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A two-step model for the localization of maternal mRNA in *Xenopus* oocytes: Involvement of microtubules and microfilaments in the translocation and anchoring of Vg1 mRNA

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Summary

In an effort to understand how polarity is established in *Xenopus* oocytes, we have analyzed the process of localization of the maternal mRNA, Vg1. In fully grown oocytes, Vg1 mRNA is tightly localized at the vegetal cortex. Biochemical fractionation shows that the mRNA is preferentially associated with a detergent-insoluble subcellular fraction. The use of cytoskeletal inhibitors suggests that (1) microtubules are involved in the translocation of the message to the vegetal hemisphere and (2) microfilaments are important for the anchoring of the message at the cortex. Furthermore, immunohistochemistry reveals that a cytoplasmic microtubule array exists during translocation. These results suggest a role for the cytoskeleton in localizing information in the oocyte.

Key words: localization, maternal mRNA, Vg1, polarity, microtubules, microfilaments, *Xenopus* oocytes.

Introduction

The animal–vegetal (A–V) axis of *Xenopus* oocytes becomes the primary axis of the embryo after fertilization. It is along the A–V axis that the only known developmental differences exist in an unfertilized egg, with ectoderm being formed from the animal hemisphere and endoderm from the vegetal hemisphere (Nakamura *et al.* 1970). The dorsal-ventral axis, established by cortical rotation after fertilization, forms orthogonal to the A–V axis (Vincent and Gerhart, 1987). The asymmetric orientation of the mitochondrial cloud opposite the chromatid attachment site in primordial germ cells is thought to presage the A–V polarity observed in oocytes (Al-Mukhtar and Webb, 1971; Heasman *et al.* 1984). This polarity is interpreted in oocytes so that organelles and maternally encoded factors, such as mRNA and protein, necessary for the development of particular regions of the early embryo will have attained their proper spatial organization by the end of oogenesis. How the A–V polarity is generated and interpreted is not understood.

Intracellular localization of specific mRNAs has been reported in a number of different cell types. Cytoplasmic actin mRNA is localized to the lamellipodia of fibroblasts in culture, where active actin protein synthesis is occurring (Lawrence and Singer, 1986). RNA encoding MAP2, a dendrite-specific microtubule-associated protein, is enriched in dendrites in the developing brain (Garner *et al.* 1988). In *Drosophila*, both zygotic and maternal mRNAs have been identified which are localized to particular regions of the syncytial blastoderm or egg. Transcripts of a number of pair-rule genes, such as *fushi tarazu*, *hairy*, and *evenskipped*, are localized apically above the nucleus in embryos before cellularization (Akam, 1987). Two localized, maternal mRNAs in *Drosophila*, *bicoid* and *nanos*, are localized at the anterior and posterior ends of the egg, respectively, and the spatial organization of their protein products is thought to be essential in setting up the basic body plan of the fly (Nüsslein-Volhard *et al.* 1987; Driever and Nüsslein-Volhard, 1988; MacDonald and Struhl, 1988; R. Lehmann, personal communication).

In *Xenopus*, several localized, maternal mRNAs have been isolated (Rebagliati *et al.* 1985). The best characterized of these is Vg1, a vegetally localized message whose protein product is a member of the TGFβ family (Melton, 1987; Weeks and Melton, 1987). Several TGFβ-like molecules have been implicated in the process of mesodermal induction (Smith *et al.* 1988; Rosa *et al.* 1988), and our recent unpublished results suggest that Vg1, like heterologous TGFβ-1 (Kimelman and Kirschner, 1987), enhances the induction of mesoderm by FGF. Distributed homogeneously in young oocytes, Vg1 mRNA is translocated in early stage IV oocytes to a tight shell along the vegetal cortex (Melton, 1987), a region approximately 5μm in depth consisting of the plasma membrane and associated internal material (Franke *et al.* 1976). Following hormonal maturation of an oocyte to an egg, Vg1 mRNA is released

from the tight cortical shell and forms a broader band in the vegetal hemisphere which remains in vegetal cells throughout early embryogenesis (Weeks and Melton, 1987). Translation of Vg1 protein product begins toward the end of oogenesis and continues until gastrulation (Dale *et al.* 1989; Tannahill and Melton, 1989). Throughout early development, Vg1 protein is largely confined to the vegetal hemisphere of embryos, where it is glycosylated and associated with membranes. Vg1 mRNA and protein are thus both located in the region important for the induction of mesoderm during the period when this induction is occurring (Nieuwkoop, 1969; Smith, 1989).

Regardless of its role in development, understanding how Vg1 mRNA is properly localized should provide information on the process of localization of mRNA in particular, and on the interpretation of polarity within cells in general. Injection of exogenous Vg1 mRNA has shown that *cis*-acting sequences in the Vg1 mRNA are important for its localization (Yisraeli and Melton, 1988). The cellular machinery involved in the localization process has not yet been elucidated. In an effort to dissect the process into its component parts, we have examined the involvement of the oocyte cytoskeleton in attaining the proper distribution of Vg1 message. Our data suggest a two-step model for the localization of Vg1 mRNA in which translocation of Vg1 message to the vegetal cortex is dependent on cytoplasmic microtubules and anchoring is achieved with the involvement of cortical microfilaments.

Materials and methods

Oocyte culture and drug treatment

Albino frogs were anesthetized with 0.5 g benzocaine (Sigma) in 1 l of water, and late stage III oocytes (0.5–0.6 mm as sized by an ocular micrometer) were manually isolated in 1X modified Barth's saline–Hepes (MBSH; Gurdon, 1968) supplemented with 0.1 g l^{-1} penicillin/streptomycin. Oocytes were then transferred in a sterile fashion to Terasaki plates (Vangard International), one to two oocytes per well, and cultured for five days at 20 °C in a humidified chamber in 0.5X Leibowitz medium supplemented with 1 mM-L-glutamine, 1 μg ml^{-1} insulin, 15 mM-Hepes (pH 7.8), 50 units ml^{-1} nystatin, 100 μg ml^{-1} gentamycin, 100 μg ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 10 % frog serum containing vitellogenin (Wallace *et al.* 1980).

Frog serum containing vitellogenin was obtained from frogs injected three weeks earlier with 0.4 ml/100 g body weight of a 10 mg ml^{-1} solution of estradiol-17 β (Sigma) suspended in propylene glycol (Wallace *et al.* 1980). Blood was collected by heart puncture, allowed to coaggulate for several hours at room temperature, and then spun in an analytical table top centrifuge (~2000 revs min^{-1}) to isolate serum. On average, 3–4 ml of green serum was obtained per frog, which was immediately aliquoted and frozen until use.

Cytoskeletal inhibitors were added when the oocytes were seeded and whenever the medium was changed (usually the first and third days after seeding) at the following concentrations: 25 μg ml^{-1} cytochalasin B, 1 μg ml^{-1} nocodazole, 1 mg ml^{-1} colchicine, 1 μg ml^{-1} tubulozole-C, and 1 μg ml^{-1} tubulozole-T (all were suspended in DMSO except for colchicine, which was dissolved in ethanol). All concentrations of inhibitors were chosen based on previous reports in which these drugs were effective (Coleman *et al.* 1981; Kimelman *et al.* 1987; Geuens *et al.* 1985), with the exception of nocodazole, which we found to be equally effective over a 10-fold range of concentrations (10 to 1 μg ml^{-1}).

Stage VI oocytes were isolated in the same way as the smaller oocytes but were cultured overnight in 1X MBSH in 96-well plates using the same concentrations of drugs as with middle stage oocytes. Maturation of stage VI oocytes was achieved *in vitro* by treating with 1 μM-progesterone (in ethanol) for approximately 8–10 h (Holwill *et al.* 1987), monitoring germinal vesicle breakdown in cocultured pigmented oocytes. All oocyte stages mentioned in this paper are according to Dumont (1972).

Oocyte extractions and RNA analysis

Using the basic protocol of Jeffery and Meier (1983), we varied the detergent, salt concentration and temperature of oocyte extractions in order to optimize conditions for the specific partitioning of Vg1 mRNA into the insoluble fraction and fibronectin mRNA into the soluble fraction. Although Brij, NP40, and Triton X-100 all solubilized fibronectin mRNA well, 0.5 % Triton gave the best recovery of Vg1 mRNA in the insoluble pellet fraction. Salt concentrations below 0.3 M-KCl do not solubilize yolk, while increasing concentrations yield higher percentages of non-localized fibronectin mRNA in the insoluble fraction. Temperatures from 4 °C to room temperature (23 °C) had little effect on the fractionation, and hence room temperature was chosen for the extractions so as not to disrupt microtubules. The optimized procedure is as follows. Ten oocytes were homogenized in 0.5 ml of buffer containing 0.5 % Triton X-100, 10 mM-Pipes (pH 6.8), 0.3 M-KCl, 10 mM-magnesium acetate, 0.5 mM-EGTA, 10 μg of yeast tRNA and 20 mM-vanadyl ribonucleoside complexes. After incubation at room temperature for 5–10 min, the lysate was spun at 13 000 revs min^{-1} for 5 min. To prepare RNA from the insoluble fraction, the pellet was resuspended in 0.4 ml of buffer A (50 mM-Tris–HCl (pH 7.5), 0.1 M-NaCl, 10 mM-EDTA, 0.5 % SDS, 10 μg of yeast tRNA and 400 μg per ml of proteinase K) and digested for 1 h at 45 °C. The RNA was then extracted twice with phenol/chloroform (1:1) and precipitated with 0.1 volumes of 3 M-sodium acetate and 2.5 volumes of cold ethanol. The RNA from the soluble fraction of the original lysate was prepared by diluting the supernatant 1:4 with buffer A and processing as described above. The relative amounts of poly (A)+ RNA in the fractions was determined by incorporation of labeled nucleotide in a reverse transcription reaction primed with oligo (dT).

RNA samples prepared from pellets, supernatants and unfractionated lysates as a control were electrophoresed in denaturing formaldehyde agarose gels and transfered to GeneScreen as described (Rebagliati *et al.* 1985). In all cases, the same number of oocyte equivalents of RNA was loaded onto each lane of the gel. Northern blot analysis was performed with ^{32}P-labeled anti-sense Vg1 (Weeks and Melton, 1987) and fibronectin probes (Krieg and Melton, 1985). Quantification of the relative amount of signal in the pellet and soluble fractions was performed by scanning the autoradiographic film with a densitometer.

In situ *hybridization*

Oocytes were fixed, sectioned and hybridized as previously described (Melton, 1987). Animal–vegetal orientation of the albino oocyte sections was determined by examining serial sections for the position of the germinal vesicle and the distribution of yolk platelets. A detailed protocol for *in situ*

hybridization is being published elsewhere (O'Keefe *et al.* in press). The probe used for all the *in situ* hybridizations presented here was a ^{32}P-antisense T7 transcript to the entire coding region of Vg1 (see Weeks and Melton, 1987). All *in situ* hybridizations shown here are dark-field photographs; the silver grains appear white under these optical conditions.

Whole-mount immunohistochemistry

Oocytes were defolliculated in 0.5% collagenase type IV (Sigma) in 1X PBS at room temperature, with gentle rocking. Oocytes were fixed and stained as described by Dent *et al.* (1989), using a horseradish peroxidase-conjugated goat anti-mouse second antibody (Cappel) to visualize the monoclonal anti-β tubulin antibody (a gift from M. Klymkowsky). Control oocytes, incubated with either the second antibody alone or in conjunction with a monoclonal antibody for a muscle-specific marker not present in oocytes (12101, a gift from C. Kintner), showed no appreciable staining.

Results

Association of Vg1 mRNA with a detergent-insoluble fraction of oocyte extracts at specific times during oogenesis

Vg1 mRNA is initially distributed homogeneously throughout the cytoplasm of small oocytes and undergoes localization to the vegetal cortex as the oocyte grows from 0.6 to 0.8 mm in diameter (early stage IV) (Fig. 1A; Melton, 1987; Yisraeli and Melton, 1988). Because injected Vg1 message, which undergoes a similar localization in cultured oocytes, and endogenous message appear to maintain steady-state RNA levels throughout localization, the accumulation of Vg1 mRNA at the vegetal cortex seems to be a translocation process rather than localized degradation of the message. Translocation of other macromolecules and organelles, in different cell types of many organisms, is known to involve cytoskeletal elements (see below). In order to determine whether Vg1 mRNA might be associated with a particular subcellular compartment of oocytes or eggs, detergent extracts of oocytes were analyzed for Vg1 mRNA.

The RNA from detergent-soluble and insoluble (pellet) fractions was isolated and analyzed by Northern blot analysis (Fig. 1B). Oocytes in which Vg1 mRNA localization has occurred (stage VI, lanes 4–6) contain a large majority of their Vg1 mRNA (approximately 80%) in the insoluble fraction (lane 4). In eggs (lanes 4–7), however, where *in situ* hybridizations show that Vg1 mRNA is released from its tight cortical shell, Vg1 mRNA is found almost exclusively in the soluble fraction (lane 8). Other messages, such as fibronectin, which are uniformly distributed, are found almost completely in the soluble fraction of extracts from both of these stages (approximately 90–95%, lanes 5 and 8). In young oocytes (stage II, lanes 1–3), both Vg1 and fibronectin mRNA are found in the detergent soluble and insoluble fractions in about equal proportions. The presence of fibronectin RNA in detergent insoluble pellets of young oocytes was repeatedly observed, but cannot be explained by the same mechanism that serves to localize Vg1 RNA since fibronectin RNA is uniformly distributed in the cytoplasm. The insoluble fraction, which is generally considered to contain most of the cytoskeletal elements of the cell (Schliwa *et al.* 1981; Jeffery and Meier, 1983), contains only 2–5% of the total poly (A)+ RNA in stage VI oocytes (see Experimental Procedures). Inasmuch as the same number of oocyte-equivalents was loaded in each lane on the gel in Fig. 1B, we can calculate that, per μg of total poly (A)+ RNA, Vg1 mRNA is enriched approximately 100-fold in the detergent-insoluble fraction of only those oocytes that contain localized Vg1 mRNA. In contrast, fibronectin mRNA shows no detectable enrichment in the detergent-insoluble fraction at this stage. These results are similar to those reported by Pondel and King (1988), although the data presented here suggest a much higher enrichment of Vg1 mRNA in the insoluble fraction.

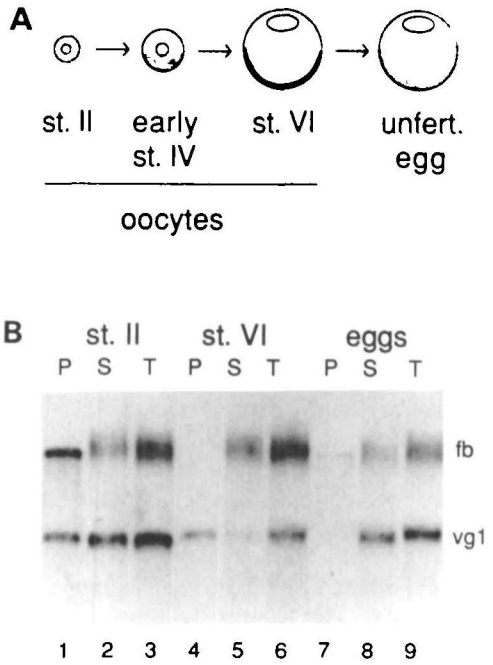

Fig. 1. Specific association of Vg1 mRNA with the detergent-insoluble fraction of extracts. (A) Schematic representation of the distribution of Vg1 mRNA in oocytes and eggs. Vg1 mRNA (dark shading in picture) is initially distributed homogeneously in early stage oocytes (stage II), undergoes a process of localization in middle stage oocytes (early stage IV), and culminates in a tight cortical shell by the end of oogenesis (stage VI; Melton, 1987). Unfertilized eggs have Vg1 mRNA distributed in a broad band in the vegetal hemisphere along the cortex (egg; Weeks and Melton, 1987). (B) Northern blot analysis of RNA detergent-extracted from oocytes and unfertilized eggs. Oocytes or eggs were homogenized and fractionated into a soluble fraction and an insoluble pellet by centrifugation, as described in Materials and methods. RNA was then purified from the pellet (P, lanes 1,4, and 7), the soluble fraction (S, lanes 2, 5, and 8) or total, unfractionated extract (T, lanes 3, 6, 9) and analyzed by Northern blot hybridization with both a fibronectin (fb) and Vg1 probe. Two oocyte-equivalents of RNA was run in each lane. Lanes 1–3, from stage II oocytes; lanes 4–6, from stage VI oocytes; and lanes 7–9, from unfertilized eggs.

Effect of cytoskeletal inhibitors on the localization of Vg1 mRNA in late stage oocytes

The biochemical studies described above and those of Pondel and King (1988) suggest that cytoskeletal elements may be involved in the localization of Vg1 mRNA. The specific effects of a number of cytoskeletal inhibitors are well established. Cytochalasin B binds to the barbed end of actin filaments and disrupts their normal organization (Cooper, 1987). Nocodazole and colchicine bind specifically to tubulin and disrupt microtubules (Wilson and Bryan, 1974). To explore further how Vg1 mRNA interacts with the cytoskeleton, late stage (stage V/VI) oocytes were treated overnight with various inhibitors, fixed the next morning, and analyzed by *in situ* hybridization (Fig. 2). Oocytes incubated in saline (Fig. 2A), nocodazole (Fig. 2C), or colchicine (data not shown) contain Vg1 mRNA distributed in a tight cortical shell in the vegetal hemisphere, just as seen in stage V/VI oocytes *in vivo* (see Fig. 1A). Cytochalasin B treatment, however, causes a release of Vg1 message from this tight distribution, resulting in a broad band of Vg1 mRNA along the cortex (Fig. 2D). The degree to which the message disperses is somewhat variable, but generally seems to be limited to the vegetal hemisphere. In late stage oocytes, actin microfilaments are found predominantly along the cortex (Franke *et al.* 1976). Our results suggest that cytochalasin B causes a release of Vg1 mRNA from its normally tight cortical distribution by disrupting cortical microfilaments and thereby allowing passive diffusion of the message.

Vg1 mRNA in unfertilized eggs is distributed in a broad band in the vegetal hemisphere (Weeks and Melton, 1987), similar to that seen after cytochalasin B treatment. To determine when this release occurs during development, stage VI oocytes were matured *in vitro* by culturing in the presence of progesterone, which releases the oocyte from its block at first meiotic prophase and, among many other changes, induces germinal vesicle breakdown (Fig. 2B). Clearly, the distribution of Vg1 mRNA in progesterone-matured oocytes is indistinguishable from that seen either in cytochalasin-B-treated oocytes (Fig. 2D) or in unfertilized eggs (Weeks and Melton, 1987). Thus, the release of Vg1 from its tight cortical position is directly connected to hormonal maturation of oocytes. The cytochalasin B induced release of Vg1 mRNA is not a result of maturation, however, as can be seen from the presence of the germinal vesicle in those sections (Fig. 2D). It is interesting to note that the few pigment granules present in the vegetal cortex undergo the same sort of inward migration upon maturation as does Vg1 mRNA (Hausen *et al.* 1985), suggesting that the cytoskeletal reorganization known to occur during maturation (Wylie *et al.* 1985; Dent and Klymkowsky, 1988) is responsible for both phenomena.

Vg1 mRNA is associated with the detergent-insoluble fraction of late stage oocytes (Fig. 1B). To test whether cytoskeletal inhibitors can affect this association, RNA was isolated from the soluble and insoluble fractions of detergent extracts of drug-treated stage VI oocytes and analyzed as above by Northern blot analysis (Fig. 3). As expected from the *in situ* hybridizations, neither nocodazole (lane 4) nor colchicine (data not shown) had any effect on the presence of Vg1 mRNA in the insoluble fraction. In accordance with its effect on the distribution of Vg1 message, cytochalasin B treatment caused a release of most of the Vg1 mRNA into the soluble fraction (lane 8). Densitometer tracings of Northern blots show that an average of 65% of Vg1 RNA is found in the detergent insoluble fraction in untreated stage VI oocytes, whereas only 28% of Vg1 RNA is found in the pellet after cytochalasin B treatment. Although the intracellular distribution of Vg1 mRNA is similar in both unfertilized eggs and cytochalasin B-treated oocytes, not all of the Vg1 message is released into the soluble fraction after cytochalasin B treatment, as appears to be the case after maturation (see Fig. 1B). This remnant of Vg1 mRNA in the detergent-insoluble fraction may imply that other cyto-

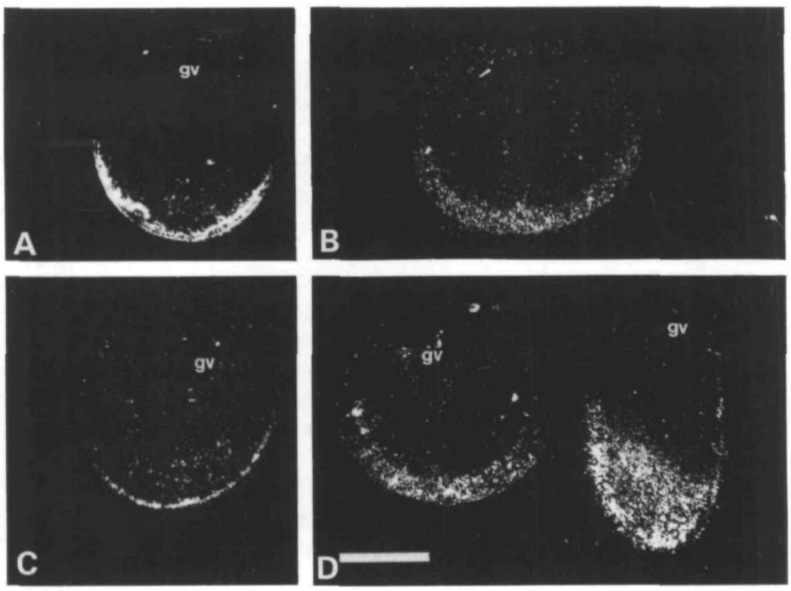

Fig. 2. Vg1 mRNA distribution in drug-treated, late-stage oocytes. *In situ* hybridization using a Vg1 probe reveals the localization of the message in oocytes incubated overnight in saline (A), progesterone (B), nocodazole (C), or cytochalasin B (D). The scale bar indicates 500 μm. The germinal vesicle (gv) is indicated in those sections where it is present.

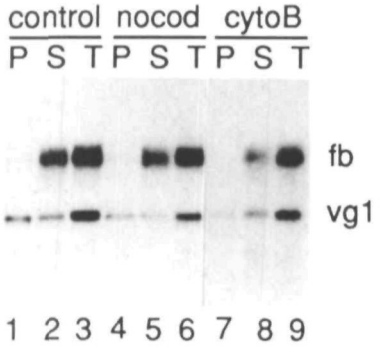

Fig. 3. Specific release of Vg1 mRNA into the soluble fraction of detergent extracts by treating with cytochalasin B. Stage VI oocytes cultured overnight in saline with no drug treatment (control; lanes 1–3), with nocodazole (nocod; lanes 4–6), or with cytochalasin B (cytoB; lanes 7–9) were homogenized and their RNA analyzed as in Fig. 1. RNA purified from the insoluble pellet was run in lanes 1, 4, and 7, from the soluble fraction in lanes 2, 5, and 8, and from total extracts in lanes 3, 6, and 9.

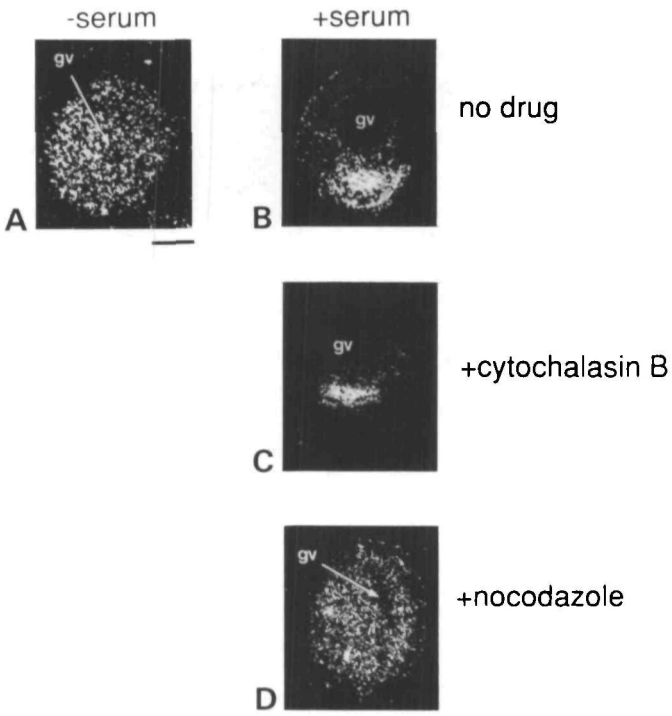

Fig. 4. The effects of cytoskeletal inhibitors on the translocation of Vg1 mRNA in middle stage oocytes. Late stage III oocytes (0.5–0.6 mm in diameter) were cultured *in vitro* for 5 days and then assayed for Vg1 mRNA distribution by *in situ* hybridization. Oocytes were grown either in medium without serum (A) or in medium with serum, either with no drug (B) or in conjunction with cytochalasin B (C) or nocodazole (D). The scale bar represents 200 μm.

skeletal elements not sensitive to cytochalasin B are associated with the message. In fact, Pondel and King (1988) have shown that the insoluble fraction from detergent extracts of late stage oocytes is enriched in cytokeratins and vimentin. Nevertheless, we can conclude that at least one component of the structure holding the Vg1 mRNA at the cortex is sensitive to cytochalasin B.

Effects of cytoskeletal inhibitors on the distribution of Vg1 mRNA in middle stage oocytes

Examining the effects of cytoskeletal inhibitors on late stage oocytes provided information about the mechanism for anchoring Vg1 message at the cortex. In order to analyze the process of translocation of the message, it was necessary to use an *in vitro* system for culturing middle stage oocytes which are just beginning to undergo localization. Immature oocytes grown *in vitro* in the presence of serum from estradiol-injected frogs increase in diameter as a result of the uptake of vitellogenin, a yolk protein precursor, from the serum (Wallace *et al.* 1980). In addition, the changes that normally occur during oogenesis *in vivo* occur in cultured oocytes as well, including the migration of the germinal vesicle to the animal hemisphere and the localization of Vg1 message to the vegetal cortex (Yisraeli and Melton, 1988).

Late stage III oocytes were cultured in the presence of various cytoskeletal inhibitors for 5 days and then analyzed by *in situ* hybridization (Fig. 4). Oocytes grown in the presence of the vitellogenin-containing serum but without any inhibitors demonstrate a marked localization of the Vg1 message at the vegetal cortex (Fig. 4B), as compared with either uncultured oocytes (data not shown) or those cultured in medium without serum (Fig. 4A). Notably, cytochalasin B treated oocytes are also capable of translocating Vg1 mRNA (Fig. 4C), although in these oocytes, the Vg1 message accumulates in the vegetal cytoplasm and not in a tight subcortical shell. A possible explanation for this atypical distribution is that the Vg1 mRNA cannot be anchored at the cortex when microfilaments are disrupted (see Fig. 2D).

The translocation of Vg1 message can be disrupted by drugs that interfere with microtubule polymerization; both nocodazole (Fig. 4D) and colchicine (data not shown) completely prevent translocation of the Vg1 mRNA. Despite the dramatic and specific effects of the microtubule inhibitors on Vg1 mRNA localization, no effect on the viability of the oocytes was detected with any of the drugs, as assayed by comparing the profile of proteins synthesized *in vitro* on the last day of the culture (data not shown). In addition, the amounts of Vg1 mRNA, as assayed on Northern blots, are constant and unaffected by any of the treatments (data not shown). Thus, cytoskeletal elements sensitive to nocodazole and colchicine, but not those sensitive to cytochalasin B, are evidently associated with the translocation of Vg1 mRNA in middle stage oocytes. In other words, these data implicate microtubules as being involved in the movement of Vg1 message to the vegetal hemisphere.

Further evidence for the specific involvement of microtubules in the movement of Vg1 mRNA comes from the use of a relatively new tubulin-binding drug

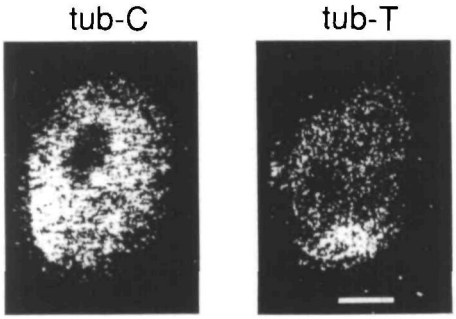

Fig. 5. The specific effect of depolymerizing microtubules on the translocation of Vg1 mRNA in middle stage oocytes. Late stage III oocytes were cultured *in vitro* in medium containing serum and either the potent microtubule depolymerizing agent tubulozole-C (tub-C) or its inactive *trans* isomer tubulozole-T (tub-T) and assayed as in Fig. 4. The scale bar represents 200 μm.

and its *trans* isomer. Tubulozole-C is a photoisomer of colchicine produced by UV irradiation, which reversibly depolymerizes microtubules; tubulozole-T, its *trans* isomer, does not inhibit microtubule polymerization even at concentrations well above those of tubulozole-C (Geuens *et al.* 1985). Oocytes cultured in tubulozole-T grow normally and show normal localization of Vg1 mRNA. Those grown in tubulozole-C, however, show no movement of the message and appear identical to oocytes treated with other microtubule inhibitors (Fig. 5). These data suggest that the inhibition of translocation is a consequence of depolymerizing microtubules, not some side effect of the drugs.

Oocytes grow in diameter, both *in vivo* and *in vitro*, by receptor-mediated endocytosis of vitellogenin from the serum (Wallace and Jared, 1976). In our earlier work, we noted a strong correlation between the size of an oocyte, grown either *in vivo* or *in vitro*, and the degree of localization of Vg1 mRNA (Yisraeli and Melton, 1988). It is formally possible, therefore, that drugs that prevent the growth of oocytes might prevent the localization of Vg1 mRNA as well. Both microtubule and microfilament inhibitors severely reduced the growth of stage III oocytes in culture (Table 1), perhaps by interfering with normal endocytosis (e.g. see Dustin, 1978). Nevertheless, it is clear from the data presented in Table 1 that it is possible to uncouple growth and Vg1 localization. Cytochalasin B-treated oocytes, despite their poor growth, translocate Vg1 mRNA. Also, oocytes cultured in the presence of fetal calf serum without any vitellogenin do not grow at all but show normal localization of Vg1 message (Table 1 and data not shown); this result further suggests that vitellogenin uptake is not important for translocation. In light of those results, it is interesting that oocytes cultured in medium alone without any serum are unable to localize Vg1 mRNA. How serum stimulates localization of the message remains a mystery. It is clear, however, that the lack of localization in oocytes cultured in the presence of microtubule inhibitors is not a result of simply preventing growth of the oocyte.

Table 1. *Growth of oocytes cultured under various conditions*

Growth medium	% increase in volume (*n*)
Saline alone	0 (12)
Medium alone	0 (18)
Medium+frog serum (containing vitellogenin)	54 (20)
Medium+frog serum+cytochalasin B	10 (20)
Medium+frog serum+colchicine	5 (23)
Medium+frog serum+nocodazole	11 (22)
Medium+frog serum+tubulozole-C	11 (23)
Medium+frog serum+tubulozole-T	36 (12)
Medium+fetal calf serum	5 (13)

Late stage III oocytes (0.5–0.6 mm in diameter) were cultured for 5 days in saline (1X MBSH), medium (supplemented Leibowitz medium), or medium with serum, with the indicated drug, as described in Materials and Methods. The increase in volume was calculated for each group of oocytes by cubing the ratio of the diameter of the oocytes at the end of the incubation period to the diameter of the oocytes at the beginning of the incubation period. The data shown represent the average from three independent experiments.

Visualization of the microtubule array in oocytes

Although the drug studies provide strong evidence for the involvement of cytoskeletal elements in both the translocation and anchoring of Vg1 mRNA, it is important to know that the appropriate cytoskeletal structures are present at the right time in the proper place. As mentioned above, actin filaments are highly enriched in the cortical region of oocytes, where they appear to be arranged in 'whirls' (Franke *et al.* 1976). Coleman *et al.* (1981), using electron microscopy, have demonstrated that cytochalasin B, but not colchicine, completely disrupts cortical microfilaments in late stage oocytes. Thus, in stage VI oocytes, not only does cytochalasin B release Vg1 mRNA from its tight localization at the cortex but also disrupts the array of cortical microfilaments.

The presence of a radial tubulin array in oocytes was first reported by Palecek *et al.* (1985) using immunohistochemistry on oocyte sections. The location and arrangement of tubulin in oocytes during oogenesis has also been visualized using the whole-mount procedure developed by Klymkowsky and colleagues (Dent and Klymkowsky, 1988). We have employed the latter technique to monitor the appearance of the array and its sensitivity to cytoskeletal inhibitors. As seen in Fig. 6A, a radial tubulin array, emanating from the germinal vesicle and extending throughout the cytoplasm to the periphery of the oocyte, is first evident in stage II oocytes, using an anti-β tubulin antibody; these structures are thus present before translocation of the Vg1 message begins. This array is fairly symmetric in the early and middle stage oocytes, but as the germinal vesicle moves up to the animal hemisphere, the staining begins to disappear from the vegetal region (Fig. 6C). (By this point in oogenesis, Vg1 mRNA is already completely localized). In stage V/VI oocytes, the radial array persists, emanating from the germinal vesicle, but

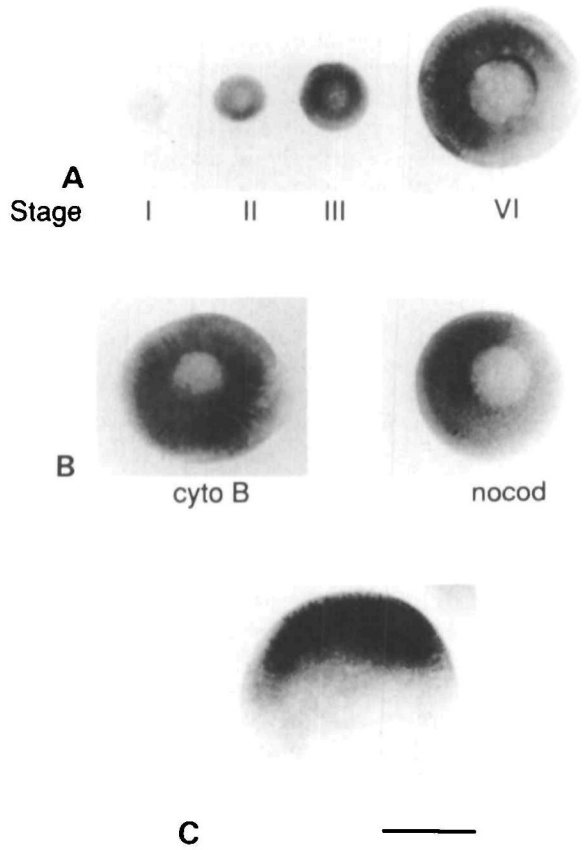

Fig. 6. Whole-mount immunohistochemistry of β-tubulin in oocytes. Oocytes were defolliculated, fixed and stained using an anti-β-tubulin antibody according to the procedure of Dent *et al.* (1989). (A) Untreated oocytes of the indicated stages were stained. The staining in stages II and III is radially symmetric. The stage VI oocyte is shown viewed from the animal hemisphere. (B) Stage VI oocytes were incubated in either cytochalasin B (cyto B) or nocodazole (nocod) overnight in saline and then processed as above. Both are shown as viewed from the animal hemisphere. The punctate staining of the nocodazole-treated oocyte, evidence for the disruption of the microtubule array, is detectable only in the animal hemisphere. The scale bar for A and B represents 500 μm. (C) A lateral view of an untreated stage VI oocyte at slightly higher magnification showing the tubulin array emanating from around the gv evident only in the animal hemisphere. The scale bar represents 500μm.

is detectable in only the animal hemisphere (Fig. 6A). The microtubule nature of the array is evident from its sensitivity to depolymerizing drugs such as nocodazole; both stage VI and stage IV oocytes treated with these drugs have completely disrupted arrays and show only punctate staining (Fig. 6B and data not shown). No effects on the tubulin array were detected when oocytes were incubated in the presence of cytochalasin B (Fig. 6B). Thus, a cytoplasmic tubulin array that is sensitive to microtubule inhibitors is present throughout the oocyte during the period of translocation of Vg1 mRNA.

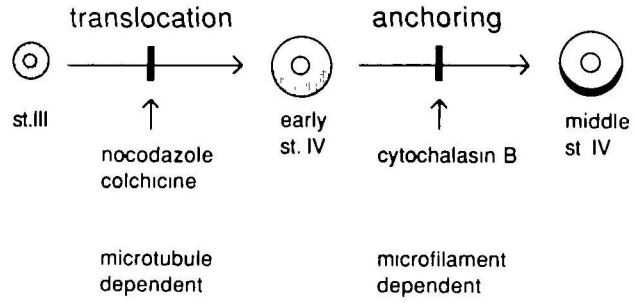

Fig. 7. A two-step model for the localization of Vg1 mRNA in oocytes. The diagram represents the simplest interpretation of the data presented in the paper. Vg1 mRNA distribution (indicated by shading) becomes progressively restricted to the vegetal cortex during normal oogenesis (indicated by the horizontal arrows). Microtubule inhibitors, such as nocodazole or colchicine, block the first part of this process, which we have termed translocation, and result in the prevention of any noticeable migration of Vg1 mRNA. Microfilament inhibitors, such as cytochalasin B, prevent the second step of the localization, which we have called anchoring, and result in the release, in late stage oocytes, of the Vg1 mRNA from its tight cortical shell into a broad band along the cortex. (In middle stage oocytes when the translocation process is occurring, cytochalasin B prevents anchoring at the cortex as well and results in the ectopic accumulation of Vg1 mRNA in the cytoplasm above the cortex; see Fig. 4C).

Discussion

The two-step model diagrammed in Fig. 7 integrates the data presented here. We propose that Vg1 mRNA is associated with microtubules in middle stage oocytes and that this association is necessary for the translocation of Vg1 mRNA to the vegetal hemisphere. The anchoring of Vg1 message at the cortex requires the involvement of cortical actin microfilaments. Once Vg1 mRNA is anchored at the vegetal cortex, its distribution is not susceptible to disruption by nocodazole or colchicine. In fact, oocytes that have only partially localized Vg1 mRNA do not release the localized RNA when the oocytes are cultured with microtubule inhibitors, even though further localization is prevented (data not shown). Previously, we noted that the anchoring process seemed to be saturated when excess, exogenous Vg1 mRNA was injected into oocytes, even though the translocation process continued to function (Yisraeli and Melton, 1988). This observation is consistent with the model presented here.

The use of inhibitors that affect specific cytoskeletal elements does not allow us to conclude that Vg1 mRNA or mRNPs interact directly with microtubules during translocation or microfilaments during anchoring. It is certainly possible that microtubules and microfilaments act indirectly through contact with other cytoplasmic components. Nonetheless, these experiments do demonstrate a requirement for intact microtubules and filaments to move and anchor Vg1 mRNA.

An association between mRNAs and cytoskeletal

elements has been noted in a number of different cell types (Lenk *et al.* 1977; Cervera *et al.* 1981; Jeffery, 1984). In general, these reports have found that the bulk of mRNA (or, in some cases, polyribosomes) is not washed away from the cytoskeleton in detergent extracts. The data presented here, however, as well as those of Pondel and King (1988), suggest that Vg1 mRNA is a member of a very small group of mRNAs (less than 5% of the total poly (A)+ RNA) that is associated with the subcellular fraction of stage VI oocytes and is insoluble in detergent. Furthermore, our results demonstrate that Vg1 mRNA can be released from this association by incubation with cytochalasin B, implying a role for microfilaments in this process. The discrepancy between the findings with oocytes and with other cells may reflect the specialized nature of oocytes, which need to spatially organize developmentally important molecules before cleavage begins.

The involvement of microtubules in intracellular transport has become an area of intensive study. Long known for their role in ciliary movement (Warner, 1979), microtubule-based motors are also responsible for both anterograde and retrograde transport in axons (Okabe and Hirokawa, 1989), chromosome segregation during mitosis (Mitchison, 1989), pigment granule aggregation in chromatophores (McNiven and Porter, 1984), and general organelle transport in such disparate organisms as amoeba (Koonce and Schliwa, 1986) and sea urchins (Pryer *et al.* 1986). In *Drosophila* oocytes intact microtubules are required for cytoplasmic streaming (Gutzeit, 1986).

The data presented here provide evidence for microtubule-mediated RNA transport. How RNA in general, and Vg1 in particular, might move along a microtubule is not understood. Making the assumption that association of Vg1 mRNA with microtubules is not rate limiting, we can roughly estimate that the rate of movement of Vg1 mRNA in late stage III oocytes is on the order of 100 μm per day. Clearly, the rates reported for fast axonal transport of proteins and organelles along microtubules are several orders of magnitude faster than that estimated for Vg1 mRNA (50–400 mm per day; Lasek *et al.* 1984). On the other hand, slow axonal transport rates, which are two to three orders of magnitude slower than fast transport, are similar to the hypothesized rate for Vg1 mRNA movement, although the molecular basis for this kind of transport is still unclear (Lasek *et al.* 1984; Vale, 1987). It is interesting to note that an RNA transport system similar to the one we observe in oocytes may exist in neurons. Using cultured hippocampal neurons pulse-labeled *in vitro* with [^{3}H]uridine, Davis *et al.* (1987) observed RNA migration specifically into dendrites, which can be identified by the presence of the microtubule-associated protein, MAP2. Detergent extraction does not remove the RNA from the dendrites, implying an association with the cytoskeleton, and the rate of RNA transport is approximately the same order of magnitude, 400–500 μm per day, as seen with Vg1. Recently, *in situ* hybridization has revealed that this transport of RNA may not be random; MAP2, but not tubulin, mRNA is found specifically localized in dendrites in the developing brain (Garner *et al.* 1988).

Movement along microtubule tracks has been suggested as a possible model for the cortical rotation that occurs in fertilized eggs before first cleavage (Elinson and Rowning, 1988). The microtubule array in fertilized eggs, however, is quite different from that seen in oocytes. In eggs, the microtubule array is much denser, and the density of the array is probably important for providing the necessary force. In addition, the array in eggs forms transiently in an oriented fashion running along the cortex. It is important to note that none of the evidence presented here proves that the microtubules in oocytes are providing the motive force for the translocation process. It is formally possible, for instance, that the microtubules might function as 'highways', facilitating movement as a result of some other force, perhaps even passive diffusion along the tubule.

The animal–vegetal axis of oocytes is determined well before the localization of Vg1 mRNA, perhaps as early as the primordial germ cell (Al-Mukhtar and Webb, 1971; Heasman *et al.* 1984). Now that some of the structural elements involved in localizing RNA have been elucidated, the question of how the polarity of the oocyte is interpreted becomes better defined. Several lines of evidence seem to implicate additional, specific factors in the process of spatially organizing the oocyte. Three mRNAs that are localized to the animal hemisphere in late stage oocytes were isolated by the same differential screening procedure that yielded Vg1 (Rebagliati *et al.* 1985). These mRNAs are initially distributed homogeneously throughout young oocytes and appear to undergo localization within more or less the same narrow period of oogenesis as does Vg1 message (O'Keefe *et al.* 1989). The movement of different RNAs in opposite directions at the same time, coupled with the ubiquitous nature of the microtubule array in stage III/IV oocytes, suggests that the directionality of the translocation process is achieved through the use of additional factors. In addition, although all of the information necessary for the specific localization of Vg1 mRNA resides in the naked RNA itself (Yisraeli and Melton, 1988), it is hard to imagine how microtubules could recognize these *cis*-acting sequences without the intervention of factors specific for the RNA. The search for these factors should be greatly aided by the identification of *cis*-acting sequences necessary for localization.

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