical localization of pair-rule transcripts requires 3′ sequences and limits protein diffusion in the *Drosophila* blastoderm embryo. *Cell* **67,** 927–940.

Davis, I., Francis-Lang, H., and Ish-Horowicz, D. (1993). Mechanisms of intracellular transcript localization and export in early *Drosophila* embryos. *Cold Spring Harbor Symp.* **58,** 793–798.

Ding, D., Parkhurst, S., Halsells, S., and Lipshitz, H. (1993). Dynamic hsp83 RNA localization during *Drosophila* oogenesis and embryogenesis. *Mol. Cell. Biol.* **13,** 3773–3781.

Dumont, J. (1972). Oogenesis in *Xenopus laevis* (Daudin): Stages of oocytes in laboratory maintained animals. *J. Morphol.* **136,** 153–180.

Forristall, C., Pondel, M., Chen, L., and King, M. L. (1995). Patterns of localization and cytoskeletal association of two vegetally localized RNAs Vg1 and Xcat2. *Development* **121,** 201–208.

Gerhart, J., Danilchik, M., Doniach, T., Roberts, S., Browning, B., and Stewart, R. (1989). Cortical rotation of the *Xenopus* egg: Consequences for anterioposterior patterning of embryonic dorsal development. *Development* **Suppl.,** 37–51.

Gurdon, J. B. (1992). The generation of diversity and pattern in animal development. *Cell* **68,** 185–199.

Harland, R. (1991). *In situ* hybridization: An improved whole mount method. *In* ''Methods in Cell Biology'' (H. B. Peng and B. Kay, Eds.), Vol. 36, pp. 685–695. Academic Press, San Diego.

Hausen, P., and Riebesell, M. (1991). ''The Early Development of *Xenopus laevis:* An Atlas of Histology.'' Springer-Verlag, New York.

Heasman, J., Quarmby, J., and Wylie, C. C. (1984). The mitochondrial cloud of *Xenopus* oocytes: The source of germinal granule material. *Dev. Biol.* **105,** 458.

Jeffery, W. (1988). The role of cytoplasmic determinants in embryonic development. *In* ''Developmental Biology: A Comprehensive Synthesis'' (L. Browder, Ed.), Vol. 5, pp. 3–56. Academic Press, San Diego.

Jongens, T., Hay, B., Jan, L. Y., and Jan, Y. N. (1992). The germ cell-less gene product: A posteriorly localized component necessary for germ cell development. *Cell* **70,** 569–584.

Kloc, M., and Etkin, L. (1994). Delocalization of Vg1 mRNA from the vegetal cortex in *Xenopus* oocytes after destruction of Xlsirt RNA. *Science* **265,** 1101–1103.

Kloc, M., and Etkin, L. (1995). Two distinct pathways for the localization of RNAs at the vegetal cortex in *Xenopus* oocytes. *Development* **121,** 287–297.

Kloc, M., Miller, M., Carrasco, A., Eastman, E., and Etkin, L. (1989). The maternal store of xlgv7 mRNA in full grown oocytes is not required for normal development in *Xenopus. Development* **107,** 899.

Kloc, M., Reddy, B., Miller, M., Eastman, E., and Etkin, L. (1991). Xl21: A localized maternal transcript in *Xenopus laevis. Mol. Rep. Dev.* **28,** 341–345.

Kloc, M., Spohr, G., and Etkin, L. (1993). Translocation of repetitive RNA sequences with the germ plasm in *Xenopus* oocytes. *Science* **262,** 1712–1714.

Klymkowski, M., and Karnovsky, A. (1994). Morphogenesis and cytoskeleton: Studies of the *Xenopus* embryo. *Dev. Biol.* **165,** 372–384.

Ku, M., and Melton, D. (1993). Xwnt11, a maternally expressed *Xenopus* wnt gene. *Development* **119,** 1161–1173.

Melton, D. A. (1987). Translocation of a localized maternal mRNA to the vegetal pole of *Xenopus* oocytes. *Nature* **328,** 80–83.

Melton, D. A. (1991). Pattern formation during early development. *Science* **252,** 234–241.

Mosquera, L., Forristall, C., Zhou, Y., and King, M. L. (1993). A mRNA localized to the vegetal cortex of *Xenopus* oocytes encodes a protein with a nanos-like zinc finger domain. *Development* **117,** 377.

Pokrywka, N., and Stephenson, E. C. (1991). Microtubules mediate the localization of bicoid mRNA during *Drosophila* oogenesis. *Development* **113,** 55–66.

Rebagliati, M. R., Weeks, D. L., Harvey, R. P., and Melton, D. A. (1985). Identification and cloning of localized maternal mRNA from *Xenopus* eggs. *Cell* **42,** 769–777.

Reddy, B., Kloc, M., and Etkin, L. (1991). The cloning and characterization of maternally expressed novel zinc finger nuclear phosphoprotein (xnf7) in *Xenopus laevis. Dev. Biol.* **148,** 107–116.

Reddy, B., Kloc, M., and Etkin, L. (1992). The cloning and characterization of a localized maternal transcript in *Xenopus laevis* whose zygotic counterpart is detected in the CNS. *Mech. Dev.* **39,** 143–150.

Raff, J., Whitfield, W., and Glover, D. (1990). Two distinct mechanisms localize cyclin B transcripts in syncytial *Drosophila* embryos. *Development* **110,** 1249–1261.

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

Theurkauf, W. (1994). Microtubules and cytoplasm organization during *Drosophila* oogenesis. *Dev. Biol.* **165,** 352–360.

Tourte, M., Mignotte, F., and Mounolou, J-C. (1981). Organization and replication activity of the mitochondrial mass of oogonia and previtellogenic oocytes in *Xenopus laevis. Dev. Growth Differ.* **23,** 9–21.

Wallace, R., and Misulovin, Z. (1978). Long-term growth and differentiation of *Xenopus* oocytes in a defined medium. *Proc. Natl. Acad. Sci. USA* **75,** 5534–5538.

Wallace, R., Misulovin, Z., and Etkin, L. D. (1980). Full grown oocytes from *Xenopus laevis* resume growth when placed in culture. *Proc. Natl. Acad. Sci. USA* **78,** 3078–3082.

Weeks, D. L., and Melton, D. A. (1987). A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGF-B. *Cell* **51,** 861–867.

Yisraeli, J. K., and Melton, D. A. (1988). The maternal RNA Vg1 is correctly localized following injection into *Xenopus* oocytes. *Nature* **336,** 592–595.

Yisraeli, J. K., Sokol, S., and Melton, D. A. (1990). A two step model for the localization of maternal mRNA in *Xenopus* oocytes: Involvement of microtubules and microfilaments in translocation and anchoring of Vg 1 mRNA. *Development* **18,** 289–298.

Received for publication June 4, 1996
Accepted August 23, 1996