Localization of Xcat-2 RNA, a putative germ plasm component, to the mitochondrial cloud in Xenopus stage I oocytes

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SUMMARY

The mitochondrial cloud is a unique cell structure found in stage I Xenopus oocytes that plays a role in mitochondriogenesis and in the distribution of germ plasm to the vegetal pole. Xcat-2 RNA specifically localizes to the mitochondrial cloud and moves with it to the vegetal subcortex in stage II oocytes. Later, in the 4-cell embryo, it is found in a pattern identical to the germ plasm. Following microinjection into stage I oocytes, synthetic Xcat-2 RNAs localize to the mitochondrial cloud within 22 hours. Transcripts are stable over this time period with very little evidence of degradation. The Xcat-2 3′ untranslated region was found to be both required and sufficient for mitochondrial cloud localization. Further deletion analysis narrowed this localization signal to a 250 nucleotide region at the proximal end of the 3′ untranslated region. This region is different from, but overlaps with, a domain previously shown to be sufficient to direct Xcat-2 to the vegetal cortex in stage IV oocytes. Examination of early stage I oocytes reveals a time when Xcat-2 is uniformly distributed, arguing against vectorial nuclear export into the mitochondrial cloud. Analysis of localization at different time points does not suggest active transport to the mitochondrial cloud. We postulate that localization occurs by selective entrapment of Xcat-2 within the cloud by localized binding sites.

Key words: Xenopus, localized maternal mRNA, germ plasm, mitochondrial cloud, Xcat-2, oocyte

INTRODUCTION

In previtellogenic oocytes, a ‘necklace’ consisting of small clusters of mitochondria surround the nucleus or germinal vesicle (GV) (Dumont, 1972; Heasman et al., 1984). One cluster, on the centriolar side of the GV, becomes distinctly larger (20-40 μm in diameter) than the others and is called the mitochondrial cloud (Billet and Adam, 1976). Later during oogenesis, it is evident that the mitochondrial cloud marks the basal or vegetal pole side of the GV. The mitochondrial cloud is a site of intense mitochondriogenesis and, together with the other clusters, constitutes the impressive maternal contribution of this organelle to the embryo (Tourte et al., 1984).

Several unique features distinguish the mitochondrial cloud from the other perinuclear mitochondrial aggregates. The mitochondrial cloud has a distinct cytoarchitecture and retains its integrity when removed from the cell with a micropipette. Immunohistochemical staining reveals microtubules and intermediate filaments surrounding as well as penetrating the cloud (Wylie et al., 1985a; Gard et al., 1995). Disruption of microtubules leaves the mitochondrial cloud intact while the perinuclear clumps disaggregate (Heasman et al., 1984). Besides Golgi complexes, lipid and smooth endoplasmic reticulum, the mitochondrial cloud is also the exclusive site of germ plasm, a distinctive association of mitochondria, lipid and germinal granules. These granules contain RNA and protein and evidence strongly supports their role as determinants of the germ cell lineage (Mahowald and Hennen, 1971; Ikenishi et al., 1986; Heasman et al., 1984). At the beginning of stage II, the mitochondrial cloud fragments, and at least half of it moves down and into the vegetal cortex. Based on these observations, another major function of the mitochondrial cloud, besides mitochondriogenesis, has been proposed: i.e. to concentrate and to distribute the germ plasm to the vegetal pole where it is eventually inherited by the primordial germ cells (Heasman et al., 1984).

At least two pathways have been identified for localizing RNAs to the vegetal cortex in Xenopus (King, 1995; Forristall et al., 1995). One occurs early during stage I/II, the other functions later, during stage III/IV. Several RNAs, including Xlsirt (Kloc et al., 1993), Xwnt-11 (Kloc and Etkin, 1995) and Xcat-2 mRNA (Forristall et al., 1995), have been mapped to the mitochondrial cloud by in situ hybridization and these RNAs move with the cloud material to the vegetal cortex early in oogenesis. Vg1 localizes much later in stage IV oocytes (Yisraeli and Melton, 1988; Forristall et al., 1995). Movement of Vg1 requires intact microtubules and most likely is actively transported to the cortex (Yisraeli et al., 1990).

It is unknown how Xcat-2 RNA concentrates within the mitochondrial cloud, although its localization is of special interest as it appears to be a component of the germ plasm (Forristall et al., 1995). Understanding the mechanism of Xcat-2 localization may shed some light on the ontogeny of the germ
plasm and hence germ cell specification. Xcat-2 encodes a putative zinc finger RNA-binding protein related to the Drosophila determinant nanos (Mosquera et al., 1993). Interestingly, nanos is also inherited by the germ plasm in pole cells (Wang et al., 1994) and is required for germ-line development. In nanos mutants, pole cells fail to migrate into the gonads (Kobayashi et al., 1996) and females lay few eggs (Lehmann and Nusslein-Volhard, 1991).

Besides active microtubular based transport, there are several other possible mechanisms to be considered for localizing Xcat-2. For example, hsp83 RNA is localized to the posterior pole of Drosophila oocytes by selective protection there and widespread degradation everywhere else (Ding et al., 1993). RNAs may accumulate at a specific site because of specific ‘receptors’ being localized there as has been suggested for nanos (Wang et al., 1994) and cyclin B RNAs (Raff et al., 1990). Yet another mechanism, vectorial nuclear transport, has been proposed for some pair-rule genes in Drosophila (Davis and Ish-Horowicz, 1991). In all these examples of RNA localization, sequences in the 3¢-UTR are required to mediate the process (reviewed in St Johnston, 1995).

Here we have analyzed Xcat-2 localization to the mitochondrial cloud by injecting tagged synthetic RNAs into stage I oocytes. The minimum cis-localization elements required and sufficient for Xcat-2 localization were mapped and include part of a smaller domain required for cortical localization (Zhou and King, 1996). Analysis of localization at different time points is most consistent with a model where Xcat-2 is sequestered within the cloud by localized binding sites.

**MATERIALS AND METHODS**

**Oocytes, oocyte injection and oocyte culture**

Stage I oocytes (50-250 µm in diameter) were dissected from an adult or juvenile ovary and defolliculated using 0.15% collagenase B (Boehringer Mannheim) as previously described (Forristall et al., 1991). Oocytes were maintained in 1× MBSH (Gurdon, 1968; Peng, 1991) supplemented with 100 unit/ml of penicillin and 100 µg/ml streptomycin prior to injection of transcripts.

Approximately 10-50 pg of a given transcript was injected into mature stage I oocytes in a volume of 0.5 nl using a Narishige PC-3000 microinjector. Oocytes were washed three times in 1× MBSH containing antibiotics and cultured in 5× Leibovitz medium supplemented with 1 mM L-glutamine, 1 µg/ml insulin, 15 mM Hepes (pH 7.8), 50 units/ml nystatin, 100 µg/ml gentamicin and 10% frog serum containing vitellogenin or 10% fetal calf serum (Wallace et al., 1980; Yisraeli and Melton, 1988). Oocytes were incubated at 20°C in a humidified chamber for up to 24 hours.

**DNA manipulation and in vitro transcription**

The LrRNA cDNA clone contains 650 nt (59-708 nt) of the mitochondrial large ribosomal RNA gene in pBluescript (Yost et al., 1993). LrRNA served as a marker for the mitochondrial cloud. The original Xcat-2 clone in pSPORT is described in Mosquera et al. (1993). The construct pNB40 Xcat-2 was a gift from Drs K. Mowry (Mowry and Melton, 1992). XRGB-340/3’ contains the Xenopus β-globin coding region fused to the Vgl 340 nt localization signal. ODC2 (ornithine decarboxylase) was a gift from Dr C. Wright (Bassez et al., 1990).

The deletion mutants tagged with the Xenopus β-globin 5’ leader sequence were constructed as follows. The Xcat-2 open reading frame (5’ORF) was isolated from pNBXcat-2 as a MluI-BstNI 470 bp fragment and ligated into the pNB40 vector at MluI and NotI sites. The Xcat-2 3’untranslated region (410 bp 5’UTR) was released from pNBXcat-2 after Ddel and NotI digestion and ligated into the pNB40 vector at MluI and NotI sites. The HindIII-SmaI 310 bp fragment from the 3’UTR clone was ligated into the pNB40 vector at the HindIII and NotI sites to make 3’UTRA1, a mutant lacking the terminal half of the 3’UTR. 3’UTRA2, missing the proximal half of the 3’UTR, was constructed by removing the Smal-MluI fragment from the 3’UTR clone and religating the ends. 3’UTRA3, missing the middle portion of the 3’UTR sequence was created by releasing the Bell-Apal (blunt-ended by nuclease) fragment from the 3’UTR clone and religating the clone. The Bell-Apal fragment was ligated into the pNB40 vector at MluI and NotI sites to create 3’UTRA4, containing only the middle portion of the 3’UTR.

The 3’UTR of Xcat-2 was inserted between the Sall and NotI sites in the pSPORT vector (BRL). Luciferase-tagged 3’UTR-Xcat-2 was produced by ligating pT3/T7-luc (Clonetech) vector fragments, KpnI-Ndel and NdeI-SmaI, with the 3’UTR of Xcat-2 bearing KpnI and blunt-ends from the pSPORT3’UTR construct. Deletion of a 1.5 kb portion of the luciferase insert between two BspMI sites produced a 413 nt luciferase fragment ligated to the 3’UTR of Xcat-2 (Luc/3’UTR). A vector (pT3/T7-luc3¢) containing the 413 nt fragment from the 3’ end of the luciferase-coding region (1479-1892) was made from the pT3/T7-luc clone by simply deleting the region between two BspMI sites. Mutant constructs are diagrammed in Fig. 4.

Probes and RNase protection assays were performed essentially as described by Melton et al. (1984) with a few modifications as outlined in Forristall et al. (1995). HindIII digestion of a pNB40 Xcat-2 subclone yielded a probe of approximately 210 nucleotides after T7 RNA polymerase transcription. This probe resulted in a protected fragment of 144 nt for 5’-end fragment of Xcat-2 clone in the pNB40 vector.

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**Probes and RNase protection assays**

For RNase protection assays, pNB40 Xcat-2 and 5’ORF probe constructs were made by deleting the region between two unique sites, SacII and NotI in the 5’ORF clone, treating with Mungbean nuclease and blunt end ligating the 2,620 nt fragment together. This clone contained the 50 nt Xenopus β-globin mRNA leader sequence and 144 nt 5’-end fragment of Xcat-2 clone in the pNB40 vector.

**In situ hybridization and autoradiography**

Non-radioactive in situ hybridizations were done according to the protocol developed by Oberman and Yisraeli (1995) and Harland (1991) with a few modifications. Defolliculated oocytes were fixed in 0.25% chromium trioxide in 95% ethanol and 5% acetic acid at 4°C for 1 hour. Paraffin sections were cut at 5 µm. Dewaxed, rehydrated sections were refixed in 4% paraformaldehyde for 20 minutes and then pretreated at 37°C for one to two minutes with a protease K solution (1.5 µg/ml in 0.1 M Tris-HCl, pH 7.5, 0.01 M EDTA; Solution proteinase K was purchased from BMB). Slides were then washed in 0.2 M HCl and 0.1 M triethanolamine pH 8.0 with 0.25% acetic anhydride,
followed by PBT (1·PBS, 2 mg/ml BSA, 0.1% Triton X-100) for 15 minutes. Slides were then covered with a solution of 20% fetal calf serum in PBT containing a 1:1000 dilution of the affinity-purified sheep anti-digoxigenin antibody coupled to alkaline phosphatase (BMB). Anti-DIG antibody incubations were carried out either at room temperature for 4 hours or at 4°C overnight. Excess antibody was removed by four 5 minute washes in PBT at room temperature. The chromogenic reaction employed BM purple AP as substrate (BMB). Slides were treated at room temperature until a signal appeared. The reaction was stopped with 10 mM Tris-HCL, 1 mM EDTA, pH 7.5. Samples were then dehydrated in a graded ethanol series and air dried at room temperature for 5 minutes. Prior to immunodetection of the probe, slides were washed in 1× PBS, 0.1% Tween-20 for 10 minutes followed by PBT (1× PBS, 2 mg/ml BSA, 0.1% Triton X-100) for 15 minutes. Slides were then covered with a solution of 20% fetal calf serum in PBT containing a 1:1000 dilution of the affinity-purified sheep anti-digoxigenin antibody coupled to alkaline phosphatase (BMB). Anti-DIG antibody incubations were carried out either at room temperature for 4 hours or at 4°C overnight. Excess antibody was removed by four 5 minute washes in PBT at room temperature. The chromogenic reaction employed BM purple AP as substrate (BMB). Slides were treated at room temperature until a signal appeared. The reaction was stopped with 10 mM Tris-HCL, 1 mM EDTA, pH 7.5. Samples were then dehydrated in a graded ethanol series to 100% xylene and mounted in Paramount (Sigma).

For autoradiographic analysis, 35S-labelled, capped and polyadenylated transcripts with specific activities around 5·10 7 dpm/μg (Krieg and Melton, 1984; Rusconi and Flick, 1993) were microinjected into stage I oocytes and the oocytes cultured, fixed and sectioned as described above. Slides were dipped in Kodak NBT-2 emulsion diluted with NaC1O4 as instructed by the company and air dried. Exposure times were for 1-3 weeks.

Computer modeling

A direct comparison was made of the nucleotide sequences of the RNA localization signal (RLS) from Xcat-2, Xsirt, Xwnt-11 and Vgl using the University of Wisconsin GCG programs COMPARE and BESTFIT. RNA secondary structures were generated by using the energy minimization methods of Zuker (1989). Mfold and FoldrRNA functions of GCG (Genetic Computer Group) program were employed.

RESULTS

Xcat-2 mRNA localizes exclusively to the mitochondrial cloud in stage I oocytes

Previous work has shown that Xcat-2 RNA is concentrated within the mitochondrial cloud present in stage I oocytes (Forristall et al., 1995). Whether Xcat-2 was also associated with the smaller mitochondrial aggregates present around the nucleus remained an open question. To clarify this point, antisense RNA probes for the large mitochondrial rRNA subunit (LrRNA), (B, D-F) Xcat-2 and (C) a mixture of both probes. (A) The LrRNA probe hybridizes to the mitochondrial aggregates surrounding the nucleus as well as to the large mitochondrial cloud. (B) Endogenous Xcat-2 RNA is present only in the mitochondrial cloud. (C) A mixture of both probes reveals that Xcat-2 and the mLrRNA co-localize with the mitochondrial cloud as no additional structures were detected. All oocytes shown in D-F were processed together on one slide. (D) Endogenous Xcat-2 in small stage I oocytes is uniformly distributed in the cell. (E) Xcat-2 partially localized to the mitochondrial cloud. (F) Mitochondrial cloud localization appears complete for Xcat-2. Bar, 80 μm.

Fig. 1. Xcat-2 mRNA progressively localizes to and is found exclusively in the mitochondrial cloud of stage 1 oocytes. In situ hybridizations with stage 1 oocytes approximately 250 μm (A-C) or 50-70 μm (D-F) in diameter. Detection was by a digoxigenin-labeled antisense RNA probe for (A) the mitochondrial large ribosomal RNA (LrRNA), (B, D-F) Xcat-2 and (C) a mixture of both probes. (A) The LrRNA probe hybridizes to the mitochondrial aggregates surrounding the nucleus as well as to the large mitochondrial cloud. (B) Endogenous Xcat-2 RNA is present only in the mitochondrial cloud. (C) A mixture of both probes reveals that Xcat-2 and the mLrRNA co-localize with the mitochondrial cloud as no additional structures were detected. All oocytes shown in D-F were processed together on one slide. (D) Endogenous Xcat-2 in small stage I oocytes is uniformly distributed in the cell. (E) Xcat-2 partially localized to the mitochondrial cloud. (F) Mitochondrial cloud localization appears complete for Xcat-2. Bar, 80 μm.
vectorially exported out of the nucleus. A more probable explanation is that, in early stage I oocytes, Xcat-2 is newly transcribed and distributed throughout the oocyte. As the oocyte and mitochondrial cloud develop, Xcat-2 is localized within the cloud.

Does Xcat-2 accumulate within the mitochondrial cloud, or is ooplasmic Xcat-2 degraded and only that RNA within the mitochondrial cloud preserved? We examined the levels of Xcat-2 during oogenesis by RNase protection and found that the total amount of Xcat-2 remained constant from late stage I to stage VI oocytes (Fig. 2B). Early stage I oocytes as depicted in Fig. 1D contained a quarter of the amount found in mature stage I oocytes (Fig. 2C). In mature stage I oocytes, Xcat-2 was never detected outside the mitochondrial cloud. Either Xcat-2 is no longer synthesized at these stages or it is synthesized and transported rapidly into the cloud. Although we cannot rule out that rapid synthesis and degradation are taking place, it seems unlikely that degradation can account for Xcat-2 mitochondrial cloud localization.

**Injected Xcat-2 RNA localizes to the mitochondrial cloud**

Synthetic transcripts of localized RNAs have generally been found to localize correctly after injection into a cell. Does Xcat-2 RNA contain cis-acting elements sufficient to target it to the mitochondrial cloud? To address this question, approximately 20 pg of 35S-labeled polyadenylated and capped Xcat-2 transcripts were injected into stage I oocytes. Transcripts were injected into the GV or on the opposite side of the nucleus from the mitochondrial cloud. Oocytes were cultured for 24 hours and localization evaluated by autoradiography. Mitochondrial clouds were identified by acid fuchsin staining and only those sectioned oocytes demonstrating a cloud were counted (Hausen and Riebesell, 1991). After 22-24 hours, the injected Xcat-2 had concentrated within the mitochondrial cloud in 81% of the cases (Figs 3A,B, 4). Vg1 mRNA does not localize to the mitochondrial cloud but moves to the vegetal cortex late during stage III/IV. Until then, it is equally distributed in the ooplasm (Melton, 1987; Forristall et al., 1995). Mowry and Melton (1992) have shown that a relatively large segment of 340 nt in the Vg1 3’UTR is sufficient for vegetal localization at stage IV. We used the Vg1 transcript as a control for specificity, and found, as expected, that it did not localize to the mitochondrial cloud (Fig. 3C). Therefore, the information required to specify mitochondrial cloud localization resides within the Xcat-2 transcript, whereas the Vg1 3’UTR vegetal signal cannot target Vg1 to the cloud. Furthermore, the anchoring sites for Xcat-2 in the mitochondrial cloud must be in excess as injected transcript levels were twofold to fivefold over endogenous and were still localized.

To assess the time periods involved in localization, oocytes were also examined after 4 and 8 hours of culture. Neither time period was sufficient for localization to be completed, indicating that injected transcripts were not rapidly translocated into the cloud material. The pattern of localization was also informative. In contrast to what is observed for Vg1 localization, Xcat-2 signal declines uniformly throughout the ooplasm and concentrates within the cloud exclusively. We never observed a perinuclear localization for Xcat-2 or Xcat-2 in transit moving up a concentration gradient; two steps typically observed during the active transport of Vg1 to the vegetal cortex in stage III/IV oocytes (Fig. 3A,B,E). These observations are most consistent with an entrapment model for Xcat-2 mitochondrial cloud localization.

**The 3’UTR of Xcat-2 is both required and sufficient for mitochondrial cloud localization**

Localization elements for RNAs have invariably mapped to regions within the 3’UTR (MacDonald and Struhl, 1988; Wang et al., 1994; MacDonald et al., 1993, Kim-Ha et al., 1993; Kislauskis et al., 1994; Mowry and Melton, 1992) with sizes varying from 35 (β-actin in fibroblast cells) to 630 nt (*Drosophila* bicoid) in length. As a first step in mapping the

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**Fig. 2.** Endogenous Xcat-2 levels remain constant throughout oogenesis. The concentration of Xcat-2 and Vg1 in stage I, II, III, IV early (E), IV late (L), V and VI oocytes was determined by RNase protection. The steady state level of Xcat-2 remains the same throughout oogenesis, arguing against RNA degradation as a mechanism for mitochondrial cloud localization. (A) Diagram of Xcat-2 mRNA with 5’ leader (open box), coding region (shaded box), 3’UTR (thick line). (B) RNase protection. Two oocyte-equivalents were loaded per lane. The probes used were Vg1 (~400 nt), Xcat-2 (240 nt) and ODC (120 nt) as indicated. Note that Xcat-2 appears to accumulate before Vg1 does in late stage I oocytes. The Vg1 antisense probe is protected from RNase digestion by Vg1 transcripts to give multiple bands as shown previously (Rabagliati et al., 1985; Melton, 1987). ODC (ornithine decarboxylase) was used as a control for RNA loading (Bassez et al., 1990) and yeast total RNA (20 µg) was used as a control for specificity. (C) RNase protection showing Xcat-2 is present in young oocytes (50-70 µm; as in Fig. 1D) and increases in amount in late stage I oocytes (250 µm) relative to the ODC control.
mitochondrial cloud localization signal, the 5’ORF and the 3’UTR deletion mutants of Xcat-2 were tested. Examples of injected oocytes are presented in Fig. 3 and quantitation of the results is presented in Fig. 4. The 3’UTR of Xcat-2 alone localized to the mitochondrial cloud 91% of the time (Fig. 3E) while the 5’ORF failed to do so in a 100% of the cases (Figs 3D, 4). To test for sufficiency of the 3’UTR in localization, 400 nt of the luciferase-coding region was fused to this region and the chimeric transcript introduced into stage 1 oocytes. In this instance, the transcript was detected by in situ hybridization using a DIG-labeled antisense luciferase probe. The luciferase-tagged transcript localized to the cloud in 74% of the cases and within the same time period as full-length Xcat-2, arguing that the 3’UTR was not only required but sufficient for localization (Fig. 3F).

In order to locate the minimum sequence in the 3’UTR required for mitochondrial cloud localization, four different deletion mutants were tested that divided this region into three parts: proximal, middle and distal (Fig. 4). The only deletion tolerated was of the distal half (3’UTRΔ1 in Fig. 4). The 3’UTRΔ2, 3’UTRΔ3 and 3’UTRΔ4 mutants all failed to localize. From this, we conclude that the proximal 250 nt are required for mitochondrial cloud targeting. The minimal localization signal sufficient to target ectopically introduced Xcat-2 to just the vegetal cortex in stage 1 oocytes has been mapped (Zhou and King, 1996). Again, the 3’UTRΔ2 and 3’UTRΔ4 mutants lack localization activity. However, whereas the mitochondrial cloud signal is broken by the BclI site in the 3’UTRΔ3 mutant, this mutant can localize to the vegetal cortex (Zhou and King, 1996). Therefore, there appear to be two localization elements within the 3’UTR with the mitochondrial cloud element larger and inclusive of part of the vegetal cortex localization signal.

![Fig. 3. Xcat-2 RNA, but not Vg1 RNA, contains a signal required and sufficient for mitochondrial cloud localization. (A-E) Oocytes were injected with 35S-labeled Xcat-2 or XβG-340/3’ transcripts on the side opposite the mitochondrial cloud (A,B) 22 hours after injection, Xcat-2 is localized within the mitochondrial cloud whereas (C) the Vg1 XβG-340/3’ transcript remains unlocalized. (D) The Xcat-2 5’ORF fails to localize but the Xcat-2 3’UTR (E) localizes as efficiently as does the full-length sequence (B). (F) Oocyte injected with a DIG-labeled chimeric transcript containing the luciferase-tagged 3’UTR of Xcat-2. The 3’UTR is sufficient for mitochondrial cloud localization. Note however that the 400 nt luciferase tag is not as efficiently localized or retained in the cloud as the smaller transcripts and this is reflected in the higher background levels. Arrowheads indicate the smaller transcripts and this is reflected in the quantitation of localization results. Bar, 80 μm.](https://example.com/fig3)

<table>
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<th>Name of constructs</th>
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<th>% Mitochondrial cloud localization</th>
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<tr>
<td>5’ORF</td>
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![Fig. 4. The localization element maps to a 250 nt region within the 3’UTR. Deletion mutations used to create mutant transcripts are shown in schematic form. The Xcat-2 ORF (shaded) and the 3’UTR (black) of Xcat-2 are shown. Deletions within the 3’UTR are indicated by interruptions in the black lines. 30 bp of the 5’ leader sequence of Xenopus β-globin cDNA (open box) or 400 bp of the luciferase-coding region (slanted lines) was used to tag the Xcat-2 3’UTR. Mitochondrial cloud localization was detected by autoradiography (for globin-tagged 35S-labeled Xcat-2) or in situ hybridization (for luciferase-tagged Xcat-2) with DIG-labeled probes. Localization of the encoded transcripts in stage 1 was scored as minus as shown in Fig. 3C or D; plus (signal was 2-fold or 4-fold over background) as represented in Fig. 3A, or plus plus (signal was >5-fold over background) as shown in Fig. 3B. Silver grains over the mitochondrial cloud and an equal area over the nucleus (background) were counted. Only oocytes with obvious mitochondrial clouds were scored (n) except for the 5’ORF construct (*) as the uniform signal made detection of the cloud difficult in this case. Data are based on two series of injections.](https://example.com/fig4)
DISCUSSION

In this work, we have examined Xcat-2 RNA localization to the mitochondrial cloud in stage I oocytes. We find that the mechanism for localization is distinctly different from that which has been described for Vg1 transport to the vegetal cortex in stage III/IV oocytes. In the case of Vg1, current evidence strongly supports directed transport as the mechanism involved (Yisraeli et al., 1990). Uniformly distributed Vg1 is moved by means of microtubules to a perinuclear position and is then again transported by microtubules to the vegetal cortex (Yisraeli et al., 1990; Forristall et al., 1995; Zhou and King, 1996). In contrast, results presented here are most consistent with the mitochondrial cloud containing specific binding sites that sequester Xcat-2 RNA to this location.Injected Xcat-2 RNA gradually accumulates within the cloud with no intermediate steps involving concentration gradients or perinuclear localization. Background levels of Xcat-2 remained uniform in the ooplasm over the course of localization. Since stage I oocytes are relatively small and yolk platelets have not yet formed in the ooplasm, diffusion alone may be sufficient to provide the necessary binding opportunities for Xcat-2 to concentrate within the large mitochondrial cloud. These binding sites must be RNA and region specific as Vg1 cannot bind to the mitochondrial cloud and Xcat-2 does not concentrate within the other mitochondrial aggregates arrayed around the nucleus, but only accumulates within the cloud. However, although the pattern of localization is most consistent with a model of localized binding sites, we cannot yet rule out a role for microtubules.

Two other possible mechanisms for localization have been described by St Johnston (1995): selective degradation (Ding et al., 1993) and vectorial nuclear transport (Davis and Ish-Horowicz, 1991). Vectorial nuclear export as a possible mechanism seems unlikely since Xcat-2 RNA was found uniformly distributed in the ooplasm of early stage I oocytes and only later entered the cloud. Selective degradation as a model is also unlikely as Xcat-2 RNA levels remain constant during oogenesis and actually increase during stage I (Fig. 2). Furthermore, injected Xcat-2 concentrated within the mitochondrial cloud without significant degradation as indicated by the lack of recycled label appearing in the nucleus (Fig. 3B). In fact, the Xcat-2 signal within the mitochondrial cloud appeared to increase as the signal found in the general ooplasm declined. Both these observations argue against selective degradation as a mechanism for localization where only the Xcat-2 in the cloud remains intact.

One general concept that has emerged from studies on RNA localization, irrespective of the organism, cell type or mechanism involved, is that the signal for localization (RLS) resides in the 3′UTR (Macdonald and Struhl, 1988; Kim-Ha et al., 1991; Mowry and Melton, 1992; Kloc et al., 1993). The results of fine deletion mapping of the bicoid (MacDonald et al., 1993; Ferrandon et al., 1994), oskar (Kim-Ha et al., 1991) and actin (Kislauskis et al., 1994) RLSs suggest that the large signal may consist of several smaller elements with different elements responsible for distinct steps in the localization pathway. Analysis of Xcat-2 deletion mutants indicated that a 250 nt region upstream of the Smal site in the 3′UTR of Xcat-2 is required for mitochondrial cloud localization. All localization activity was lost in the 3′UTRΔ3 mutant in which this region is broken. Interestingly, in related but separate experiments, the 3′UTRΔ3 mutant was both required and sufficient to target Xcat-2 to the vegetal cortex in stage IV oocytes (Zhou and King, 1996). Since the signal sufficient for vegetal cortical localization in Xcat-2 (3′UTRΔ3) and Vg1 (XβG-340/3′) fails to localize to the mitochondrial cloud when tested in stage I oocytes, our results define two elements in the Xcat-2 3′UTR that have different functions or binding abilities. The localization requirements for the mitochondrial cloud may require additional and/or different RNA-protein interactions compared to vegetal cortical localization. These differences in RNA cis-localization signals could also underlie the different cortical binding properties that have been observed for Xcat-2 and Vg1 (Forristall et al., 1995; King, 1995; Kloc and Etkin, 1994).

The mitochondrial cloud appears to contain binding sites for a number of RNAs in addition to Xcat-2. These include Xsirts (Kloc et al., 1993; Kloc and Etkin, 1994) and Xwnt-11 (Ku and Melton, 1993). We have isolated an additional four RNAs that all localize to the mitochondrial cloud bringing the total to seven. These new mitochondrial cloud RNAs will be reported on elsewhere (King, 1995; Zhang and King, unpublished). Xsirts are non-coding RNAs that may play a role in RNA binding to the vegetal cortex (Kloc and Etkin, 1994). Xwnt-11 is a member of the Wnt-family of growth factors and its protein can partially rescue a dorsal axis in UV-irradiated embryos (Ku and Melton, 1993). No localization elements for Xwnt-11 have been mapped yet, but, when we compared the 250 nt mitochondrial cloud localization signal in Xcat-2 with the 3′UTR of Xwnt-11, we could not find any significant homologies. However, it is difficult to prove that similarities in RNA sequence or secondary structure are significant without further mutant analysis.

What is the underlying purpose of localizing mRNAs to the mitochondrial cloud? Heasman et al. (1984) have suggested that one of the important functions of the mitochondrial cloud is to accumulate and localize germ plasm to the vegetal pole. We have previously shown that Xcat-2 appears to be a component of the germ plasm (Forristall et al., 1995) and two other mitochondrial cloud RNAs that we have isolated co-localize with Xcat-2 (Zhang and King, unpublished). It is well known that primordial germ cells must lie in the endoderm to migrate properly into the developing gonads (Wylie et al., 1985b). In Drosophila the genetic pathway that specifies the PGC lineage is linked to that which specifies abdomen, the correct position for PGC development in flies (Lehmann and Ephrussi, 1994). In an analogous fashion, the genetic pathway that specifies the PGCs may be linked to that which specifies endodermal fate in frogs. The physical link could be the co-localization of determinants to the vegetal cortex by means of the mitochondrial cloud. If this is true, we would predict that mRNAs involved in specifying dorsal and/or endoderm would also be found in the mitochondrial cloud. Xwnt-11 would be a candidate for such an RNA.

The overlap in localization signals between the early and late pathways revealed by the deletion mutant analysis of Xcat-2 may also be significant. Perhaps the early pathway evolved from the late one as a specialized transport system for localizing germ plasm material early, before the localization of most other RNAs involved in somatic cell differentiation like Vg1. It is interesting to speculate that the posterior pole of Drosophila and the vegetal pole of Xenopus, both sites where the germ cell determinants become localized, localize some RNAs using a similar mechanism. In this regard, nanos, cyclin B and germ cell-less mRNAs are thought to accumulate at the
posterior pole because of specific binding sites localized in this domain (Wang et al., 1994; Raff et al., 1990). Clearly, the mechanism that targets mRNAs to the mitochondrial cloud plays an important role in establishing polarity in the oocyte and, as a consequence, cell fate in the embryo.

We thank Kim Mowry for generously providing the XJG-340/3’ cDNA clone and Joseph Yost for providing the LrRNA clone. We thank Dr Joel Yisraeli for sharing his in situ hybridization protocol with us. We also acknowledge Dr Bubunenko for many helpful discussions regarding this work. This work was supported by an NIH grant (GM 33932) to M. L. King.

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