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Biochemical Characterization of a Cellular Structure Retaining Vegetally Localized RNAs in *Xenopus* Late Stage Oocytes

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Abstract Two pathways operate during *Xenopus* oogenesis to localize a small number of RNAs to the vegetal cortex. Correct localization of these RNAs is essential to normal development as the proteins they encode are involved in specifying cell type and in patterning the early embryo. Binding these RNAs to the vegetal cortex and thus preserving their localized condition is a critical step, although little is known about how this is achieved. In this study, we have used a biochemical approach to examine the anchoring step. *Xlsirts,* an abundant localized RNA (locRNA), was selectively enriched in a detergent-insoluble fraction (DIF) prepared from oocytes that had completed the RNA localization process. These putative RNA-anchoring complexes were analyzed by density gradient centrifugation and in RNA-protein binding assays. Cortical *Xlsirts* and other localized RNAs are specifically found in the heavy region of sucrose gradients and in the pellet, quite different from other cellular RNPs. Four proteins were identified by UV-crosslinking that bound the *Xlsirts* localization signal in the cortex, but not in the soluble fraction. These are likely members of the anchoring complex and appear to include vera, a characterized *Vg1* RNA binding protein. Vera was found to co-sediment with other locRNAs found in the vegetal cortex, suggesting that it is a common component of locRNPs. Finally, we found that locRNPs extracted into the soluble fraction had the same buoyant density as typical ooplasmic RNPs. We propose that locRNAs are organized and anchored in the cortex as typical RNPs. J. Cell. Biochem. 80:560–570, 2001.
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Key words: RNA localization; *Xenopus* oocytes; cytoskeleton; RNA-binding proteins; cortical retention of localized RNPs

The wide use of RNA localization by both somatic and germ cells to localize specific proteins has revealed an important mechanism for regulating gene expression at the post-transcriptional level [Bashirullah et al., 1998; Oleynikov and Singer, 1998]. Recent studies in *Drosophila*, *Xenopus*, and ascidians have shown that correct RNA localization is essential for early development [Lasko, 1999; King et al., 1999; Mowry and Cote, 1999; Fujimura and Takamura, 2000]. In these organisms, localization of maternal RNAs in the oocyte is used to asymmetrically distribute regulatory proteins into different cells during cleavage and thereby initiate regional and cellular identity in the embryo. For example, localized RNAs are involved in specifying the future germ cells [Williamson and Lehmann, 1996; Houston and King, 2000], primary germ layers [Zhang et al., 1998], and embryonic axes [Marikawa and Elinson, 1998]. Therefore, understanding the mechanism of RNA localization in oocytes represents a key problem in development.

One general concept emerging from work on RNA localization is that RNAs appear to be transported as components of large RNA/protein particles [Wang and Hazelrigg, 1994; Barbarese et al., 1995; Kloc and Etkin, 1995]. Such particles have been visualized in living cells using confocal laser scanning microscopy. The composition of these granules is an area of active investigation, and results so far suggest

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that some components play a role in translational control and cytoskeletal binding [Barbarese et al., 1995; Bashirullah et al., 1998]. Most recently, hnRNP proteins have been shown to bind RNA localization signals and be required for localization, revealing a novel role for these nuclear proteins [Havin et al., 1998; Deshler et al., 1998; Cote et al., 1999]. What components are required to anchor localized RNAs at their final destination remains unknown.

At least two RNA localization pathways have been described in *Xenopus* oocytes contributing to the distribution of constituents along the animal–vegetal axis [Forristall et al., 1995; Kloc and Etkin, 1995]. Both pathways have distinct temporal and spatial modes of regulation and localize different RNAs [reviewed in Mowry and Cote, 1999; King et al., 1999]. During the early pathway in Stage I oocytes, a set of RNAs concentrate in the mitochondrial cloud. Many of these RNAs, such as *Xcat*2 [Kloc et al., 1998; MacArthur et al., 1999] and *Xlsirts* [Kloc et al., 1998] accumulate in the forming germ plasm, a structure containing germ cell determinants. Unlike the early pathway, the late pathway uses microtubules to actively transport RNAs, *Vg1* and *VegT*, to the vegetal cortex. Both of these late-localizing RNAs are involved in primary germ layer formation.

The cis-acting signals involved in *Xcat*2 [Zhou and King, 1996a, 1996b; Bubunenko, Vempati, and King, unpublished observations] and *Vg1* localization have been characterized [Gautreau et al., 1997; Deshler et al., 1997; Havin et al., 1998]. Two transacting factors that bind the *Vg1* signal and are required for localization have been identified. Vera/*Vg1*RBP protein belongs to a family of highly conserved vertebrate proteins [Deshler et al., 1998; Havin et al., 1998] and the VgRBP60 protein is a member of an hnRNPI family [Cote et al., 1999]. Both were shown to co-localize with *Vg1* RNA in the vegetal cortex and showed identical patterns of distribution with *Vg1* RNA during localization [Cote et al., 1999; Zhang et al., 1999]. Taken together, these results strongly suggest that vera/*Vg1*RBP and VgRBP60 are in a complex with *Vg1* at each step during translocation to the vegetal cortex. They are candidates for involvement in RNA targeting, cortical anchoring, and/or translational control.

In this paper, we used a biochemical approach to study the anchoring step in RNA localization. Taking advantage of the fact that localized RNAs can be selectively enriched in the detergent-insoluble fraction (DIF) of oocyte extracts [Pondel and King, 1988], we prepared the DIF from stages V–VI oocytes and further analyzed it by density gradient centrifugation. Two main localized RNA (locRNA)-containing fractions were identified and their characterization provides the first information about the biochemical properties of the RNA anchoring complex in *Xenopus* oocytes.

METHODS

Oocyte Isolation

Sexually mature female frogs were anesthetized with 5 mg/ml MS-222 (3-aminobenzoic acid ethyl ester) and whole ovaries were surgically removed. Ovaries were washed in OR2 (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic] acid (HEPES), pH 7.8), and dissociated into individual oocytes by treatment with 0.5% solution of collagenase B (Serva) in phosphate-buffered saline (PBS). The oocytes were staged by passing them through Nytex nylon filters of different mesh sizes. Stages V–VI oocytes of 1.0–1.2 mm were selected and immediately frozen at −80°C until use.

Preparation of Oocyte Extracts

To enrich for locRNAs, oocytes were extracted in a high salt buffer (HSB) 10 mM PIPES, pH 6.8, containing 500 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, 1% Triton X-100, 3 mM β-mercaptoethanol, 0.5 mM PMSF, 5 μg/ml leupeptin and 0.15 μg/ml DNaseI. Extraction was carried out for 15–20 min at 0°C by pipetting with a Pasteur pipette followed by several strokes in a tightly fitted Potter homogenizer. The extract was then centrifuged at 16,000*g* for 15 min to separate the detergent-insoluble (DIF) from detergent-soluble (DSF) fraction. The pellet was resuspended in HSB and subjected to further analysis.

Centrifugation in Sucrose Gradients

Subcellular fractions containing locRNAs were analyzed in two types of sucrose gradients, both of which were made in HSB but without Triton X-100. In one case, the DIF was analyzed in a heavy preformed 40–85% sucrose gradient, centrifuging it in a SW41 rotor at

35,000 rpm for 90 min at 4°C using a L5 Beckman ultracentrifuge. Fractions of 0.8 ml were collected from the bottom of the tube and processed immediately or stored at −80°C until further analysis. The relative density D was estimated from the weight of 50 μl gradient fractions at 20°C, comparing it against the weight of the HSB. For CsCl centrifugation, the DSF was first centrifuged in the standard 20–55% sucrose gradient in the same rotor at 36,000 rpm for 3.5 h at 4°C and 0.6 ml fractions were collected for RNA analysis.

Centrifugation in CsCl and $CsSO_4$ Gradients

All Cs salt gradients were made in HSB without Triton X-100 and centrifuged in a SW41 rotor at 35,000 rpm, 20°C for the indicated times. The relative density D was estimated as described for the heavy sucrose gradient. For CsCl centrifugation, DIF, DSF or fractions containing locRNAs in the heavy sucrose gradient were fixed in 6% formaldehyde for 1 h at 0°C with light stirring and centrifuged in the preformed 25–100% CsCl gradient for 68–70 h. Seventeen fractions were collected from the bottom of the gradient, digested with 0.5 mg/ml Proteinase K in PK-buffer [Forristall et al., 1995] for 2 h at 50°C, extracted twice with phenol/chloroform and chloroform, respectively, and analyzed for RNA content as described below. An intact pellet fraction from the sucrose gradient was centrifuged in the preformed 20–50% $CsSO_4$ gradient for 40 h and the collected fractions were analyzed by SDS-electrophoresis and RNA dot blotting for the protein and RNA content, respectively.

RNA Analysis

Partitioning of *Xcat*2, *Xlsirts* and *Cx*1 RNAs to the DIF and DSF was analyzed by Northern blot hybridization as previously described [Elinson et al., 1993; Kloc et al., 1993]. The distribution of specific RNAs in the gradient fractions was determined by dot blot hybridization [Kobayashi et al., 1991]. All hybridizations were done with the following [^{32}P]-labeled DNA probes made by random priming: the full length *Xcat*2 cDNA [Mosquera et al., 1993], pR11 clone containing repeated sequences of *Xlsirts* [Kloc et al., 1993], 2.3 kb *Bam*HI-*Hind*III fragment of the *Vg1* coding region [Weeks and Melton, 1987], 2.1 kb *Pvu*II-*Xba*I fragment of *Veg*T [Zhang and King, 1996]; 1.5 kb fragment of *DEADSouth*, pDS3UTR, [MacArthur et al., 2000], and the 2.3 kb *Cx*1 clone [Elinson et al., 1993]. The hybridized membranes were exposed and quantitated using the PhosphoImager (Molecular Dynamics).

Protein Electrophoresis and Immunoblotting

The protein content of the 40–85% sucrose gradient fractions was analyzed by 10% PAGE-gels stained with Coomassie R250 (Sigma) or by silver staining (Bio-Rad Silver Stain). For immunoblotting, gels were washed in the transfer buffer and transferred onto nitrocellulose membrane (Schleicher and Schuell, 0.45 μm) in a semi-dry blotter (Idea Scientific, Inc.) for 30 min at 25 V or in a conventional blotter (Bio-Rad) at 15 V overnight according to the manufacturers' recommendations. Part of the membrane was stained with Amido Black 10B (Sigma) and the other part was washed in PBS, blocked in 4% dry milk in PBS for 60 min at 50°C and allowed to react with the primary antibodies overnight at room temperature. The following primary mouse monoclonal antibodies were diluted in 2% blocking solution as indicated and used: anti-actin (against microfilament actin N350, Amersham), 1/1,000; anti-β-tubulin (Amersham), 1/300; and 1h5 anti-type II cytokeratin (DSHB) 1/40. After incubation with 1/500 secondary goat anti-mouse antibodies (BMB) for 2 h, the membranes were developed using the alkaline phosphatase reaction. To detect the vera protein, the membranes were incubated with polyclonal rabbit anti-vera serum diluted 1/3–5,000 (a gift from Dr. W. Taylor) at 4°C overnight. The membrane was washed, incubated with 1/100,000 goat anti-rabbit HRP-conjugated secondary antibodies for 1 h at room temperature and the immunoreactive proteins detected using ECL with SuperSignal West substrate (Pierce).

In Vivo Analysis of Specific RNA-Binding Proteins

The sense strand of the *Xlsirts* RNA [clone *Xlsirtp*-11; Kloc et al., 1993] was transcribed in vitro in the presence of [^{32}P]-UTP and $6–7 \times 10^3$ dpm was injected into manually defolliculated late Stage III oocytes. The oocytes were incubated for 4 days in a culture medium [50% L15 medium, 1 mM glutamine, 15 mM HEPES (pH 7.6), 10% vitellogenin-enriched frog serum, 1 μg/ml insulin, 0.5% Fungizone (BRL), 100 μg/ml gentamicin,

50 μg/ml streptomycin, 50 U/ml penicillin] [Yisraeli and Melton, 1988]. Oocytes were then extracted in HSB as described above and the whole extract was irradiated in the Stratalinker™ at maximum dose in an immunological 96-well plate (Falcon) placed on ice. Oocyte extracts were then separated into DIF and DSF using the MicroMax microfuge (IEC) for 15 min at 16*g* at 4°C, treated with a mixture of RNase A (2 mg/ml) and RNase T1 (100,000 U/ml). RNase-treated samples were analyzed by 10% PAAG SDS-electrophoresis followed by autoradiography.

RESULTS

DIF is Enriched in locRNAs and Cytokeratins

LocRNAs at the vegetal cortex of Stage VI oocytes, unlike non-localized RNAs, preferentially sediment under conditions of medium speed centrifugation after high salt and detergent extraction [Pondel and King, 1988; Mosquera et al., 1993]. This observation suggested that locRNAs are organized into unusually large cellular structures within the oocyte, strikingly different from free ooplasmic or polysomal RNPs. A reasonable assumption that follows is that the DIF contains the RNA anchoring domain or its elements. Therefore, to characterize these RNA anchoring complexes further, the DIF was isolated and analyzed by Northern and Western blot hybridization for the presence of locRNAs and major cytoskeletal proteins, respectively. *Xcat*2 is a locRNA that segregates with the germ plasm [Mosquera et al., 1993] and *Cx*1 is a non-localized RNA originally selected as a false positive in a screen for locRNAs [Zhang and King, unpublished observation]. The *Cx*1 RNP is used throughout this study as a control because of its ubiquitous distribution in oocytes and cortices of all stages and its solubility in high salt and detergent [Elinson et al., 1993]. As expected, the *Xcat*2 RNA was found exclusively in the DIF (Fig. 1A), while the *Cx*1 RNA was enriched in the DSF by more than 90%. Trace amounts of *Cx*1 were detected in the DIF only after overexposure of the autoradiogram. *Xlsirts*, a non-coding family of repetitive RNAs, also localizes to the vegetal cortex [Kloc et al., 1993] and was found in the DIF (see below). The Western blots show (Fig. 1B) that more than 90% of the oocyte pool of cytokeratins was found in the DIF, compared to 5–10% for cytoplasmic actin and

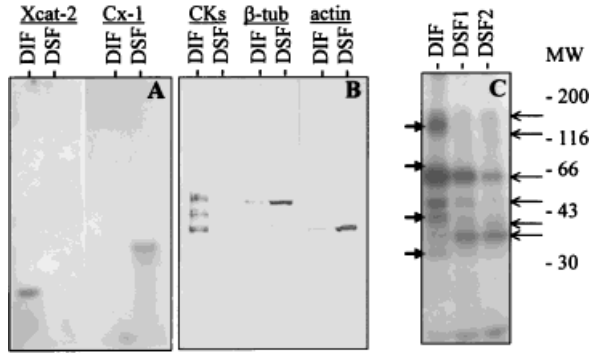


Fig. 1. Localized RNAs are enriched in a DIF along with cytoskeletal proteins and specific RNA binding proteins in late stage *Xenopus* oocytes. Stages V–VI *Xenopus* oocytes were extracted in HSB with Triton X-100 and separated into DIF and DSF by centrifugation. Distribution of localized *Xcat2* RNA versus non-localized *Cx1* RNA was analyzed by Northern blotting (**A**). Distribution of the major cytoskeletal proteins was analyzed by Western blotting with monoclonal antibodies raised against actin, β-tubulin and cytokeratins, respectively (**B**). For analysis of specific RBPs (**C**), late stage III oocytes were injected with the ^{32}P-labeled *Xlsirts* RNA localization signal. The oocytes were incubated for 3 days [Kloc et al., 1993], lysed and irradiated with UV-light to crosslink proteins. Equal amounts of DIF and DSF were digested with RNases and analyzed by SDS-electrophoresis followed by radioautography to reveal the ^{32}P-labeled proteins. Cross-linked proteins unique to the DIF *Xlsirts* are indicated by a thick arrow; DSF proteins are indicated by a thin arrow.

β-tubulin. This work confirms and extends our previous observations [Pondel and King, 1988].

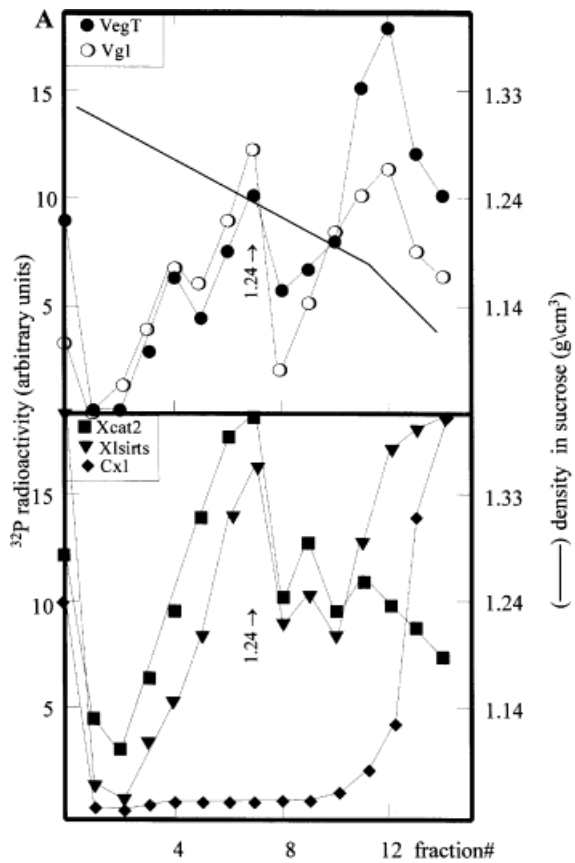
Xlsirts-Binding Proteins Specific to the DIF

Xlsirts transcripts injected into Stage IV oocytes will localize to the vegetal cortex within 3 days of culture [Kloc et al., 1993]. It follows that injected *Xlsirts* must associate with transacting factors sufficient for correct cortical localization and anchoring. We examined the proteins that specifically associate with localized *Xlsirts* in the DIF, and therefore, with the putative RNA anchoring domain, and compared it with proteins that bind *Xlsirts* still in the DSF. *Xlsirts* were selected for this analysis because of their abundance and the likelihood that specific *Xlsirts*-binding proteins would also be abundant. Oocytes were injected with [^{32}P]-labeled *Xlsirts* repeats [*Xlsirtp*-11R clone described in Kloc et al., 1993] containing only the vegetal localization signal. After incubation for 3 days as required to localize *Xlsirts* [Kloc et al., 1993], the injected oocytes were homogenized in HSB, the extract UV-irradiated, and centrifuged. The resulting DIF and DSF were

digested with a mixture of RNase A and T1. RNase treatment of the UV-irradiated samples leaves only the short, labeled segments of RNA protected by the covalently bound protein. Such RNA-labeled proteins were resolved by SDS-electrophoresis and visualized by autoradiography. In a parallel experiment, whole oocytes were UV-irradiated to test for nonspecific interactions created at the homogenization step. The same proteins were identified in both experiments, although, less intensely for whole oocytes, arguing that the binding proteins are binding in vivo (data not shown). Comparison of the RNA-binding proteins of the DIF and DSF (Fig. 1C, DSF1) revealed that *Xlsirts* are organized into RNPs associated with at least 10 proteins. Four of these 10 (Mr~125, 74, 41, and 32 kD) were specifically associated with *Xlsirts* in the DIF and greatly reduced or missing from the DSF *Xlsirts*. The 74 kD protein is somewhat hidden by the very abundant 64 kD protein, and on lighter exposures is more obvious. The second extraction of the DIF (Fig. 1C, DSF2) led to the release of *Xlsirts* RNPs into the DSF with the same pattern of six crosslinked proteins as found for the original DSF RNPs. After extraction, the DSF2 *Xlsirts* are missing the four proteins unique to *Xlsirts* in the DIF. These results strongly suggest that the DSF pool of locRNAs forms from RNPs originally associated with the DIF structures. Furthermore, the four proteins specific to the DIF are likely involved in the anchoring step.

Fractionation of DIF by Centrifugation in a Sucrose Gradient

To further characterize the putative RNA-anchoring complex, the DIF was fractionated on a 40–85% sucrose gradient and the position of each locRNA in the gradient determined by dot blot hybridization. Figure 2A shows the



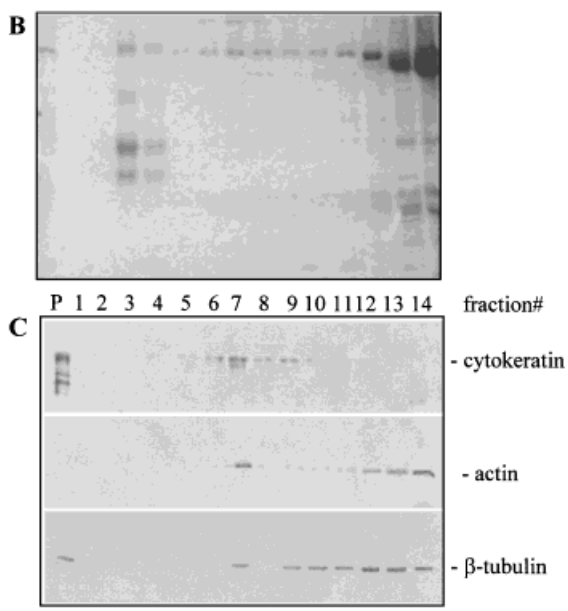


Fig. 2. Localized RNAs fractionate as large cytoskeletal structures on sucrose gradients, distinctly different from ooplasmic RNPs. (**A**) DIF was centrifuged in a 40–85% sucrose gradient and distribution of localized RNAs and nonlocalized *Cx1* RNA analyzed by dot blot hybridization followed by quantitation of autoradiograms using the Phosphoimager. (**B**) Protein composition of sucrose gradient fractions analyzed by SDS-electrophoresis. (**C**). Western blot analysis of sucrose gradient fractions with monoclonal antibodies against actin, β-tubulin, and cytokeratin.

simplest pattern of peaks obtained from five experiments. All four localized RNAs that were examined (*Vg1*, *VegT*, *Xcat2*, and *Xlsirts*) were distributed into three main regions of the gradient besides the pellet (Fig. 2A). Strikingly, *Cx1* RNA is found floating at the top of the gradient, clearly very different from the locRNAs. The locRNAs in the low density region of the gradient most likely dissociated from the DIF during centrifugation as serial washings of the DIF could generate this peak. There was considerable variability in the intensity of a given peak when batches of oocytes from different females were compared, probably reflecting the inherent variability in the stability of complexes during fractionation. However, the position of the control *Cx1* RNA was invariant and always in the top two fractions of the gradient (Fig. 2A). These experiments show that locRNAs fractionate quite differently from other cellular RNPs and are specifically found in the heavy region of the gradient.

Most of the protein component of the gradient fractions was at the top of the gradient (Fig. 2B). Only vitelline envelope proteins were found in the 1.26 g/cm^3 peak of the gradient (fractions 3,4), overlapping with the minor locRNA peak ($d = 1.25$ g/cm^3, fractions 3,4,5). In comparison, the protein content of the major RNA peak (fractions 6,7) was so low that no specific proteins were detected, even after silver staining (data not shown). Western blotting, however, showed that all three major cytoskeletal proteins, actin, β-tubulin, and cytokeratins, were in the major RNA peak, the latter two were also in the pellet (Fig. 2C). Surprisingly, these proteins were below detectable levels in the minor, 1.25 g/cm^3 RNA peak.

Co-sedimentation of the Vera/Vg1RBP Protein with locRNAs

*Vg1*RBP (also known as vera) is a microtubule-associated protein [Elisha et al., 1995] known to specifically interact with the UUCAC RNA sequences in the *Vg1* RNA localization signal [Deshler et al., 1997]. Vera co-localizes with *Vg1* in the vegetal cortex [Zhang et al., 1999]. Replacement of the UUCACs with noncognate sequences prevents the localization of the *Vg1* RNA [Deshler et al., 1997], as well as the transport and anchoring of the *VegT* RNA [Bubunenko et al., in preparation]. Based on these data, a role for vera in the transport and perhaps anchoring of the late locRNAs is suggested. Using antibodies against the vera protein, we asked if vera co-sedimented with locRNAs in late stage oocytes when such RNAs are anchored in the vegetal cortex. In preliminary experiments, vera protein was not detected in the DIF, although it was easily detected in the oocyte lysate and the DSF corresponding to 0.01 oocyte equivalents. The most likely interpretation of these results is that vera is a relatively abundant oocyte protein that is weakly bound to locRNAs. Therefore, we applied whole oocyte lysates to sucrose gradients (40–80%), and the resulting fractions were analyzed by Western blotting with anti-vera antibodies [Zhang et al., 1999]. The results show that vera co-sediments with locRNAs, being clearly enriched in the pellet and in the 1.24 g/cm^3 peak (Fig. 3B), the same fractions enriched in *Xcat*2, but not in the control *Cx*1 RNA (Fig. 3A). An additional cross-reacting protein of unknown origin was detected in the top gradient fractions, ruling out an involvement in RNA localization for this protein (Fig. 3B). These results raise the possibility that vera may be involved in the anchoring of locRNAs in the late stage oocytes.

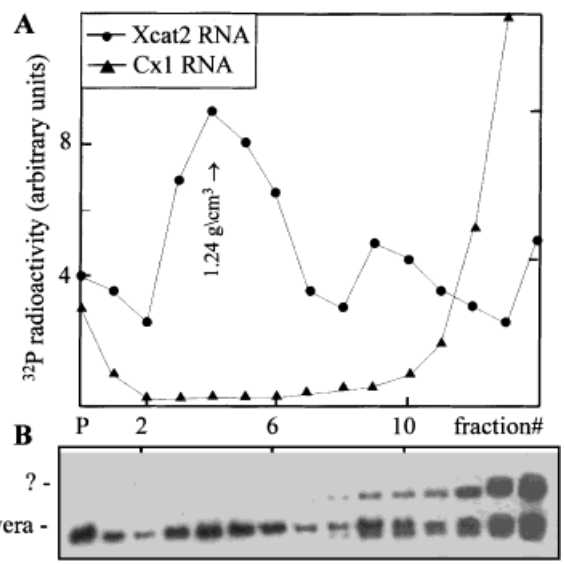


Fig. 3. Co-sedimentation of vera protein with localized RNAs in a sucrose gradient. (**A**) 40–80% sucrose gradient of whole oocyte lysates showing the distribution of the *Xcat*2 and *Cx1* RNAs. Analysis as described in the legend to Fig. 2. (**B**) Distribution of the vera protein in fractions of the same sucrose gradient analyzed by Western blotting.

CsCl Density Centrifugation of Fractions Containing locRNAs

To analyze the relative RNA protein composition of locRNPs found in the heavy region of the sucrose gradients, the pellet and the major RNA peak fractions were fixed in formaldehyde and centrifuged in a 25–100% CsCl gradient. These locRNPs are thought to be part of the cortical anchoring complex as all three cytoskeletal proteins were present in this fraction (Fig. 2C). The locRNPs, detected by hybridization with *Xlsirts*, were found at the top of the gradient (Fig. 4) migrating at a density (1.36 and 1.30 g/cm^3) indicating a higher protein content relative to typical RNPs (1.43 g/cm^3) [Spirin, 1969]. Pure protein is known to have a density of 1.25 g/cm^3 under these conditions [Perry and Kelley, 1966]. However, the observed density values may be artificially low because of entrapped pigment, the majority of which was found at the bottom of the gradient ($d > 1.8$ g/cm^3). Pigment granules are a natural component of the cortex.

Analysis of the Pellet Structure by Centrifugation in $CsSO_4$ Gradient

The pellet from the sucrose gradient was uniquely enriched both in locRNAs and proteins (Fig. 2). To analyze proteins associated with locRNAs in the pellet, the pellet was further centrifuged in a 20–50% $CsSO_4$ gradient. As indicated by the position of the *Xlsirts* RNA, locRNAs were found in two closely positioned peaks with $d = 1.45$ and 1.37 g/cm^3, respectively (Fig. 5A). Only *Xlsirts* were abundant enough to be detected in this second gradient. The two peaks contained most of the total protein (Fig. 5B), with the exception of the vitelline envelope proteins that were found in the lighter gradient peak ($d = 1.28$ g/cm^3). It is very likely that these two peaks are due to the different amount of entrapped pigment granules, as they were intensely brown in color. This interpretation would also explain the unusually high density of the peaks compared to the 1.36 g/cm^3 density of the same structure in CsCl gradient (Fig. 4). Taken together, the evidence argues that the sucrose gradient pellet contains cortical material in general, including the anchoring domain for locRNAs and pigment granules. From previous results, it is known that the RNA anchoring domain is strongly attached to the oocyte cortex, as they can be manually isolated together as an integral stable structure [Elinson et al., 1993].

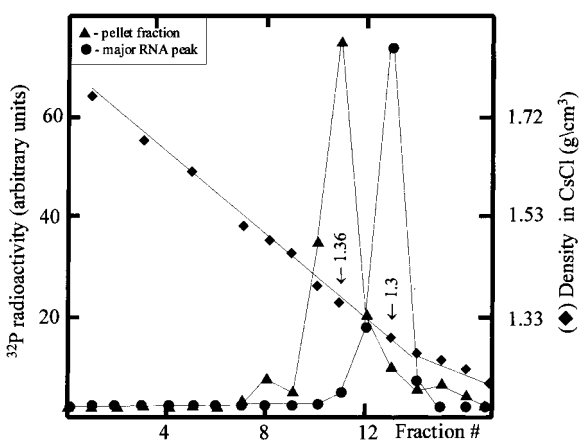


Fig. 4. CsCl gradient analysis showing that localized RNA-containing structures are rich in proteins. The DIF pellet and the major locRNA-containing peak of the heavy sucrose gradient were fixed with formaldehyde and centrifuged in a 25–100% CsCl gradient. Distribution of the *Xlsirts* RNA in gradient fractions was analyzed as described in Figure 2.

Localized RNAs in the DSF

Previous work has shown that up to 60% of the *Vg1* RNA can be extracted, intact, into the DSF [Pondel and King, 1988] and that a substantial amount of *Vg1*, *Xlsirts* and *DEAD-South* RNAs dissociate during centrifugation in a sucrose gradient (i.e., like DSF2 in Fig. 1C) (Table I). Since the sedimentation and density characteristics of the *Xenopus* ooplasmic RNPs are well known [Richter and Smith, 1984], a comparison of localized and non-localized RNPs in the DSF is possible. The centrifugation of the DSF in 20–65% sucrose is presented in Figure 6A. It shows that *Vg1*, the control *Cx*1 RNAs and *Xlsirts* (not shown) have similar profiles in the light region of the gradient (fractions 13–18). *Cx*1 demonstrates the typical polysomal profile (fractions 1–11). The signal for the locRNAs is slightly different, however, with increasing amounts of RNA in the densest region of the gradient. As predicted, no RNA signal was detected at the top of the gradient, where degraded RNA would be found. Although the origin of the larger locRNPs in the DSF has not been investigated in this work, we suspect that they are the products of further fragmentation of the RNA anchoring domain, and similar to the EDTA-insensitive particles detected by Schroeder and Yost [1996].

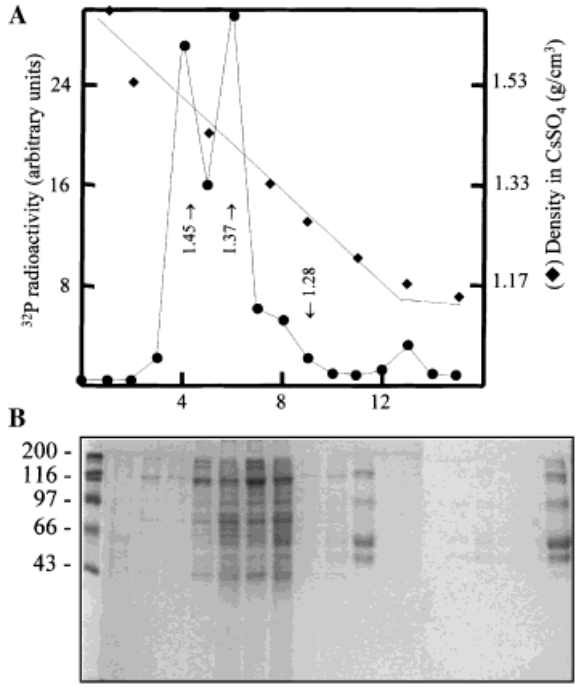


Fig. 5. Centrifugation in a $CsSO_4$ gradient showing that the major protein-containing structure of the DIF also contains localized RNAs. The pellet fraction of the heavy sucrose gradient was centrifuged in the 20–50% $CsSO_4$ gradient. Distribution of the *Xlsirt* RNA (**A**) and protein content (**B**) was analyzed as described in Fig. 2. Molecular weight standards are shown in the left most lane. Fractions are numbered 1–15, excluding the pellet.

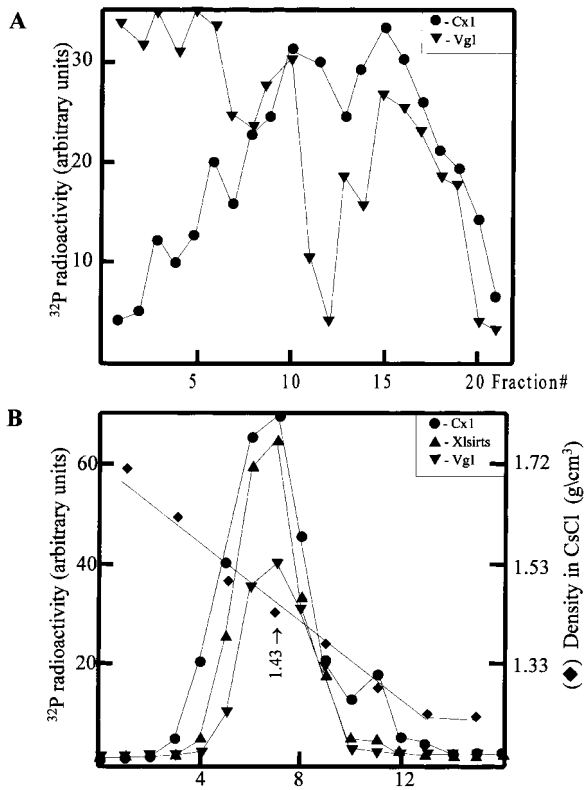


Fig. 6. Similar properties of DSF-containing locRNPs and ooplasmic RNPs. The DSF was centrifuged in a 20–65% sucrose gradient (**A**) or fixed with formaldehyde and centrifuged in a 20–75% CsCl gradient (**B**). Distribution of the localized *Vg1* and *Xlsirt* RNAs versus ooplasmic *Cx1* RNA was analyzed and quantitated as described in Fig. 2.

Similar properties of the localized and regular RNPs have been further confirmed by CsCl centrifugation of formaldehyde-fixed DSF. Figure 6B shows that localized *Vg1* and *Xlsirts* RNAs and non-localized *Cx*1 RNA possess the same density ($d = 1.43\,g/cm^3$), in good agreement with the published results for the ooplasmic RNPs of *Xenopus* [Richter and Smith, 1984].

TABLE I. Retention of Different Localized RNAs with the Anchoring Complex After Gradient Analysis

RNA	% RNA bound[a]	% RNA released[b]	RNA bound/ released
VegT	60	40	1.5
Vg1	59	41	1.44
Xcat-2	76	24	3.13
DEADSouth	36	64	0.56
Xlsirts	66	34	1.92
Cx-1	12	88	0.14

[a]% total RNA detected in the pellet and fractions 1–10.
[b]% total RNA detected in the top fractions 11–14.
Non-localized *Cx*-1 RNA served as a control for free RNPs.

DISCUSSION

RNA localization during *Xenopus* oogenesis consists of several steps including RNA selection, transport, and anchoring within the vegetal cortex. Virtually nothing is known about the last step, although clearly elements of the cytoskeleton are involved and the process is complete in late stage oocytes [reviewed in King et al., 1999]. In this work, we have begun a biochemical characterization of a locRNA cortical anchoring domain. In this analysis, the DIF of Stages V–VI oocytes, an oocyte fraction enriched in cortical locRNAs, was isolated and analyzed by a series of density gradient centri-

fugations and by RNA–protein binding assays. We found that cortical locRNAs fractionate quite differently from other cellular RNPs as shown by *Cx1* RNA. While the latter is invariably found at the top of the high sucrose gradient, locRNAs are found in the heavy region of the gradient ($d = 1.24\,g/cm^3$) and in the pellet (Fig. 2A). *Cx1* RNA is a particularly good control in these experiments as it is present in hand-isolated animal and vegetal cortices, but recovered entirely in the soluble fraction. The large size of these locRNA-containing structures, their low buoyant density in gradients of Cs salts together with their high protein content (Figs. 4 and 5), their co-sedimentation with cytoskeletal proteins (Fig. 2B) and vera/*Vg1*-RBP protein (Fig. 3) clearly indicate a novel type of RNA-containing structure.

Based on the following evidence, we propose that the pellet structure is a complex of the RNA anchoring domain and the oocyte cortex. First, locRNAs are in tight association with the cortex as demonstrated by their retention in cortices hand-isolated from Stage VI oocytes [Elinson et al., 1993]. Furthermore, after extraction with the detergent, locRNPs remain in the pellet with cytoskeletal proteins, predominantly cytokeratins. Therefore, the locRNPs' structure can tolerate extraction under harsh conditions and the shear forces generated during centrifugation in concentrated sucrose. Third, protein material in the unfixed pellet continued to co-purify with locRNAs in the $CsSO_4$ gradient, indicating an integral RNA-protein structure (Fig. 5). Furthermore, pigment granules, organelles that are an integral part of the cortex, co-localized on these $CsSO_4$ gradients with locRNAs. Fourth, both the pellet and the major RNA peak at $1.24\,g/cm^3$ contain quite different ratios of RNA to protein, but both contain cytoskeletal proteins and vera, the *Vg1* RNA binding protein (Figs. 2B, 3). Vera has been shown to co-localize with *Vg1* RNA in the cortex [Deshler et al., 1997; Havin et al., 1998; Zhang et al., 1999]. The most likely interpretation of the latter observation is that the $1.24\,g/cm^3$ RNA peak is a product of further but selective fragmentation of the anchoring domain–cortex complex. Consistent with this interpretation, up to 60% of the locRNAs analyzed can be extracted into the soluble fraction during preparation of the DIF [Pondel and King, 1988] or by further extraction of the DIF. Perhaps most convincing, however, is the finding that additional extraction of the DIF resulted in the release of *Xlsirts* RNPs with the same pattern of crosslinked proteins as found for the DSF RNPs.

Comparison of the proteins bound to the *Xlsirts* localization signal (LS) in the cortex (DIF) but not in the soluble fraction (DSF) revealed four proteins (Mr~125, 74, 41, and 32 kD) that are candidate members of the putative *Xlsirts* anchoring complex. It is possible that some of these proteins are in common with those that bind the *Vg1* LS. For example, five proteins, p78, p69, p60, p36, p33, specifically bind the *Vg1* LS. Two of these have been sequenced and identified as vera/*Vg1*RBP (p69) and hnRNPI (p60) [Cote et al., 1999]. Vera or the 78 kD *Vg1* RNA binding protein may be one of the proteins cross-linked to *Xlsirts* in Figure 1C. Vera was found to co-sediment with locRNAs in whole oocyte lysates, being clearly enriched in the pellet and in the $1.24\,g/cm^3$ peak (Fig. 3B), the same fractions enriched in *Xcat*2, but not in control *Cx1* RNA [Zhang et al., 1999] (Fig. 3A). These results suggest that vera may be involved in the anchoring of locRNAs in the late stage oocytes. Significantly, vera was not detected in the DIF without a prior UV-crosslinking step, indicating its relatively weak association with locRNPs. The 125 kD protein appears to be distinct from those proteins that bind the *Vg1* LS and may reflect the differences in the *Vg1* and *Xlsirts* localization pathways [King et al., 1999]. It will be important to further identify and characterize this protein.

The discovery of four *Xlsirts*-crosslinked proteins specific to the DIF (Fig. 1c) may provide the basis for strong and complex binding of *Xlsirts* to the cortex. Significantly, the *Xlsirts* complex could not be reconstituted in vitro by simple incubation with the DIF under various ionic conditions (data not shown). This finding is consistent with the anchoring complex being formed through multiple protein–protein and RNA–protein interactions, providing kinetic stability. Such is certainly the case for many macromolecular complexes, e.g., ribosomes [Nierhaus, 1991] or Qβ replicase [Blumenthal and Carmichael, 1979]. At the same time, not all the RNAs are bound to the cortex with equal affinity. If the ratio of sedimenting-to-dissociated RNPs in the heavy sucrose gradient is compared (Table I), it can be seen that *Xcat*2 is the most strongly (3.13) and *DEADSouth* the most weakly (0.56) bound RNA in the anchor-

ing domain. This observation may reflect the difference in transacting factors involved in anchoring and, secondarily, in the regulation of their expression.

In situ hybridization results suggested that oocyte locRNAs were transported as part of large particles or complexes. It is formally possible that such complexes represent unique RNP structures that could be isolated on density gradients. However, we found that locRNAs in the soluble fraction are present in the form of RNPs with the same buoyant density as average ooplasmic RNPs, 1.43 g/cm^3 (Fig. 6). This observation is consistent with results found for a number of other locRNAs such as the B2 RNA in neurons [Kobayashi et al., 1991] and MBP RNA in oligodendrocytes [Colman et al., 1982]. Therefore, the large size of locRNPs suggested by in situ hybridization results, cannot be explained by the large size of a localization particle containing a unique RNP [Kloc et al., 1996; Zhou and King, 1996b]. We suggest that locRNAs are most likely organized and anchored (and may be transported as well) as typical RNPs which are locally compartmentalized in patches in particular regions of the RNA anchoring domain. Distribution of different, but overlapping RNA patches in the cortex seems most likely as all of the six locRNAs studied in this work had the same sedimentation profile in the heavy sucrose gradient (Fig. 2a). This was true even when the DIF was resolved into three or more additional minor peaks (data not shown). However, from our results we cannot say whether RNPs are anchored individually or in complexes as has been shown for MBP RNA which is associated with components of the translational machinery in oligodendrocytes [Barbarese et al., 1995].

Characterization of the locRNA-containing structures in the oocyte DIF as described in this paper provides the first biochemical analysis of the RNA anchoring step in *Xenopus* oocytes. It appears that RNAs are strongly anchored as 1.43 g/cm^3 RNPs in overlapping regions of the cortex. The anchoring of *Xlsirts* in the cortex appears to be mediated by several proteins that could promote kinetically stable and complex interactions with the cortical cytoskeleton. Presently, we are trying alternative ways to isolate the major RNA peak identified in the heavy sucrose gradient in amounts sufficient for further investigation of the anchoring domain. These studies should provide valuable information about the mechanism of RNA anchoring.

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