Two distinct pathways for the localization of RNAs at the vegetal cortex in *Xenopus* oocytes

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**SUMMARY**

We found that there are two major pathways by which RNAs are localized at the vegetal cortex during oogenesis of *Xenopus laevis*. One of these, through which Xslsirts, Xcat2 and Xwnt11 are localized, involves transport during stages 1 and 2 of oogenesis via a region of the mitochondrial cloud that we call the message transport organizer or METRO. This pathway involved three steps, transport of RNA from the GV to the mitochondrial cloud, sorting of the RNAs to specific regions of the METRO, and translocation to and anchoring at the vegetal cortex. These three RNAs exhibit a distinct pattern of spatial localization within the METRO when they approach the vegetal cortex. The other pathway is used by Vg1. We detected Vg1 throughout the oocyte cytoplasm during stages 1 and 2. During stage 3 it was translocated to the vegetal cortex and associated with the cortex overlapping the region at which the Xslsirt, Xcat2, and Xwnt11 RNAs are anchored. Our results also showed that anchoring of these RNAs was dependent in part on actin microfilaments, but was independent of microtubules. These results demonstrate a novel mechanism of translocation and RNA sorting used by RNAs several of which may be involved in the establishment of the embryonic body axis.

Key words: axis formation, RNA localization, vegetal cortex, *Xenopus laevis*, oogenesis, oocyte, METRO

**INTRODUCTION**

Normal embryonic development requires the proper temporal and spatial patterns of expression and localization of maternal transcripts. Therefore, it is important to identify the localized gene products that play a critical role in early development and to define the mechanisms responsible for their localization in the oocyte. In *Drosophila*, the anterior-posterior body axis is determined by localized maternal RNAs such as bicoid (St. Johnston et al., 1989) at the anterior pole and nanos (Wang and Lehman, 1991) at the posterior pole of the oocyte. The oskar mRNA localized at the posterior pole, likewise, plays a crucial role in the organization of the germ plasm in this region (Kim-Ha et al., 1993; Ephrussi et al., 1991; Ephrussi and Lehman, 1992).

Analysis of the molecular anatomy of the radially symmetrical *Xenopus* oocyte reveals populations of maternal RNAs that are localized in the animal and vegetal regions (Melton, 1991). Indeed, RNAs such as Vg1 and Xwnt11 (Weeks and Melton, 1987; Ku and Melton, 1993) localized at the vegetal cortex may function in the patterning of the early embryo as demonstrated by their ability to induce secondary axes or correct axial deficiencies when synthetic Xwnt11 and Vg1 mRNAs were injected into the fertilized eggs (Ku and Melton, 1993; Thomsen and Melton, 1993). Another mRNA, Xcat2, which encodes a nanos-like molecule that may be involved in axial patterning of the embryo, was also reported localized at the vegetal cortex (Mosquero et al., 1993). Hence, it is clear that localized RNAs play an important role in determining the future dorsal-ventral (D/V) and anterior-posterior (A/P) axes of the *Xenopus* embryo.

Both oocyte cytoskeletal elements and *cis*-acting sequences on the RNA are involved in the localization of transcripts. Yisraeli et al. (1990) demonstrated that the localization of Vg1 is a two-step process dependent on microtubules for translocation to the vegetal pole and on actin microfilaments for anchoring to the cortex. Pondel and King (1988) also found cytokeratin filament involvement with movement and anchoring of Vg1, although Klymkowsky et al. (1991) showed that only a fraction of Vg1 was anchored by this component of the cytoskeleton. Several vegetally localized mRNAs, including Xcat2, were isolated on the basis of their association with the intermediate filament fraction of stage 6 oocytes, further supporting the role of cytoskeletal elements in their localization. Cytoskeletal elements also are involved in the localization of RNA in *Drosophila* since drugs that affect microtubule polymerization abolish or alter the localization of bicoid mRNA in cultured egg chambers (Pokrywka and Stephenson, 1991; Wang and Hazelrigg et al., 1994)). Thus it appears that, in both *Drosophila* and *Xenopus*, cytoskeletal elements are important in RNA localization during oogenesis.

Mowry and Melton (1992) showed that a *cis*-acting 340-nt region in the 3′ untranslated region of Vg1 mRNA is required for localization. Several *Drosophila* mRNAs such as bicoid...
(Macdonald and Struhl, 1988), nanos (Gavis and Lehman, 1992), orb (Lantz and Schedl, 1994) and tutor (Golumbeski et al., 1991) contain signals in the 3′ untranslated regions that are required for proper spatial distribution during oogenesis. Recently, a 30-nt region of the bicoid 3′ untranslated sequence called the bicoid localizing element (BLE) was identified that is required for proper localization of this RNA (Macdonald et al., 1993). Because there are no apparent overall sequence similarities between the 3′ domains of different localized RNAs, the secondary structure is probably important in their function (Macdonald, 1990; Macdonald et al., 1993). It is likely that there are similar mechanisms utilized in the localization of RNAs in Xenopus and Drosophila oocytes and that their localization involves both cis-acting elements within the transcripts and cytoskeletal elements present in the cells.

In addition to Vg1, Xcat2, and Xwnt11, we reported the presence of a nontranslatable short interspersed repeat sequence transcript (Xlsirt) that was also localized at the vegetal cortex of Xenopus stage 3 oocytes (Kloc et al., 1993). Xlsirts were translocated to the vegetal cortex through a distinct pathway involving the mitochondrial cloud (Balbiani body). Recently, it was demonstrated that Xcat2 also is detected within the mitochondrial cloud (Forristall et al., 1994). We showed that the translocation of Xlsirts was dependent upon the presence of a cis-acting tandemly repeated 79 nt sequence contained within each Xlsirt RNA. The Xlsirt RNAs may play a structural role in the vegetal cortex since destruction of these RNAs by injection of antisense oligodeoxynucleotides (ODN) (Kloc and Etkin, 1994) resulted in the release of localized Vg1 mRNA but not Xcat2 into the cytoplasm. We suggested that the nontranslatable Xlsirt RNAs are an integral part of the network of cytoskeletal elements anchoring Vg1.

In the present study, we examined the precise spatial patterns of distribution of several RNAs localized at the vegetal cortex of Xenopus oocytes. We found that Xlsirts, Xcat2 and Xwnt11 localized as a discrete disk-like structure at the apex of the vegetal pole cortex, while Vg1 was distributed throughout the entire vegetal cortex from the apex of the vegetal pole to the future marginal zone. During stage 1 (Dumont, 1972) of oogenesis, Xlsirts, Xcat2 and Xwnt11 RNAs accumulated in distinct patterns within a region of the mitochondrial cloud and were translocated to the cortex within this structure, while Vg1 was excluded from the mitochondrial cloud and translocated to the vegetal cortex via a different pathway. We suggest that a region of the mitochondrial cloud serves as a messenger transport organizing center (METRO) that facilitates the translocation of a population of RNAs to the vegetal cortex. We also suggest that the Xlsirt-containing disk-like structure at the apex of the vegetal pole cortex serves as a center to which the translocating Vg1 transcripts are directed, after which they slide along the inner cortical layer and anchor at their final position. Association of the RNAs with the mitochondrial cloud was not sensitive to cytochalasin B or nocodazole and the RNAs were anchored at the cortex in a sequential hierarchical manner. The proper anchoring of all of these RNAs at the vegetal cortex was partially dependent upon actin microfilaments, while the integrity of the inner cortical layer that contains most of the Vg1 mRNA in stage 3 oocytes was dependent upon microtubules.

**MATERIALS AND METHODS**

**Animals and oocytes**

Oocytes were surgically removed from anesthetized wild-type and albino Xenopus laevis frogs and stored in 1× Barth solution. Oocytes were either defolliculated manually or pieces of ovary were treated with collagenase for 1 hour at 27°C [1 mg/ml of collagenase (Sigma)] in calcium-deficient buffer (1× OR). After extensive washing in 1× Barth oocytes were fixed in MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM MgSO₄, 3.7% formaldehyde) for 1-2 hours on a rotating platform and transferred to 100% methanol and stored at −20°C.

**Probes for in situ hybridization**

Complementary RNA probes were synthesized from linearized plasmid templates in the presence of digoxigenin-11-UTP or fluorescein-12-UTP (both from BMB). Xlsirt clone R-U (Kloc et al., 1993) containing three repeats was linearized with EcoRI and the RNA was synthesized using T3 RNA polymerase. pSPORT-Xcat2 plasmid containing 789 bp of coding sequence from Xcat2 (Mosquero et al., 1993) was linearized with SalI, and RNA was synthesized using Sp6 RNA polymerase. Xenopus Xwnt11 was a gift from Kim Mowry, Brown University. Plasmid pSP73/Xl-849 containing 849 bp of coding region of Xwnt11 was linearized with EcoRI, and RNA synthesized using T7 RNA polymerase. The size of transcripts was reduced by 40 mM sodium bicarbonate and 60 mM sodium carbonate hydrolysis at 60°C for 30 minutes. RNA was precipitated with 4 M LiCl and ethanol and resuspended in hybridization buffer.

**Whole-mount in situ hybridization**

All procedures were done in 6-well and 24-well culture plates using 50 to 100 oocytes per well with shaking on a rotating platform. Whole-mount in situ hybridization was done according to Harland (1991). In brief, fixed oocytes stored in 100% methanol were rehydrated with 1× PBS with 0.1% Tween-20, treated with 7 minutes in proteinase K (10 µg/ml), and postfixed in 4% paraformaldehyde for 20 minutes. After extensive washing in PBS with Tween-20, oocytes were transferred from 6-well to 24-well plates, and prehybridized at 50°C in 2 ml of hybridization buffer (50% formamide, 1× Denhardt’s, 1 mg/ml of yeast RNA, 0.1% CHAPS, 5× SSC, heparin at 100 µg/ml, 5 mM EDTA, 0.1% Tween-20).

After 10 hours, the prehybridization buffer was replaced with 0.5 ml of fresh buffer containing 1 µg/ml of digoxigenin-labeled probe, plates were placed in ziplock bags and oocytes were hybridized overnight at 50°C. The next day oocytes were washed in 2× SSC with 0.3% CHAPS, treated with RNAse A (20 µg/ml) at 37°C for 30 minutes, and washed extensively in PBS-Tween 20.

The detection procedure and reagents were from a Genius 3 kit (Boehringer). Oocytes were incubated in alkaline-phosphatase-conjugated anti-digoxigenin antibody (1:5000 dilution) at 4°C overnight on a rotating platform. After extensive washing in Genius buffer 1, oocytes were incubated in color substrate solution according to the Genius 3 protocol. The color reaction was developed for 1 hour in the dark and oocytes were postfixed in MEMFA overnight, and stored in 100% methanol.

**Double whole-mount in situ hybridization**

All procedures were done as above with the exception that a mixture of two differently labeled RNA probes was added to the hybridization solution. One probe was digoxigenin-labeled RNA and the second was RNA labeled with fluorescein-UTP (BMB). Each probe was added at a concentration of 1 µg/ml. Sequential detection with alkaline phosphatase- or horseradish peroxidase-conjugated anti-digoxigenin and anti-fluorescein antibodies were done according to Jowett and...
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Lettice (1994). In sequential detection, oocytes were incubated first with 1:2000 dilution of sheep anti-fluorescein™ alkaline phosphatase antibody at 4°C overnight and, after extensive washing in PBS-Tween 20, were stained using Vector Red kit (Vector Laboratories). After development of the color, the reaction was stopped by fixing in 4% paraformaldehyde. Alkaline phosphatase was inactivated by heating for 30 minutes at 65°C. After being washed and blocked, oocytes were incubated in second antibody (anti-digoxigenin-alkaline phosphatase antibody, 1:5000 dilution or anti-digoxigenin-horseradish peroxidase, 1:2000 dilution) at 4°C, overnight. After extensive washing, second color reaction was done using Vector Black kit or Vector SG kit (Vector Laboratories), respectively. The reaction was stopped by fixing in MEMFA overnight and oocytes were stored in methanol.

Whole-mount sections

After whole-mount in situ hybridization and color detection, oocytes were fixed in MEMFA, washed 2× in 100% methanol (1 hour each), 2× in 100% ethanol (1 hour each), cleared in HistoClear II (National Diagnostic) and embedded in paraplast. 10 µm sections were deparaffinated in HistoClear and mounted in Permount.

Inhibitors

Collagenase-treated or manually defolliculated oocytes were incubated in vitellogenin supplemented medium (Wallace et al., 1980) for 16 to 72 hours in the presence of cytoskeletal inhibitors: Cytochalasin B (Sigma) (25 µg/ml) or nocodazole (Sigma) (1-4 µg/ml). Both inhibitors were dissolved in DMSO.

RESULTS

Vg1 is distributed differently at the vegetal cortex than either Xlsirts, Xcat2, or Xwnt11 in stage 4 oocytes

Previously published reports indicated that Xcat2 (Mosquera et al., 1993), Xlsirts (Kloc et al., 1993), Xwnt11 (Ku and Melton, 1993) and Vg1 (Rabegliati et al., 1985; Weeks and Melton, 1987; Melton, 1987; Yisraeli and Melton, 1988) were all localized at the vegetal cortex in stage 4-6 oocytes. These analyses were all performed by in situ hybridization of sectioned oocytes. To gain a broader perspective of the spatial distribution of these RNAs, we reinvestigated their localization in stage 4 oocytes by which time they were anchored at the cortex using whole-mount in situ hybridization. We found that the pattern of hybridization to Vg1 mRNA was to the cortex throughout the entire vegetal hemisphere from the apex of the vegetal pole to the future marginal zone of the embryo (Fig. 1A). In contrast, Xlsirts, Xcat2 and Xwnt11 exhibited a much more limited distribution than Vg1 and were all localized as a disk at the apex of the vegetal pole region (Fig. 1B-D). Thus, it appears that there are two basic patterns of spatial distribution of transcripts at the vegetal cortex: the Vg1-like and Xlsirt-like patterns.

The movement of Xlsirts and Vg1 define two major pathways for RNA translocation to the vegetal cortex

Kloc et al. (1993) and Kloc and Etkin (1994) reported that Xlsirts were translocated to the vegetal cortex via the mitochondrial cloud prior to the localization of Vg1 and coincident with the localization of Xcat2. We examined the distribution of Xlsirts, Xcat2, Xwnt11 and Vg1 RNAs during their translocation to the oocyte vegetal cortex by whole-mount in situ hybridization.

Fig. 1. Localization of Vg1, Xlsirts, Xcat2 and Xwnt11 at the vegetal cortex in stage 4 oocytes. Stage 4 oocytes were analyzed by whole-mount in situ hybridization for the presence of Vg1, Xlsirts, Xcat2 and Xwnt11 RNAs according to the procedures described in methods. (A) Oocyte hybridized with the Vg1 probe; (B) oocyte hybridized with the Xlsirt probe; (C) oocyte hybridized with the Xcat2 probe; (D) oocyte hybridized with the Xwnt11 probe. The bar in D represents 200 µm.
In stage 1 oocytes, Xlsirts, Xcat2 and Xwnt11 RNAs were localized in the mitochondrial cloud, which was located adjacent to the GV (Fig. 2A, panels a,d,g). All of the RNAs were detected in a specific region of the cloud that faced toward one side of the oocyte. Based on the presence of the cloud at the vegetal pole region of later stage oocytes, we assume that the localized RNAs are on the side of the cloud facing the future vegetal pole. This pattern of localization was seen in oocytes ranging in size from 90 to 230 µm in diameter, which correlates with early to mid-stage 1 (Dumont, 1972). The distribution of the transcripts within the cloud can be clearly seen in sections of oocytes (Fig. 2B, panels c,e,i). The arrows point to the mitochondrial cloud. GV, germinal vesicle. The bars represent 100 µm in g and 125 µm in i.

In larger oocytes (size range 230-300 µm, equivalent to Dumont mid to late stage 1), Xlsirt, Xcat2 and Xwnt11 RNAs showed distinct patterns of distribution within the cloud (Fig. 2A, panels b,e,h). At this time, instead of being oval in shape, the cloud appears as a flattened pancake-like structure that contains groups of fragmented islands of mitochondria and electron-dense material (Heasman et al. 1984). When viewed from the vegetal pole region (Fig. 2A, panels b,e,h) Xlsirt RNA was detected as a ring around the outside and Xwnt11 mRNAs were localized within the center of the cloud. These patterns of RNA distribution can be clearly seen in sections of these larger oocytes (Fig. 2B, panels b,d,f). The bars represent 100 µm in b, d and f.
Two pathways for vegetal localization were observed in hundreds of oocytes in ovaries from different females. In oocytes of the size range 275-350 μm (Dumont stage 2), when the mitochondrial cloud was near the vegetal cortex and vitellogenesis began, all three of these RNAs tended to overlap to some extent (Fig. 2A, panels c,f,i). They were anchored at the cortex by stage 3 as seen in Fig. 2B, panels b,d,f. Since several RNAs are localized to this organelle as they translocate to the vegetal cortex, we refer to this region of the cloud as the messenger transport organizer (METRO). It should be noted that there is some variation in oocyte size and mitochondrial cloud morphology between oocytes from different frogs resulting in the overlapping size ranges above.

In late stage 1 to early stage 2 oocytes, Vg1 was distributed throughout the cytoplasm and was conspicuously absent from the mitochondrial cloud (Fig. 3A, panel a; Fig. 3B, panel a). During late stage 2 to early stage 3, after the localization of Xlsirt, Xcat2 and Xwnt11, the Vg1 RNA was detected in the cortex at the apex of the vegetal pole, overlapping with the localized Xlsirt, Xcat2 and Xwnt11 RNAs (Fig. 3A, panel b). In sections of these oocytes, Vg1 transcripts appeared to be channeled toward the apex of the vegetal cortex to the position...
Fig. 3. (A) Localization of Vg1 mRNA during oogenesis. Different stage *Xenopus* oocytes were analyzed by whole-mount in situ hybridization using the Vg1 probe. (a) Stage 1 oocyte. The arrow points to the mitochondrial cloud; (b) Stage 2 oocyte. The arrow points to the position of the translocating Vg1 RNA that is accumulating over the localized Xlsirts, Xcat2, and Xwnt11 RNAs at the vegetal cortex. (c) Stage 4 oocyte showing the distribution of the Vg1 RNA from the apex of the vegetal pole region to the future marginal zone. A, animal pole, V, vegetal pole. Bars represent 100 µm.

(B) Sections showing the localization of Vg1 RNA in different stage oocytes. (a) Section through a late stage 1 oocyte showing the distribution of Vg1 RNA throughout the entire cytoplasm. (b) Section through stage 2 oocyte showing the accumulation of Vg1 RNA at the vegetal cortex at the position of the METRO localized RNAs. The arrows show the apparent movement of the Vg1 mRNA along the cortical layer towards the future marginal zone. (c) Section through a stage 3 oocyte showing the continued streaming of the Vg1 mRNA toward the vegetal cortex.
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of the METRO-localized RNAs (Fig. 3B, panel b). In sections of mid-stage 3 oocytes, the Vg1 RNA was detected as cords within the cytoplasm connecting with the cortex (Fig. 3B, panel c). By stage 4, Vg1 reached its final position extending uniformly from the apex of the vegetal pole to the future marginal zone (Fig. 3A, panel c).

**Xlsirts, Xcat2 and Xwnt11 form overlapping layers when anchored at the vegetal cortex**

Fig. 4 shows oocytes hybridized with combinations of two probes for Xlsirts and Xcat2; Xlsirts and Xwnt11; and Xlsirts and Vg1. When the mitochondrial cloud was located adjacent to the vegetal cortex in late stage 1 or early stage 2 oocytes, we detected Xcat2 transcripts tightly opposed to the cortex while the Xlsirt RNA was layered above (Fig. 4A). In slightly older oocytes co-hybridized with Xlsirts and Xwnt probes, we found the Xlsirts more closely opposed to the cortex than was the Xwnt11 RNA (Fig. 4B). In late stage 3 oocytes co-hybridized with Xlsirts and Vg1 probes, the Vg1 transcripts were associated with the cortex at the point of Xlsirt localization (Fig. 4C and D; see also Fig. 3A,B). In all oocytes analyzed at stages 1 and 2, we detected a similar order of RNA layering in which the Xcat2 mRNA was closest to the outermost cortical layer followed by Xlsirts and Xwnt11. By stage 4 when the Xcat2, Xlsirts and Xwnt11 RNAs were at their final position in the disk-like structure at the vegetal cortex, this layering was not as evident (data not shown).

**Nocodazole and Cytochalasin B do not affect the association of RNAs with the mitochondrial cloud**

To determine the role of actin microfilaments and microtubules in the association of Xlsirts, Xcat2 and Xwnt11 with the mitochondrial cloud, we incubated stage 1 and 2 oocytes for up to 3 days in nocodazole to destroy microtubules and in cytochalasin B to destroy the actin microfilaments. Treated oocytes were analyzed by in situ hybridization for any changes in the localization of these RNAs. We found that the association of Xlsirts with the cloud was not affected by 3-day treatment with either nocodazole or cytochalasin B (Fig. 5). There was also no effect on either Xcat2 or Xwnt11 (data not shown), suggesting that the association of these three transcripts with the mitochondrial cloud was not dependent upon either microtubules or actin microfilaments.

**Anchoring of RNAs at the vegetal cortex relies on several cytoskeletal components**

We had previously shown that destruction of Xlsirt RNA with antisense ODNs resulted in the complete release of Vg1 mRNA from the cortex in stage 4 oocytes (Kloc and Etkin, 1994) suggesting that there may be an interaction between the Xlsirt RNA and cytoskeletal elements anchoring Vg1. Therefore we examined the role of actin microfilaments and microtubules in the anchoring of Xlsirts, Xcat2, Xwnt11 and Vg1.

Treatment of stage 3 and 4 oocytes with cytochalasin B resulted in the detachment of Xlsirts (Fig. 6a,b), Xcat2 (Fig. 6d,e), Vg1 (Fig. 6g,h) and Xwnt11 (Fig. 6j,k) RNAs from their tight association with the outer cortical shell, but it did not release them freely into the cytoplasm. We also noticed that all of these RNAs stained much more intensely when analyzed by whole-mount in situ hybridization, indicating a change in the nature of the outer cortical layer of the oocytes.

Treatment of stage 3 oocytes with nocodazole had no effect on the anchoring of either Xlsirts (Fig. 6c), Xcat2 (Fig. 6f), or Xwnt11 (Fig. 6l), which are anchored at the outermost cortical layer.

![Fig. 4. Detection of Xwnt11, Xcat2, Xlsirts and Vg1 RNAs by double in situ hybridization.](image-url)
shell layer. Analysis with the Vg1 probe using in situ hybridization on whole mounts and on sectioned material showed that, after nocodazole treatment, a small fraction of Vg1 was still tightly anchored at the outer cortical shell along with the other three RNAs (Fig. 6i). However, the majority of the Vg1 mRNA was released from the cortex in the form of cortical blebs (Fig. 6i), rather than being free in the cytoplasm. In older stage 4 oocytes treated with nocodazole, Vg1 RNA remained tightly associated with the outer cortical shell (data not shown). In addition, we found that treatment of oocytes with a combination of both cytochalasin B and nocodazole did not completely release any of these RNAs (data not shown).

The results with nocodazole and cytochalasin B treatments suggest that actin microfilaments partially anchor all of the RNAs to the outermost cortical shell and when destroyed result in a detachment but not a complete release of the RNAs from this layer. In contrast, microtubules did not play an important role in the anchoring of Xlsirts, Xcat2 or Xwnt11; however, their destruction in stage 3 oocytes resulted in the peeling away of an inner cortical layer that contained the majority of the Vg1 transcripts. In stage 4 oocytes, however, nocodazole had very little effect on localized Vg1 RNA. Thus, destruction of microfilaments and microtubules alone or together did not completely release the RNAs into the cytoplasm, suggesting that there is a network of several different types of cytoskeletal elements as well as Xlsirt structural RNAs involved in the anchoring of these RNAs at the vegetal cortex.

DISCUSSION

We have demonstrated that there are two major pathways through which RNAs are translocated and anchored at the vegetal cortex during oogenesis in *Xenopus laevis* (Fig. 7). Xlsirts, Xcat2 and Xwnt11 are translocated within a region of the mitochondrial cloud that we call the messenger transport organizer (METRO), while Vg1 is localized through a cytoplasmic pathway outside the mitochondrial cloud. Our observation on the localization of Xcat2 in the mitochondrial cloud is in agreement with a recent report by Forristall et al. (1995). In addition, our data suggest that: (1) Vg1 appeared to be channeled toward the apex of the vegetal cortex to the position of the METRO-localized RNAs; (2) the METRO-localized RNAs anchor at the vegetal cortex in an overlapping layered pattern, with Xcat2 anchoring closer to the outer cortex followed by Xlsirts and Xwnt11, respec-

![Fig. 5. The effect of cytochalasin B and nocodazole on the Xlsirt RNA in the migrating mitochondrial cloud. (A) Control untreated stage 2 oocytes that were cultured for 2 days. (B) Stage 2 oocytes that were cultured for 2 days in the presence of cytochalasin B (25 µg/ml). (C) Stage 2 oocytes that were cultured in nocodazole (1 µg/ml) for 2 days.](image-url)
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the oocyte involves an intrinsic *cis*-acting signal or groups of signals within the RNA and an association with *trans*-acting components in the cytoplasm and/or nucleus. In addition, there must also be an anchoring mechanism that holds the RNA at the proper position. Yisraeli et al. (1990) demonstrated that for Vg1 the translocation process involves association with microtubules, while anchoring of Vg1 to the cortex is dependent upon actin microfilaments.

Treatment of stage 1 or 2 oocytes with either cytochalasin B or nocodazole alone or together did not affect the association of the RNAs with the METRO structure within the mitochondrial cloud, suggesting that their association with the cloud during translocation to the vegetal cortex was not dependent upon actin microfilaments or microtubules. Also, in our hands, destruction of the microfilaments with cytochalasin B in stage 3 and 4 oocytes did not completely release Vg1 RNA into the cytoplasm as was reported previously (Yisraeli et al., 1990). This treatment, however, did result in the movement of Vg1, Xcat2, Xwnt11 and Xlirts away from their tight association with the outermost cortical shell, which may have been interpreted as release by Yisraeli et al. (1990) since they used lower resolution autoradiography.

We discovered that Vg1, but not Xcat2 RNA, was released from the cortex when the Xlirt RNA was destroyed by injection of antisense ODNs (Kloc and Etkin, 1994). This suggested that Xlirts are a structural component of the cortex and that Vg1 anchoring is dependent upon their integrity. We believe that, instead of a direct interaction between Vg1 and Xlirts, other components link the two. It is likely that other protein components integrated into the cytoskeletal architecture are involved in anchoring these transcripts. Our results suggest that anchoring RNAs at the vegetal cortex involves a network of elements that include among others actin microfilaments and putative structural RNAs such as Xlirts and that there are differences in how the various RNAs are anchored, since Xlirt destruction only affects Xlirts but not Xcat2. We

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**Fig. 6.** The effect of cytochalasin B and nocodazole on the anchoring of Xlirts, Xcat2, Xwnt11 and Vg1 RNAs. Stage 3 oocytes were treated with either cytochalasin or with nocodazole for 2 days in culture. The treated oocytes were analyzed using whole-mount in situ hybridization and sectioned to determine the effect of the drugs on the localized RNAs. Panels a,d,g and j are untreated control oocytes cultured for 2 days and hybridized with the Xlirt, Xcat, Vg1 and Xwnt11 probes respectively. Panels b, e, h and k are oocytes treated with cytochalasin B for 2 days and analyzed with the same probes. Panels c, f, i and l are oocytes treated with nocodazole for 2 days and also analyzed with the same probes as above.
have not yet tested the effect of loss of Xlsirts on Xwnt11 anchoring nor have we determined its effect on the translocation step of any of these RNAs.

Nocodazole treatment of stage 3 oocytes had no effect on the anchoring of either Xcat2, Xlsirts or Xwnt11, but it did result in the peeling away of a large portion of the inner cortical layer from the outer cortical shell. We believe that the Vg1 RNA associated with this inner cortical layer was in the process of migrating through the cortex (or being moved by the stretching of the cortex) toward the future marginal zone. This is supported by our observation that nocodazole treatment of stage 4 oocytes, when Vg1 was completely localized, did not affect the association of Vg1 with the cortex. This result was also consistent with similar observations by Yisraeli et al. (1990).

Our results also showed that localization of RNAs through the METRO pathway involved at least three steps: the movement of the transcripts from the GV to the mitochondrial cloud, the sorting of the individual transcripts within the cloud to their unique positions within the METRO, and their translocation to the cortex. Therefore, an important question concerns the nature of the cis-acting signals within the RNAs that directs them towards this pathway and their subsequent sorting within the METRO. Kloc et al. (1993) showed that cis-acting signals in the Xlsirt repeat sequences are necessary and sufficient for translocation to the vegetal cortex. In addition, these sequences also directed the Xlsirt RNAs to the cloud; however, we do not yet know if they are sufficient to direct the proper spatial sorting of the Xlsirt RNA within the METRO.

We also observed that Xcat2, Xlsirts and Xwnt11 were associated with the cortex in stage 2 oocytes in a layered configuration with the Xcat2 mRNA directly adjacent to the outermost cortical layer followed by Xlsirts and Xwnt11. These data suggest the possibility that there is a hierarchy in the association of the METRO-localized RNAs with the vegetal cortex with Xcat2 RNA associating first, followed by Xlsirts and then Xwnt11. Later the Vg1 RNA is translocated. Parallels may be drawn between this potential hierarchy of RNA localization at the vegetal cortex and the hierarchy involved in the localiza-

tion of RNAs at the two poles in Drosophila (see Ding and Lipshitz, 1993).

We also show that the translocating Vg1 RNA was channeled towards the cortex through a narrow zone overlapping with the site of the METRO-localized RNAs at the cortex. Although we are analyzing a static situation in fixed oocytes, careful analysis of different staged oocytes suggests that Vg1 is following the pathway used by the METRO-localized RNAs as they translocated to the cortex. This suggests the intriguing possibility that the migrating METRO establishes a pathway that is used by other RNAs that localize later during oogenesis. Therefore, the proper localization of Vg1 and Vg-like RNAs may depend upon the establishment of a cytoskeletal pathway by the mitochondrial cloud. Since evidence suggests that several of these RNAs such as Vg1 (Thomsen and Melton, 1993) and Xwnt11 (Ku and Melton, 1993) play a role in axial patterning of the embryo, the knowledge of how these pathways function will be important in our understanding of how the embryonic axis is established.

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REFERENCES

localization and cytoskeletal association of two vegetally localized RNAs Vg1 and Xcat2. Development 120, 201-208.


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