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The process of localizing a maternal messenger RNA in *Xenopus* oocytes

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Summary

The maternal mRNA Vg1 is localized to the vegetal pole during oogenesis in *Xenopus*. We have cultured oocytes *in vitro* to begin to understand how this localization occurs. Endogenous Vg1 mRNA undergoes localization when oocytes are cultured *in vitro*, and synthetic Vg1 mRNA injected into such oocytes is localized in the same fashion. Vg1 mRNA is associated with a detergent-insoluble fraction from homogenized oocytes, suggesting a possible cytoskeletal association. The use of cytoskeletal inhibitors reveals a two-step process for localizing Vg1 mRNA. Microtubule inhibitors such as nocodazole and colchicine inhibit the localization of Vg1 mRNA in late stage III/early stage IV oocytes, but have no effect on Vg1 mRNA once it is localized. The microfilament inhibitor cytochalasin B, however, has little effect on the translocation of Vg1 mRNA in middle-stage oocytes but causes a release of the message in late-stage oocytes. We propose a model for the localization of Vg1 mRNA in which translocation of the message to the vegetal cortex is achieved via cytoplasmic microtubules and the anchoring of the message at the cortex involves cortical microfilaments.

Key words: Vg1, TGF-β, RNA localization, polarity, microtubules, microfilaments, *Xenopus*.

Introduction

Xenopus oocytes have a clearly defined animal–vegetal (a–v) polarity. In previtellogenic oocytes, the mitochondrial cloud and the chromosomal attachment site in the germinal vesicle (gv) define a polarity that is proposed to be the future a–v axis (Al-Mukhtar and Webb, 1971; Heasman *et al.* 1984). Pigment granules migrate to the animal hemisphere in middle-stage, vitellogenic oocytes (Dumont, 1972), followed by the asymmetric deposition of dense yolk platelets in the vegetal hemisphere (Danilchik and Gerhart, 1987). Around this same time, the germinal vesicle migrates to near the animal pole from its location in the center of the oocyte. After fertilization, the dorsal–ventral axis is generated orthogonally to the a–v axis (Vincent and Gerhart, 1987), and it is along these primary axes that the germ layers become defined.

In addition to these morphological markers, numerous molecular asymmetries exist (see Gerhart, 1979). In particular, a small class of RNAs have been identified which are unevenly distributed in oocytes and eggs (Rebagliati *et al.* 1985; King and Barklis, 1985). The best studied of these RNAs is Vg1, a vegetally localized mRNA whose protein product is a member of the TGF-β family (Weeks and Melton, 1987). Vg1 is initially synthesized in previtellogenic oocytes and is uniformly distributed until the oocyte reaches the end of stage III (Melton, 1987). By the middle of stage IV, Vg1 mRNA is found almost exclusively in a tight shell at the vegetal cortex (Melton, 1987; Yisraeli and Melton, 1988). The RNA is released from its tight localization after maturation but remains in the vegetal hemisphere, presumably as a result of cellularization, throughout embryogenesis (Weeks and Melton, 1987).

We are interested in understanding how polarity is generated and interpreted in oocytes and eggs. By culturing oocytes *in vitro*, we have been able to analyze and interfere with the localization of Vg1 mRNA. Injected, *in vitro* synthesized Vg1 mRNA can be localized in cultured oocytes in a manner identical to the localization of the endogenous message, suggesting that all the information necessary for proper localization is encoded by the RNA itself. Insoluble pellets of detergent extracts of oocytes, which maintain most of the cytoskeletal elements of the cell, preferentially retain Vg1 mRNA. Using cytoskeletal inhibitors in middle- and late-stage oocytes, we have been able to distinguish two separate steps in the translocation process. The movement of Vg1 RNA to the vegetal hemisphere is inhibited by drugs which depolymerize microtubules, but not by those that affect microfilaments. Anchoring of the Vg1 mRNA, however, can be disrupted by microfilament inhibitors, but not microtubule inhibitors. Thus, at least in the case of Vg1 RNA, the oocyte utilizes common cytoskeletal elements to help generate specific asymmetries in the cell.

Endogenous Vg1 mRNA is localized in cultured oocytes

Large oocytes cultured in the presence of viteliogenin-

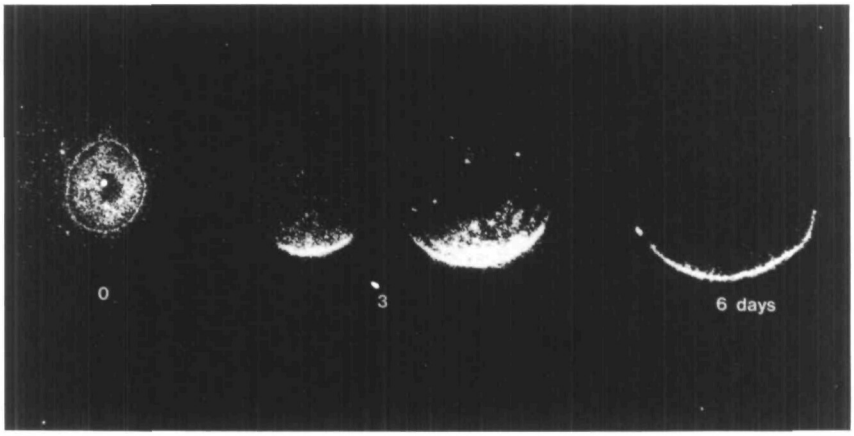

Fig. 1. Localization of endogenous Vg1 mRNA in cultured oocytes. Oocytes were grown in Leibowitz medium supplemented with vitellogenin-containing frog serum as previously described (Yisraeli and Melton, 1988). After the indicated time in culture, the oocytes were fixed and hybridized with a Vg1 probe made to the coding region (Melton, 1987; Yisraeli and Melton, 1988). In these dark-field photographs, silver autoradiographic grains appear white.

containing serum continue to grow and incorporate vitellogenin (Wallace *et al.* 1980). In order to make use of this *in vitro* system for our studies, it was important to show that smaller oocytes retained their ability to localize endogenous Vg1 message in culture. As shown in figure 1, oocytes grown *in vitro* in the presence of frog serum containing vitellogenin localize endogenous Vg1 mRNA in the same pattern as oocytes grown *in vivo*. Oocytes incubated in either saline or medium without serum demonstrate no localization of the endogenous mRNA (data not shown). Only those oocytes grown in the presence of vitellogenin increase in diameter, a result of micropinocytosis of vitellogenin, the precursor for the yolk proteins (Wallace *et al.* 1970). *In situ* hybridization to oocytes of different stages (Melton, 1987; Yisraeli and Melton, 1988; unpublished observations) suggests that localization of Vg1 RNA occurs within a small window of oogenesis between the end of stage III and the middle of stage IV. *In vivo*, this period of growth probably requires several weeks to a month (Keem *et al.* 1979). Oocytes cultured under the conditions described here grow at least three to four times faster. Nevertheless, the progressive accumulation of Vg1 mRNA along the vegetal cortex coupled with the graded loss of the mRNA from the cytoplasm in an animal-to-vegetal direction is identical *in vivo* and *in vitro*.

Localization of exogenous Vg1 mRNA

The endogenous, steady-state level of Vg1 mRNA remains constant from previtellogenesis until after maturation (Melton, 1987). In addition, as mentioned above, localization of Vg1 mRNA occurs in a particular pattern, regardless of whether that movement takes five to six days or three to four weeks. Finally, the accumulation of Vg1 message at the vegetal cortex is accompanied by a graded disappearance of message, first in the animal hemisphere and then throughout the oocyte, as opposed to a general loss of message everywhere. These observations suggested that the localization of Vg1 mRNA is not the result of specific degradation of the message away from the vegetal cortex, but rather the accumulation of Vg1 message at its proper location. In order to explore this possibility

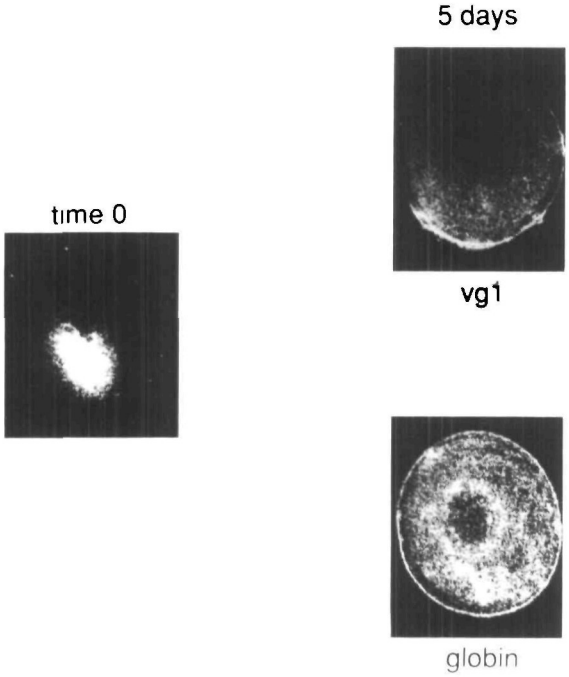

Fig. 2. Localization of exogenous *in vitro* synthesized Vg1 mRNA injected into oocytes. Capped, radioactively labeled Vg1 and globin transcripts were synthesized as described (Krieg and Melton, 1987). Oocytes injected with message were cultured as above for 5 days. *time 0*, the initial site of injection; *5 days*, oocytes injected with the indicated message fixed after 5 days in culture.

further, and to begin to determine how the specificity of localization is achieved, we synthesized Vg1 and globin RNAs in the presence of both radioactively labelled UTP and GpppG (a cap analog whose incorporation at the 5′ end of synthetic RNA transcripts prevents 5′ exonucleolytic degradation). Late stage III oocytes injected with synthetic Vg1 message were capable of localizing the exogenous message when cultured in vitellogenin-containing serum for five days (figure 2). The kinetics and pattern of this localization are virtually identical to those of the endogenous message and the fact that the injected RNA is relatively stable throughout the entire incubation period further argues that localization is not due to degradation or differential

stability (Yisraeli and Melton, 1988). Injected globin RNA, however, becomes distributed throughout both the animal and vegetal hemispheres over the course of the culture period. The information necessary for the specific localization of Vg1 mRNA is thus present in the naked Vg1 RNA molecule itself. Studies are now under way to define further the *cis*-acting sequences which are involved in the process. Initial studies using a deleted Vg1 RNA lacking 62 nucleotides from the 5′ end, including the start codon and putative signal sequence, have shown that this region is not necessary for the proper localization of the RNA (Yisraeli and Melton, 1988).

Association of Vg1 mRNA with the detergent-insoluble fraction of oocyte extracts

The striking distribution of Vg1 mRNA at the vegetal cortex suggested that cytoskeletal elements might be involved in its anchoring. A large number of RNAs have been shown to be associated with a detergent-insoluble fraction of extracts from different cells, a fraction that has been shown to contain cytoskeletal elements such as microtubules, microfilaments, and various intermediate filaments (e.g. Lenk *et al.* 1977; Jeffery, 1984). If Vg1 message is specifically associated with the cytoskeleton in these late-stage oocytes one would expect that Vg1 but not non-localized RNAs would be associated with the detergent-insoluble pellet. As shown in figure 3, Vg1 mRNA is highly associated with the cytoskeletal pellet, with less than 10% of the total Vg1 mRNA in the soluble fraction. This association is not a result of non-specific trapping, because fibronectin mRNA, which is approximately twice the size of the Vg1 message, is found almost exclusively in the soluble fraction. Less than 2% of the total oocyte poly(A)+ RNA is found in the detergent-insoluble pellet, and equal oocyte equivalents of pellet and soluble RNA are compared on the gel. Translatability of a message appears not to be important for its association with the cytoskeletal pellet because Vg1, fibronectin, and several other RNAs are all translated in oocytes but only Vg1 mRNA is found in the pellet (data not shown). These results are similar to those recently reported by Pondel and King (1988).

Microtubule and microfilament involvement at different stages of the localization process

The above results suggested that Vg1 mRNA is associated with cytoskeletal elements at the cortex in late-stage oocytes. In order to determine what these elements are and how they are interacting with Vg1 mRNA, we treated late-stage oocytes with various cytoskeletal inhibitors overnight and then looked at the localization of the Vg1 message by *in situ* hybridization. Cytochalasin B treatment, which depolymerizes microfilaments, had a dramatic effect on the distribution of Vg1 mRNA, releasing it from its tight shell and

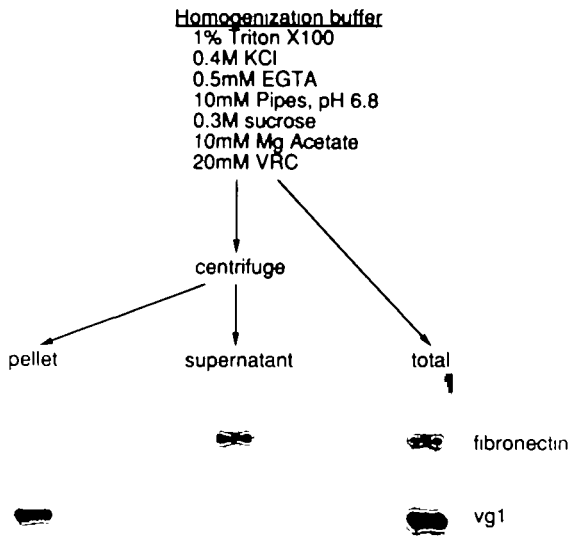

Fig. 3. Attachment of Vg1 mRNA to the detergent-insoluble pellet of extracts. Stage V/VI oocytes were homogenized in the indicated buffer at room temperature. After removing an aliquot and extracting the RNA (*total RNA*), the homogenate was centrifuged and RNA was prepared separately from the supernatant (*soluble*) and insoluble (*pellet*) fractions and analyzed by Northern blot hybridization. The positions of fibronectin and Vg1 messages on the blot are indicated at right. The RNA from two oocytes prepared in this way was run in each lane.

allowing it to diffuse (fig. 4); this distribution is very similar to that observed in unfertilized eggs (Weeks and Melton, 1987). Nocodazole and colchicine, drugs that bind tubulin and cause depolymerization of microtubules, have no effect on the localization of Vg1 message (fig. 4 and data not shown). Interestingly, analysis of the partitioning of RNA in detergent extracts of the treated oocytes only partially reflects the effects of the drugs. As expected from the *in situ* results, nocodazole treatment does not affect the association of Vg1 mRNA with the cytoskeletal pellet (fig. 4). Cytochalasin B treatment, however, despite releasing Vg1 mRNA from its cortical localization, has only a slight effect on Vg1 mRNA distribution in extracts. Presumably, although the depolymerization of microfilaments in late-stage oocytes allows Vg1 mRNA to diffuse away from the cortex, the mRNA remains bound with other factors that may cause it to be pelleted during the detergent extraction.

The experiments with late-stage oocytes address the question of how Vg1 mRNA is tethered at the vegetal cortex. To look at how the message is translocated to this site, we studied the effects of the cytoskeletal inhibitors on middle-stage oocytes undergoing localization. Cytochalasin B had little if any effect on the translocation of Vg1 mRNA, although the message seemed to accumulate further from the cortex than in untreated oocytes (fig. 5). Nocodazole, however, completely inhibits localization (fig. 5), and colchicine has

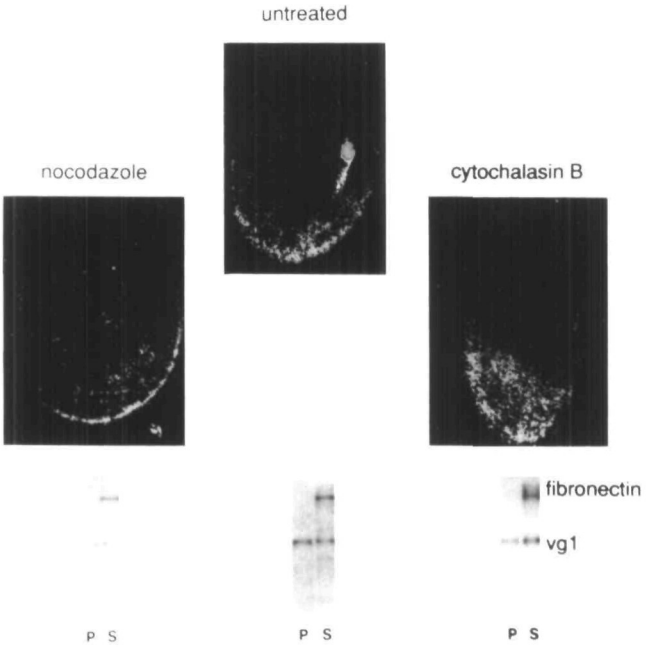

Fig. 4. Effect of cytoskeletal inhibitors on Vg1 mRNA in late-stage oocytes. Stage V/VI oocytes were incubated in saline (*untreated*), cytochalasin B (25 $\mu g\,ml^{-1}$, *cytochalasin B*), or nocodazole (10 $\mu g\,ml^{-1}$, *nocodazole*) overnight at 20°C and then analyzed by *in situ* hybridization for the localization of Vg1 mRNA. In addition, detergent extracts of the oocytes were performed for each treatment and the corresponding Northern blots are shown below each section. *p*, pellet; *s*, soluble fraction.

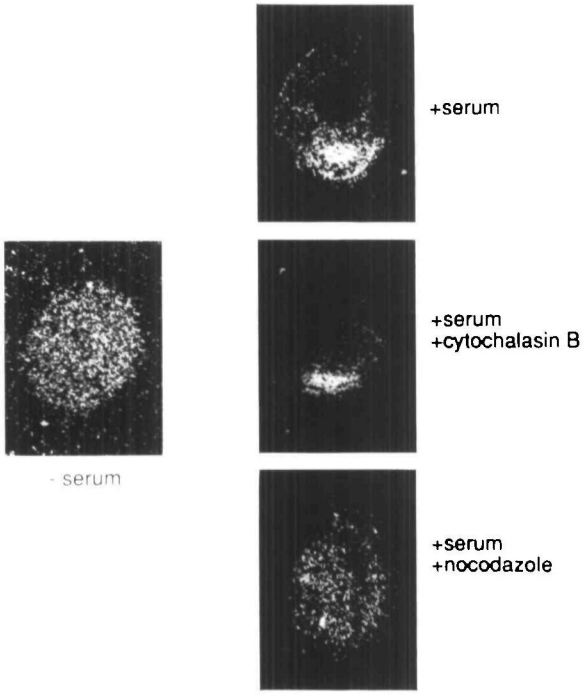

Fig. 5. Effect of cytoskeletal inhibitors on Vg1 mRNA in middle-stage oocytes. Late stage III oocytes were cultured for 5 days in the presence of medium and serum alone (*+serum*), medium and serum with cytochalasin B (*+serum +cytochalasin B*), and medium and serum with nocodazole (*+serum +nocodazole*). *In situ* hybridization visualized the location of the Vg1 mRNA in the oocyte sections. At left is a section from an oocyte cultured in medium alone for 5 days, showing no localization whatsoever (*−serum*).

an identical effect (data not shown). None of the drug treatments had any effect on the stability of Vg1 mRNA or on the synthesis of protein over the five day culture period (data now shown). All of the drugs severely inhibited growth of the oocytes in culture, perhaps by inhibiting the uptake of vitellogenin from the medium. Nonetheless, Vg1 mRNA was translocated in cytochalasin B-treated oocytes, indicating that growth and translocation are independent and separable phenomena, and that the inhibition of translocation by microtubule inhibitors is not a result of the lack of growth of the oocyte.

Discussion

The data presented here demonstrate that microtubule and microfilament inhibitors have different and stage-specific effects on the Vg1 localization machinery. The simplest model to explain these results is the two-step process diagrammed in figure 6, with microtubules involved in the translocation process and microfilaments involved in anchoring the message at the cortex. Although the data described above appear to argue against a specific degradation or instability of non-localized Vg1 mRNA, it is hard to distinguish between active, as opposed to passive, movement of Vg1 mRNA. The graded disappearance of Vg1 message during its localization both *in vivo* and *in vitro* in the

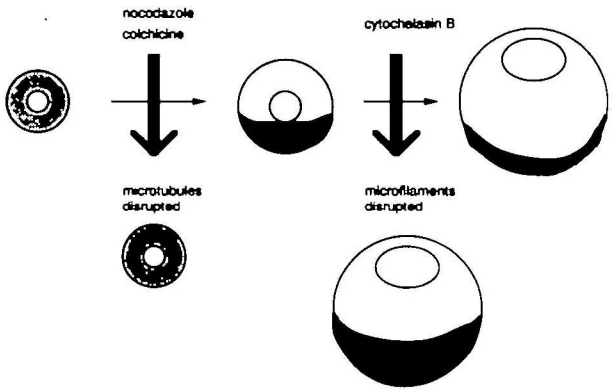

Fig. 6. A two-step model for the localization of Vg1 mRNA in oocytes. The horizontal arrows indicate the normal process of oogenesis, with the black dots and black shading indicating the location of Vg1 mRNA and its progressive localization. The larger, vertical arrows show where the various cytoskeletal inhibitors are thought to interrupt this process and point to the results of treating oocytes with these drugs. Thus, translocation of the Vg1 mRNA to the vegetal cortex is thought to be associated with microtubules and the anchoring of the message is thought to involve microfilaments.

animal to vegetal direction may be the result of an active localization of the message. Alternatively, reversible binding of Vg1 mRNA to a membrane-bound receptor would result in the same 'window shade' effect, even if movement of the mRNA were passive. The apparent involvement of microtubules in the translocation of the message may indicate an active movement of the message. A number of different types of cell make use of microtubule-mediated motors to actively direct the transport of macromolecules and organelles (see Vale, 1987). Indeed, calculated rates for slow axonal transport, which is mediated by microtubules, are similar to the estimated rate of localization of the Vg1 mRNA *in vitro* (0·1 mm/day; Lasek, 1982). Alternatively, however, microtubules may act simply as 'highways' or tracks, expediting the otherwise random movement of Vg1 mRNA without actually propelling it.

The model in figure 6 implies that cytoplasmic microtubules are disrupted in middle-stage oocytes by nocodazole or colchicine, and that cortical microfilaments are depolymerized by cytochalasin B in late-stage oocytes. Microtubule arrays begin to form early in oogenesis; tubulin staining appears first perinuclearly and then in radial arrays extending completely around the oocyte from the germinal vesicle (gv) to the cortex (Palecek *et al.* 1985; Dent and Klymkowsky, 1988; manuscript in preparation). As oogenesis proceeds and the gv migrates to the animal hemisphere, the microtubule arrays become less easily detectable in the vegetal hemisphere but remain radially aligned, emanating from the gv to the animal cortex. In both middle- and late-stage oocytes, these arrays are depolymerized by nocodazole and colchicine. Microfilaments, by comparison, appear mainly along the cortex early in oogenesis and remain there until maturation, when there is a reorganization of cytoskeletal elements throughout the oocyte (Franke *et al.* 1976; Dent and Klymkowski, 1988). Thus, the temporal and spatial organization of microtubules and microfilaments is precisely what would be expected from the two-step localization scheme outlined in figure 6.

The association of Vg1 mRNA with the detergent-insoluble fraction of extracts from late-stage oocytes is consistent with the release of Vg1 mRNA after cytochalasin B treatment. A similar detergent extract of oocytes has been shown to contain significant amounts of several intermediate filament proteins (Pondel and King, 1988), although there is no evidence at present for any Vg1 mRNA association with these proteins. The data presented here clearly indicate that Vg1 mRNA is somehow associated with microfilaments in the cortex but do not rule out interactions with other proteins as well. The association of Vg1 mRNA with the cytoskeletal pellet even in oocytes where the message has been released from its tight cortical shell by cytochalasin B may suggest a continued association of other detergent-insoluble proteins with the message. Presumably, the specificity for the localization process is a combination of the presence of *cis*-acting signals in the RNA itself, specific factors that recognize these sequences, and the cytoskeletal framework within which the RNA moves and is anchored properly. By more precisely defining the RNA signals and identifying the cytoskeletal elements involved in the localization, it should be possible more fully to understand how oocytes generate and interpret intracellular polarity.

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