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Two copies of a subelement from the Vg1 RNA localization sequence are sufficient to direct vegetal localization in *Xenopus* oocytes

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SUMMARY

Localization of mRNA has emerged as a fundamental mechanism for generating polarity during development. In vertebrates, one example of this phenomenon is Vg1 RNA, which is localized to the vegetal cortex of *Xenopus* oocytes. Vegetal localization of Vg1 RNA is directed by a 340-nt sequence element contained within its 3′ untranslated region. To investigate how such *cis*-acting elements function in the localization process, we have undertaken a detailed analysis of the precise sequence requirements for vegetal localization within the 340-nt localization element. We present evidence for considerable redundancy within the localization element and demonstrate that critical sequences lie at the ends of the element. Importantly, we show that a subelement from the 5′ end of the Vg1 localization element is, when duplicated, sufficient to direct vegetal localization. We suggest that the Vg1 localization element is composed of smaller, redundant sequence motifs and identify one such 6-nt motif as essential for localization. These results allow insight into what constitutes an RNA localization signal and how RNA sequence elements may act in the localization process.

Key words: RNA localization, Vg1 RNA, polarity, oocyte, *Xenopus*

INTRODUCTION

Establishment of polarity in the egg can be viewed as the earliest step in embryonic patterning. Thus, molecules that trigger certain developmental programs are specifically sequestered in defined regions of the egg cytoplasm and inherited differentially after cleavage. While evidence for the existence of localized cytoplasmic determinants is available in many systems (reviewed in Davidson, 1986; Gurdon, 1992), the molecular mechanisms underlying the localization process are only now being unraveled.

Localized information can in principle be stored as RNA or protein, and localized maternal mRNA has been described in a number of organisms (St Johnston, 1995). In *Drosophila*, for instance, the basis for patterning along both the anterior-posterior and dorsal-ventral axes is provided by maternal mRNAs that are localized during oogenesis. Maternal mRNAs encoding the anterior and posterior determinants, *bicoid* and *nanos*, are localized to opposite poles of the oocyte (Nüsslein-Volhard et al., 1987; Berleth et al., 1988; Wang and Lehmann, 1991), and the transcript of the *gurken* gene is localized to the dorsal side of the oocyte (Neuman-Silberberg and Schüpbach, 1993). Examples of localized RNA have also been described in certain polarized somatic cell types such as neurons and fibroblasts (Sundell and Singer, 1991; Steward and Banker, 1992).

Unequal distribution of mRNA has been detected along the animal-vegetal (AV) axis of *Xenopus* eggs and oocytes, and mRNAs have been identified that localize to either the animal pole or the vegetal pole of the oocyte (Sagata et al., 1981; Carpenter and Klein, 1982; Phillips, 1982; King and Barklis, 1985; Rebagliati et al., 1985; Weeks and Melton, 1987a,b; Gururajan et al., 1991; Kloc et al., 1993; Ku and Melton, 1993; Linnen et al., 1993; Mosquera et al., 1993). The AV axis coincides with an axis of developmental potential that recapitulates the fate map of the three primary germ layers, the ectoderm, the mesoderm and the endoderm. The animal hemisphere gives rise to ectodermal cell types, while the vegetal hemisphere contains prospective endoderm, and mesoderm results from an inductive signal emanating from the vegetal blastomeres that is received by the overlying cells in the marginal zone (Nieuwkoop, 1969). Studies probing the molecular nature of the mesoderm inducer(s) have implicated certain peptide growth factors (reviewed in Kessler and Melton, 1994). One particular member of the Transforming Growth Factor β family that has been implicated in mesoderm induction is Vg1 (Dale et al., 1993; Thomsen and Melton, 1993; Kessler and Melton, 1995), which is encoded by an mRNA that is localized to the vegetal hemisphere (Weeks and Melton, 1987b).

Vg1 RNA is found evenly distributed in young oocytes (stages I-II) and is localized later during the middle stages (III-IV) of oogenesis to the vegetal hemisphere, where it remains in fully grown oocytes (stage VI) and unfertilized eggs (Melton, 1987). During the time course of localization, Vg1 mRNA has been reported to be associated with cytoskeletal elements (Pondel and King, 1988; Yisraeli et al., 1990). A two-step model for Vg1 localization has been proposed whereby

microtubule-dependent translocation to the vegetal hemisphere is followed by microfilament-dependent anchoring at the vegetal cortex (Yisraeli et al., 1990). After fertilization, Vg1 RNA and protein are inherited preferentially by the vegetal blastomeres (Dale et al., 1989; Tannahill and Melton, 1989). Mis-expression of the processed Vg1 peptide in the animal hemisphere leads to induction of mesoderm in cells that would normally form ectoderm (Dale et al., 1993; Thomsen and Melton, 1993), underscoring the importance of localizing Vg1 specifically to the vegetal pole.

Vegetal localization of Vg1 RNA is mediated by a 340-nucleotide (nt) *cis*-acting localization signal contained within the 3′ UTR of the mRNA (Mowry and Melton, 1992). An increasing number of localized mRNAs have been shown to contain discrete sequences mediating localization within their 3′UTRs (Macdonald and Struhl, 1988; Davis and Ish-Horowicz, 1991; Dalby and Glover, 1993; Kim-Ha et al., 1993; Kloc et al., 1993; Kislauskis et al., 1994; Lantz and Schedl, 1994; Serano and Cohen, 1995; Gavis et al., 1996; Zhou and King, 1996). In most cases, these sequence elements are rather large, as might be expected in order to generate the complex patterns of localization adopted by these RNAs; however, this has complicated efforts to understand how such sequences act to promote localization.

The size and complexity of many localization elements may in part be explained by the use of multiple elements to orchestrate multiple steps in complex localization pathways. In some cases such elements have been suggested to be structural in nature, as with *bicoid* mRNA, where the 625-nt localization element is predicted to fold into a complex secondary structure involving long-range base pairing interactions (Macdonald, 1990; Seeger and Kaufman, 1990), and *K10* mRNA, where a small stem loop structure of 44 nucleotides (nts) mediates localization in the *Drosophila* oocyte (Serano and Cohen, 1995). Detailed dissection of the primary sequence requirements for *bicoid* localization identified a 53-nt element designated BLE1 that can direct the early steps of the *bicoid* localization process (Macdonald et al., 1993). One or more elements must mediate late events, as is implied by the existence of a deletion outside of BLE1 that interferes with a later step in localization, and other deletions that impair localization. Multiple, potentially redundant, elements that are themselves relatively large have also been implicated in mediating steps in the localization pathways of both *oskar* (Kim-Ha et al., 1993) and *orb* (Lantz and Schedl, 1994) mRNAs in the *Drosophila* oocyte. Multiple elements have also been identified in localization of actin mRNA in fibroblasts (Kislauskis et al., 1994) and *nanos* mRNA in *Drosophila* oocytes (Gavis et al., 1996), but these elements appear to be redundant and do not mediate discrete steps in the localization pathways. Elements within RNA localization signals may function by providing recognition motifs for *trans*-acting localization factors (Macdonald et al., 1995) but, in spite of the available information on sequence elements that specify localization from a variety of organisms, it remains generally unclear how RNA localization sequences act in the localization process.

To address this question, we have performed a detailed analysis of the sequence requirements for vegetal localization of Vg1 mRNA. We find evidence for considerable redundancy within the 340-nt localization element; small deletions throughout the element have little effect on localization. Critical sequence elements lie at the ends of the element and the first 85 nts of the localization element, when duplicated, is sufficient to direct vegetal localization. Thus, this subelement contains all of the information necessary for localization, at least some of which must be reiterated for proper function. Moreover, at least part of this information is present as a repeated 6-nt motif that may act as a recognition element for a *trans*-acting localization factor.

MATERIALS AND METHODS

Construction of mutants

A series of 15-nt deletions, replaced by a 6-nt *Apa*I restriction site, were created using site-directed mutagenesis, according to the procedure of Deng and Nickoloff (1992). The mutagenic oligonucleotides each consisted of an *Apa*I restriction site imbedded in 24-30 nts of flanking sequence from the deletion site. The template used for mutagenesis, pSP73-340, contained the entire 340-nt localization sequence. The positions of the deletions along the localization element are presented schematically in Fig. 1. After mutagenesis and DNA sequence analysis, the localization element inserts bearing the desired deletions were cloned downstream of the *Xenopus* β-globin coding region in pSP64XβM (Krieg and Melton, 1984) to facilitate in vivo localization studies.

Internal deletions of 40-nt, 65-nt, 90-nt and 115-nt, as depicted in Fig. 1, were constructed by replacing the *Apa*I-*Eco*RI fragment (3′ to the deletion site) from the appropriate upstream 15-nt deletion with the *Apa*I-*Eco*RI fragment from the corresponding downstream 15-nt deletion. The 2X1-85 and 2X1-135 chimeras were constructed by inserting a fragment containing β-globin (βG) fused to Vg1 sequences 5′ of the *Apa*I site (from either the Δ86-100 or Δ136-150 chimeric constructs) into the polylinker upstream of the Δ86-100 or Δ136-150 constructs in pSP73. The 2X201-340 and 2X251-340 chimeras were constructed by inserting duplications of *Apa*I-*Eco*RI fragments from the Δ186-200 and Δ236-250 constructs downstream of the *Xenopus* β-globin coding region. The VM1 mutant (pSP732X1-135/M1) was constructed by PCR amplification of pSP73-340 using a downstream primer specific for nts 115-135 and an upstream primer containing the desired base changes. An upstream primer without base changes was similarly used to amplify wild-type 1-135 sequences. The wild-type and VM1 mutant 1-135 PCR fragments were cloned as tandem duplications either directly into pSP73 for RNase footprint analysis, or downstream of β-globin for in vivo localization assays.

RNA transcripts

In vitro RNA transcription reactions were performed as described in Krieg and Melton (1987). For microinjection, capped RNA was transcribed from either the chimeric β-globin/Vg1 constructs (above) or pSP64-XβM. β-globin-specific antisense probe RNA for whole-mount in situ hybridization and for RNase protection was transcribed from pSP73-Xβ5′ (Mowry and Melton, 1992). The transcription reactions contained either 350 μM digoxigenin-UTP (Boehringer), 650 μM UTP and 1mM each ATP, CTP and GTP for in situ hybridization probes, or 5 μCi/μl of [^{32}P]UTP (800 Ci/mmole, DuPont/NEN) and 0.5 mM each of ATP, CTP and GTP for protection probe synthesis. For RNase footprinting, transcripts were synthesized from either pSP732X1-135 or pSP732X1-135/M1 (above) in the presence of 5 μCi/μl of [^{32}P]UTP, 50 μm GTP, and 0.5 mM each of CTP, ATP and diguanosine triphosphate.

Microinjection

Oocytes were obtained surgically from albino female *Xenopus laevis* frogs (Nasco) and placed in MBSH buffer [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$. 0.82 mM $MgSO_4 \cdot 7H_2O$, 0.33 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.41 mM $CaCl_2 \cdot 6H_2O$, 10 mM Hepes (pH 7.6)]. Stage III-IV oocytes, removed manually from the ovary, were microinjected according to

standard procedures (Kay, 1991), with ~5 nl of capped in vitro transcribed RNA at 10 ng/μl. The injected oocytes were cultured for 4 days in oocyte culture medium [50% L15 medium, 15 mM Hepes (pH 7.6), 1 mM glutamine, 1 μg/ml insulin, 100 μg/ml gentamicin, 50 U/ml nystatin, 50 U/ml penicillin, 50 μg/ml streptomycin and 5-10% frog serum containing vitellogenin], as described in Wallace and Misulovin (1978) and Yisraeli and Melton (1988).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as originally described by Harland (1991), with modifications from Kloc and Etkin (1995). Briefly, oocytes were fixed in MEMFA [0.1 M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7% formaldehyde] for 2 hours at room temperature. Fixed oocytes were removed to 100% methanol for storage at –20°C, and rehydrated in PBS-Tween (1× PBS, 0.1% Tween-20). The oocytes were prehybridized for 4 hours at 50°C in 1 ml of hybridization (HYB) buffer (50% formamide, 5× SSC, 0.1% Tween-20, 0.1% CHAPS, 5 mM EDTA, 1× Denhardts, 100 μg/ml heparin, 1 mg/ml yeast tRNA), which was replaced with 0.5 ml of HYB buffer containing 0.5 μg of digoxigenin-labelled probe for hybridization overnight at 50°C. The oocytes were washed in 50% HYB / 50% 2× SSC-CHAPS (2× SSC, 0.3% CHAPS), followed by 25% HYB / 75% 2× SSC-CHAPS and 2× SSC-CHAPS. The oocytes were then treated with RNase A (20 μg/ml in 2× SSC-CHAPS for 30 minutes at 37°C) and washed, sequentially, with 2× SSC-CHAPS, 0.2× SSC-CHAPS, PBS-Tween/0.3% CHAPS and PBS-Tween. Antibody incubation and detection was performed using reagents from the Genius 3 kit (Boehringer). Oocytes were incubated overnight at 4°C with a 1:5000 dilution of alkaline-phosphatase-conjugated anti-digoxigenin antibody, washed in maleate buffer [100 mM maleic acid (pH 7.5), 150 mM NaCl], and incubated in color substrate solution. After 30-60 minutes, the color reaction was stopped by washing in 1× PBS with 1 mM EDTA and the oocytes were stored at 4°C.

For sectioning, oocytes were refixed in MEMFA for 90 minutes at room temperature. The fixed oocytes were washed twice, for 60 minutes each, in 100% methanol and 100% ethanol, 10 minutes each in ethanol/xylene (50:50) and 100% xylene, and imbedded in Paraplast. 10 μm sections were cut using a rotary microtome and Paraplast was removed by soaking the slides in xylene.

Scoring of in vivo localization phenotypes

For each experiment, injected oocytes were grouped by injected transcript and photographed. To assign a phenotype for a particular transcript, the results of multiple experiments were then compared, along with the positive (vg340) and negative (β-globin) controls for each experiment. For each transcript, the total number of injected oocytes assayed was 45-150. The injected transcripts were grouped into four categories (+++, ++, –/+, –), dependent upon both the intensity of the hybridization signal and the frequency with which a vegetal hybridization signal was detected. Localization was scored as normal if strong vegetal localization was reproducibly observed (+++), although the strong localization was slightly less reproducible in some cases (++). Those transcripts giving only very weak, but reproducible, vegetal localization were scored as strongly impaired (–/+). Localization was scored as abolished for those transcripts consistently exhibiting no detectable hybridization signal (–).

RNase protection analysis

RNase protection analysis was performed as described in Krieg and Melton (1987). RNA was extracted from injected oocytes immediately after injection (day 0), and at the end of the culture period (day 4). RNA samples were probed with a β-globin probe (Mowry and Melton, 1992) to detect the injected RNA, and were also probed for histone H4 RNA (Jamrich et al., 1984) to control for efficiency of RNA isolation. The percent of injected RNA recovered at day 4 relative to day 0 (see Table 1) was determined by densitometry of the globin-protected fragments on autoradiograms.

RNase footprint analysis

RNase footprinting was performed as described in Mowry (1996). Briefly, RNA-protein complexes, formed in the presence of *Xenopus* oocyte extract and ^{32}P-labelled RNA transcripts, were subjected to limited cleavage with T_1 ribonuclease (Pharmacia). The RNase-resistant complexes were resolved on a nondenaturing polyacrylamide gel in which protein-bound RNA fragments exhibit retarded mobility.

RESULTS

A minimal *cis*-acting localization element for Vg1 RNA has been mapped to a 340-nt sequence element within the 3′ UTR

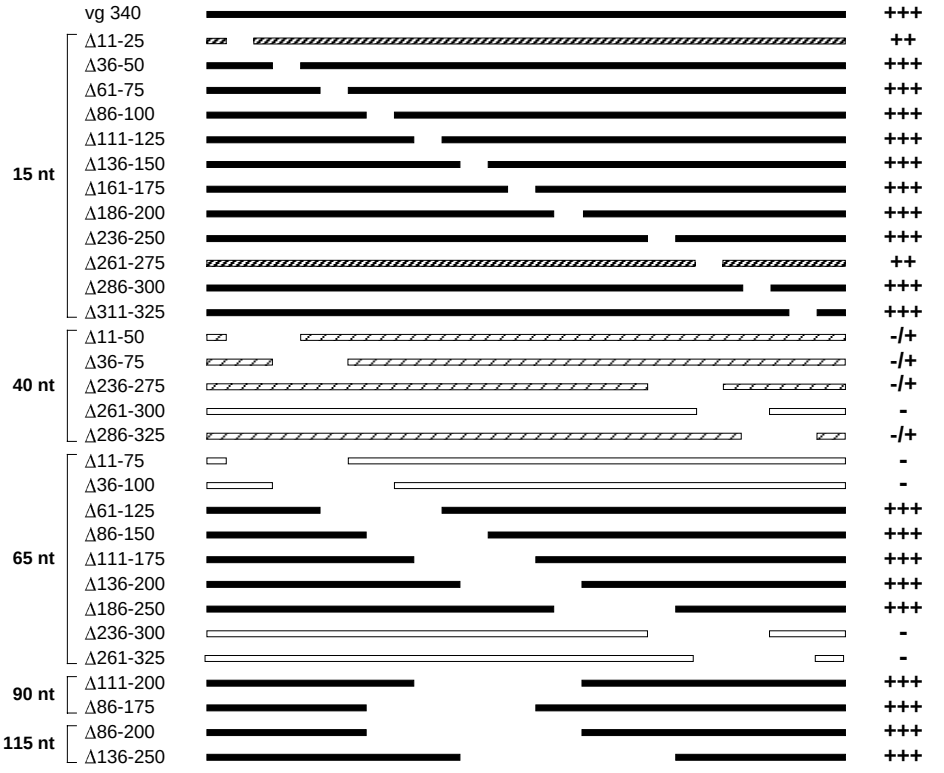

Fig. 1. Constructs tested for RNA localization. The Vg1 sequences from chimeric β-globin/Vg1 constructs tested for localization are diagrammed. The positions of deletions within the Vg1 localization element are depicted, and the in vivo localization phenotypes are indicated as follows: black, normal (+++); thick hatches, slightly diminished (++); thin hatches, strongly impaired (–/+); white, no detectable localization (–).

Table 1. Recovery of injected RNA transcripts

transcript	% recovery
β-globin	84
βG/vg340	71
βG/vgΔ11-75	84
βG/vgΔ36-100	75
βG/vgΔ236-300	75
βG/vgΔ261-325	78
βG/vg2X1-135	70
βG/vg2X1-135/M1	70

Results of RNase protection analyses were used to determine the percent of injected RNA that was recovered after culture for each of the indicated RNA transcripts.

(Mowry and Melton, 1992). To investigate the precise sequence requirements within the localization element (vg340) for vegetal localization of Vg1 mRNA, we constructed within the element, an extensive set of deletions ranging in size from 15 to 115-nts (Fig. 1). To test localization in vivo for each of these mutations, synthetic RNA transcripts were injected into middle stage oocytes, which were cultured to allow localization to proceed. After culture, localization of the injected RNAs was assayed by whole-mount in situ hybridization. Those transcripts that consistently showed little (–/+) or no (–) hybridization signal were scored as defective. Transcripts exhibiting strong hybridization signals (+++, or ++) were scored as normal. The in vivo localization phenotype for each deletion transcript is indicated in Fig. 1.

Small deletions along the entire localization element have little or no effect on localization in vivo

We began our analysis by constructing 15-nt deletions, replaced by a 6-nt restriction site, centered every 25 nts along the entire element, as diagrammed in Fig. 1. Rather surprisingly, we found that these deletions had little or no effect on localization. Two of the deletions (Δ11-25 and Δ261-275) did have a very marginal effect (++), whereby localization was slightly less reproducible than with the control vg340 transcript (+++). Shown in Fig. 2 are in situ hybridization results obtained for two 15-nt deletion transcripts, Δ36-50 (Fig. 2E) and Δ286-300 (Fig. 2I). Both appear to localize normally, as evidenced by comparison with the intact localization element, vg340 (Fig. 2A), which localizes to the vegetal pole, and β-globin (Fig. 2B), which is not normally present in the oocyte and does not localize. These results demonstrate that much of the localization element can be eliminated as small pieces and without affecting function, suggesting redundancy within the element.

Larger deletions at either end of the element abolish or impair localization in vivo

Because the small deletions had essentially no effect on localization in vivo, we next tested as series of 65-nt deletions across the entire element. These larger deletions destroyed localization, but only if situated near either the 5′ or 3′ end of the element, as summarized in Fig. 1. Deletions of 40 nts near the ends were likewise deleterious, and either strongly reduced or destroyed localization (Fig. 1). The two 40-nt deletions near the 5′ end, Δ11-50 (Fig. 2F) and Δ36-75 (Fig. 2G), are both strongly impaired for localization. Significant impairment is evident from comparison of the very light whole-mount in situ hybridization signals obtained for Δ11-50 and Δ36-75 (Fig. 2F,G) with the strong signal of the intact 340-nt Vg1 localization element (Fig. 2A). In this same region, removal of the entire 65-nt sequence (Δ11-75) encompassing these two 40-nt deletions abolishes localization; no signal is

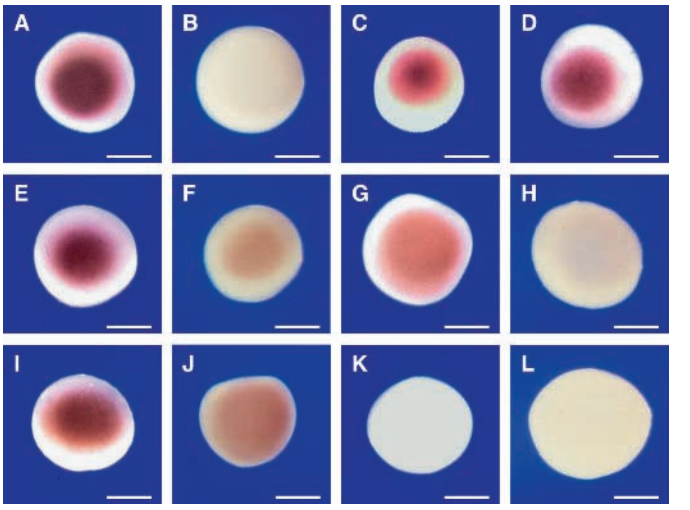

Fig. 2. Whole-mount in situ hybridization to detect localization of injected Vg1 deletion transcripts. Vegetal views are shown of oocytes that were injected with following chimeric transcripts: (A) βG/vg340 (+++), (B) β-globin (–), (C) β-globin/vgΔ86-200 (+++), (D) βG/vgΔ136-250 (+++), (E) βG/vgΔ36-50 (+++), (F) βG/vgΔ11-50 (–/+), (G) βG/vgΔ36-75 (–/+), (H) βG/vgΔ11-75 (–), (I) βG/vgΔ286-300 (–), (J) βG/vgΔ236-275 (–/+), (K) βG/vgΔ261-300 (–), (L) βG/vgΔ236-300 (–). The injected RNAs were detected by whole-mount in situ hybridization using a β-globin probe. The scale bars represent 200 μm.

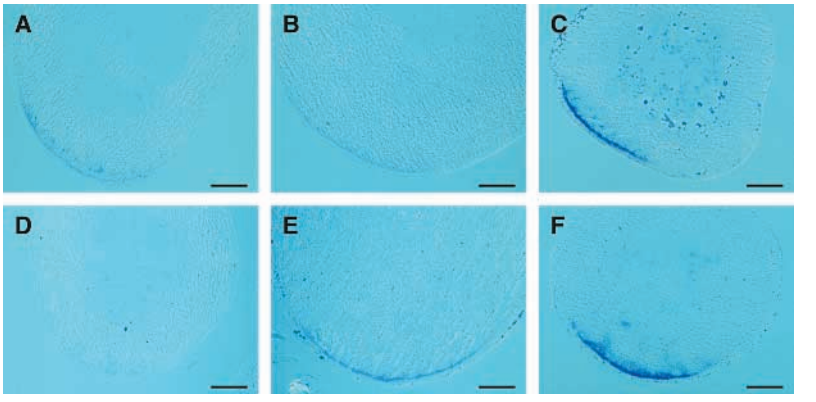

Fig. 3. Sections of oocytes to determine the distribution of injected RNA transcripts. Sections are shown of oocytes injected with the following transcripts: (A) βG/vgΔ11-50 (–/+), (B) βG/vgΔ36-75 (–/+), (C) βG/vg340 (+++), (D) βG/vgΔ236-275 (–/+), (E) βG/vgΔ261-275 (++), (F) βG/vgΔ136-250 (+++). The oocytes were sectioned after whole-mount in situ hybridization to detect the injected RNAs. The oocytes are oriented with the vegetal pole towards the bottom, and the scale bars represent 50 μm.

detectable (Fig. 2H). Localization is also destroyed by Δ36-100, but Δ61-125 gives normal localization (not shown). Deletions near the 3′ end of the element are generally more deleterious than those near the 5′ end. Of the 40-nt deletions, only Δ261-300 (Fig. 2K) abolishes localization, while Δ236-275 (Fig. 2J) and Δ286-325 (not shown) are strongly impaired. As with the 65-nt deletions at the 5′ end, both Δ236-300 (Fig. 2L) and Δ261-325 (not shown) near the 3′ end abolish localization. No effect on localization is apparent with deletions further away from the 3′ end; for example, Δ186-250 (not shown) directs normal vegetal localization. To rule out the possibility that apparent defects in localization might instead be a result of changes in RNA stability, we also analyzed the injected RNAs using RNase protection, which revealed no differences in RNA stability (Table 1). The results of in vivo analysis of these deletions suggest that key sequences specifying Vg1 localization are at the ends of the 340-nt Vg1 localization element.

Mutations that impair localization show no apparent defects in cortical anchoring

In light of the two-step model for Vg1 localization proposed by Yisraeli et al. (1990), we next asked whether any of the mutations that affect localization do so by disrupting anchoring of the RNA at the cortex. To address this, we sectioned injected oocytes after whole-mount in situ hybridization. From this analysis, no defects are revealed in cortical anchoring of Vg1 RNA with any of the mutations tested. With the 40-nt deletions from the 5′ end that reduce localization, RNA is evident at the cortex. Shown in Fig. 3, are sections of oocytes that were injected with Δ11-50 (Fig. 3A) or Δ36-75 (Fig. 3B) RNA transcripts. While the signal is much lighter than the full-length vg340 element (Fig. 3C), a cortical signal is apparent for both Δ11-50 and Δ36-75 (Fig. 3A,B). The situation with the 3′ deletions is less clear. Sections of oocytes injected with the 40-nt 3′ deletions (Δ236-275 and Δ286-325) showed barely detectable cortical signals, as shown in Fig. 3D for Δ236-275. However, these deletions cause more deleterious effects on the localization process, resulting in a very faint signal detected by whole-mount in situ hybridization (see for example, Fig. 2J). To explore this further, oocytes injected with Δ261-275, a 15-nt deletion that very slightly diminishes localization, were also sectioned. Once again, a cortical signal is evident, as shown in Fig. 3E. Because it was possible that the whole-mount assay could fail to detect RNA that was stalled in some intermediate step of the localization pathway, sectioning was also performed for all of the 65-nt deletions (not shown). All of the internal deletions that gave normal localization by the whole-mount assay also exhibited normal cortical signals in sections, whereas the 5′ and 3′ end deletions that gave no localization in whole-mounts also showed no signal in sections. Thus, no mutations are apparent that uncouple cortical anchoring from translocation to the vegetal pole.

Large deletions from the middle of the element have no effect on localization in vivo

Because the 65-nt deletions from the middle of the element Δ61-125, Δ86-150, Δ111-175, Δ136-200, Δ186-250 (Fig. 1) had no effect on localization, we constructed larger deletions of 90 nts (Δ86-175 and Δ111-200) and 115 nts (Δ86-200 and Δ136-250) within this region. All of the 90-nt and 115-nt deletions give normal cortical localization, as shown for Δ86-200 and Δ136-250 in Figs 2C,D and 3F, indicating that sequences from the middle of the element are largely dispensable. Moreover, these results reveal that it is not necessary to maintain spacing between the important sequences at the 5′ and 3′ ends; the critical sequences from the ends of the 340-nt element may act as independent subelements in the localization process.

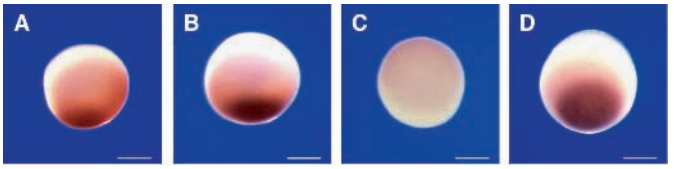

Fig. 4. Whole-mount in situ hybridization to detect localization directed by the 5′ and 3′ subelements. Oocytes were injected with the following transcripts: (A) βG/vg2X1-85, (B) βG/vg2X1-135, (C) βG/vg2X201-340, (D) βG/vg340. The injected RNAs were detected by whole-mount in situ hybridization using a β-globin probe. The oocytes are oriented with the vegetal pole towards the bottom and the scale bars represent 200 μm.

Two copies of the 5′ subelement are sufficient to direct localization

To ask whether the 5′ and 3′ subelements are functionally equivalent, we cloned tandem repeats of nts 1-85 (2X1-85), 1-135 (2X1-135), 201-340 (2X201-340) and 251-340 (2X251-340) downstream of β-globin, and tested them as above for localization in vivo. We tested two versions of each subelement because the boundaries of the subelements were suggested to lie between nts 85-135 for the 5′ subelement and nts 201-251 for the 3′ subelement. As shown in Fig. 4, RNA transcripts carrying duplications of the 5′ subelement, 2X1-85 or 2X1-135 (Fig. 4A,B), are vegetally localized. The ability of the 5′ subelement to direct vegetal localization diminishes as the element is truncated from the 3′ end. The 2X1-135 transcript

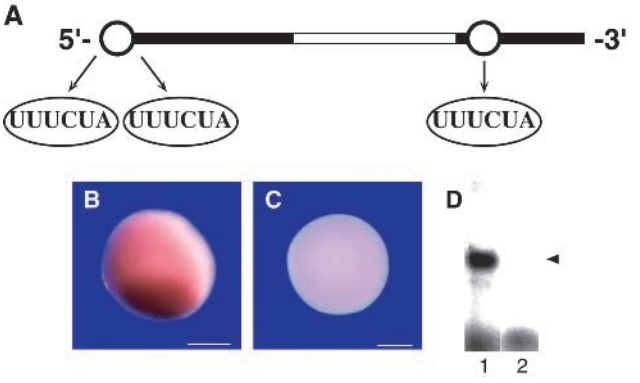

Fig. 5. Site-directed mutagenesis of VM1. (A) A schematic of the 340-nt localization element is shown, with the 5′ and 3′ subelements shaded in black. The three copies of VM1 (UUUCUA) are positioned at nts 2-7, 13-19 and 261-266. Protein binding sites (Mowry, 1996) containing VM1 are indicated by circles. (B) Oocyte injected with βG/vg2X1-135 RNA. (C) Oocyte injected with βG/vg2X1-135/M1 RNA. For B and C, injected RNA was detected by whole-mount in situ hybridization using a β-globin probe and the scale bars represent 200 μm. (D) RNase footprint analysis of vg2X1-135 (lane 1) and vg2X1-135/M1 (lane 2). The position of the expected protected RNA fragment is indicated by the arrowhead.

is somewhat more active than is 2X1-85 (compare Fig. 4A and 4B), and a 2X1-60 transcript does not localize (not shown). Neither 1-85 nor 1-135 alone support vegetal localization (not shown) indicating that a minimum of two copies of the 5′ subelement, or one copy each of the 5′ and 3′ subelements are necessary. However, the 5′ and 3′ subelements are not equivalent; tandem copies of the 3′ subelement are not competent for localization, as shown in Fig. 4C for 2X201-340. These results indicate that the 5′ and 3′ subelements are partially redundant to one another with respect to function, and that a duplicated 5′ subelement is sufficient to direct vegetal localization.

A small reiterated motif is critical for RNA localization

The finding that one copy of 5′ subelement is insufficient while two copies will suffice, could suggest the existence of one or more motifs within the element, of which at least two copies are necessary to promote localization. Inspection of the sequence reveals several potentially redundant sequence motifs with at least one copy in each of the 5′ and 3′ subelements, but not within the sequences that are dispensable for localization (nts 136-200). Of particular interest is the reiterated motif UUUCUA (designated VM1, Vg1 Motif 1). Two copies are present at the 5′ end, and one additional copy is found in the 3′ subelement (Fig. 5A). The positioning of VM1 is intriguing; the two 15-nt deletions that weakly affect localization, Δ11-25 and Δ261-275, each remove one copy of this sequence, and all copies of VM1 are contained within protein binding sites on the localization element that we have previously mapped by RNase footprinting (Mowry, 1996).

To test whether VM1 might have a central role in Vg1 RNA localization, we performed site-directed mutagenesis on both copies of the motif present in the 2X1-135 construct. In the mutant construct, 2X1-135/M1, three of the Us within both copies of the UUUCUA sequence were changed to Gs (GUGCGA). The result is striking: In vivo localization directed by the 2X1-135 element (Fig. 5B) is completely abolished by mutation of VM1, as shown in Fig. 5C. Because all copies of VM1 lie within protein binding sites that we had previously discovered (Mowry, 1996; see also Fig. 5A), we next tested whether defects in protein binding were apparent with the VM1 mutant. For this, we used the RNase footprint analysis to reveal changes in protein binding, as shown in Fig. 5D. Two copies of the 5′ footprint site are contained within the wild-type 2X1-135 transcript, and a protected RNA fragment of the expected mobility is observed (lane 1). In striking contrast, the footprint is abolished with the VM1 mutant transcript (lane 2). These results indicate that protein binding is altered by a specific mutation of VM1 that also destroys localization in vivo.

DISCUSSION

We have carried out a detailed analysis of the Vg1 RNA localization element. We have found that, while sequences from the middle of the element are largely dispensable, deletions within sequences at the ends of the element have profound effects on localization in vivo. We propose that there are subelements at both the 5′ and 3′ ends of the localization element. Unexpectedly, we find that two copies of the 5′ subelement are sufficient to direct vegetal localization. Within the critical sequences we have identified a sequence motif, VM1 (UUUCUA), that is crucial for localization and we suggest a role for VM1 as a binding site for an essential *trans*-acting localization factor.

The deletion analysis of the Vg1 localization element reveals that much of the 340-nt element can be removed, 15-nt at a time, with essentially no impact on localization (Fig. 1). It is unlikely that the positioning of these deletions is such that each removes a site on the element without function. Instead, it is possible that the function of each deleted region was compensated for by sequences elsewhere in the element. Larger deletions were necessary to uncover the existence of key sequences at either end of the element. Deletions of 40 and 65 nts near the ends of the element abolish or impair localization, and two 115-nt deletions from the middle of the element (Δ86-200 and Δ136-250) have no impact (Figs 1, 2). Thus, the 5′ and 3′ ends of the previously mapped 340-nt localization element represent key subelements that are critical for function. The boundaries of these subelements are dependent upon one another, as all of the sequences contained within the overlapping 115-nt fragments (nts 86-250) cannot be deleted [localization is abolished by a 153-nt deletion (Δ100-252) that was tested previously (Mowry and Melton, 1992)]. These results raise the key questions of how the subelements participate in the localization process and how our deletions act to disrupt localization.

Multiple sequence elements have been identified in several localized RNAs and have, in some cases, been shown to mediate distinct steps in complex localization pathways (Kim-Ha et al., 1993; Macdonald et al., 1993; Kislauskis et al., 1994; Lantz and Schedl, 1994; Gavis et al., 1996). From our analysis, this does not appear to be the case for the Vg1 localization element. Our results identify two subelements within the 340-nt localization element, but these elements apparently have similar functions. Two copies of the 5′ subelement (2X1-85 or 2X1-135, Fig. 4A,B) or one copy each of the 5′ and 3′ subelements (Δ86-200 or Δ136-250, Fig. 2C,D) are sufficient to direct vegetal localization. This indicates that a second copy of the 5′ subelement can functionally substitute for the 3′ subelement, suggesting similar roles in the localization pathway for the 5′ and 3′ subelements.

The localization pathway for Vg1 RNA consists of at least two steps: microtubule-dependent translocation to the vegetal pole, followed by anchoring at the oocyte cortex (Yisraeli et al., 1990). We have identified no deletions within the localization element that appear to uncouple these steps, suggesting that the deletions that disrupt localization do so by interfering with translocation rather than anchoring. Our data indicate that mutations that reduce localization still allow anchoring of injected RNA to the cortex. Previous analyses of cortical anchoring (Yisraeli et al., 1990; Kloc and Etkin, 1995) have used cytochalasins to release endogenous oocyte RNAs from the cortex and, in these cases, the released RNA could be detected by in situ hybridization. It is, however, possible that our failure to detect defects in anchoring is a result of mutations that never allow the RNA to associate with the cortex, possibly causing the translocated RNA to diffuse throughout the oocyte prior to fixation, thus escaping detection and appearing as translocation defects. A second, more attractive possibility is that mutations in cortical anchoring were not uncovered because the same sequences that specify translocation are also required for anchoring. It has been suggested that formation of

a translocation complex may represent a commitment step in the RNA localization pathway (Mowry, 1996; Zhou and King, 1996). Our data support such a model, in which recognition of sequences within the RNA localization element by *trans*-acting localization factors is necessary only to form a translocation-competent RNP complex. RNA recognition may not play a part in anchoring; cortical anchoring may follow translocation without scrutiny of the RNA sequence and rely on interactions with previously bound translocation factors.

It is apparent that the subelements that we have defined contain overlapping sets of information and that the minimal requirement to direct vegetal localization can be provided by two copies of the 5′ subelement. The 3′ subelement contains some, but not all of the information contained within the 5′ subelement, as two copies of the 5′ subelement, or one copy each of the 5′ and 3′ subelements, but not two copies of the 3′ subelement will direct localization. The finding that one copy of 5′ subelement is insufficient while two copies will suffice, suggests the existence of motifs within the element, of which two or more copies are necessary to promote localization. Such redundancy within the localization element may also provide an explanation for the inability of any of the 15-nt deletions across the 340-nt sequence to affect localization. Inspection of the sequence does reveal several potentially redundant sequence motifs found in both the 5′ and 3′ subelements, but not within the sequences that are dispensable for localization (nts 136-200). Of these, the reiterated motif UUUCUA (designated VM1), is particularly intriguing as all copies of VM1 lie in previously defined protein binding sites (Mowry, 1996). Two copies are contained in a footprint site at the 5′ end, and one additional copy is found in a footprint site in the 3′ subelement (Fig. 5A). We have uncovered a critical role for VM1 in Vg1 RNA localization by engineering specific base changes in both copies of VM1 within a 2X1-135 localization element. Localization is completely abolished in the VM1 mutant, underscoring the importance of this sequence motif in the localization process. Clues as to how the VM1 motif might act to promote localization of Vg1 RNA are provided by the results of RNase footprint analysis, showing that mutation of VM1 abolishes binding of protein to the VM1 footprint site. Thus, we propose that VM1 may act by providing a binding site for a *trans*-acting protein factor that is essential for Vg1 localization.

In addition to the essential VM1 motif, the 5′ subelement likely contains other sequence motifs that act in localization. This is illustrated by Δ36-100, which does not localize (Fig. 1) and removes sequences that do not contain VM1, but do contain other potentially redundant motifs that are present in both the 5′ and 3′ subelements. Consistent with this, is the work of Deshler et al. (1997), which has implicated other redundant sequence motifs in Vg1 RNA localization. Three different repeated elements, including one that contained VM1 were suggested to be important for binding of a 78-80×10^3 M_r protein to the Vg1 localization element. However, we do not believe that VM1 provides a binding site for the RNA binding protein known both as p78 (Mowry, 1996) and vera (Deshler et al., 1997), as we do not observe the VM1 mutant to be defective in p78 binding (C. A. C. and K. L. M., unpublished results).

We propose that the localization element of Vg1 RNA is composed of relatively small, redundant sequence motifs, and that two or more copies of at least one of these motifs are required for correct functioning of the element. Our data indicate that all sequence motifs critical for localization are contained within the 5′ subelement, and we have identified one such motif, VM1, as necessary for localization. We suggest that VM1 acts in the localization process by providing a recognition site for binding of a *trans*-acting localization factor to Vg1 mRNA. While the 5′ subelement represents a minimal requirement to execute the localization pathway for Vg1 RNA, it is evident that copies of essential motifs must also be present elsewhere in the 340-nt element, as localization was unaffected by any of the 15-nt deletions, including those that removed portions of the 5′ subelement. Redundancy within an RNA localization element may act to tightly regulate localization in order to ensure proper spatial expression of positional information. These results support a model for Vg1 localization element function in which critical sequence motifs act in concert, perhaps by directing assembly of an RNP transport complex that is targeted to the vegetal cortex.

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REFERENCES

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., Noll, M. and Nüsslein-Volhard, C. (1988). The role of localization of *bicoid* RNA in organizing the anterior pattern of the *Drosophila* embryo. *EMBO J.* **7**, 1749-1756.

Carpenter, C. D. and Klein, W. H. (1982). A gradient of poly(A)+ sequences in *Xenopus laevis* eggs and embryos. *Dev. Biol.* **91**, 43-49.

Dalby, B. and Glover, D. M. (1993). Discrete sequence elements control posterior pole accumulation and translational repression of maternal cyclin B RNA in *Drosophila*. *EMBO J.* **12**, 1219-1227.

Dale, L., Matthews, G. and Colman, A. (1993). Secretion and mesoderm-inducing activity of the TGF-β-related domain of *Xenopus* Vg1. *EMBO J.* **12**, 4471-4480.

Dale, L., Matthews, G., Tabe, L. and Colman, A. (1989). Developmental expression of the protein product of Vg1, a localized maternal mRNA in the frog *Xenopus laevis*. *EMBO J.* **8**(4), 1057-1065.

Davidson, E. H. (1986). *Gene Activity in Early Development*. Orlando, FL: Academic Press.

Davis, I. and Ish-Horowicz, D. (1991). Apical localization of pair-rule transcripts requires 3′ sequences and limits protein diffusion in the *Drosophila* blastoderm embryo. *Cell* **67**, 927-940.

Deng, W. P. and Nickoloff, J. A. (1992). Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. *Anal. Biochem.* **200**, 81-88.

Deshler, J. O., Highett, M. I. and Schnapp, B. J. (1997). Localization of *Xenopus* Vg1 mRNA by vera protein and the endoplasmic reticulum. *Science* **276**, 1128-1131.

Gavis, E. R., Curtis, D. and Lehmann, R. (1996). Identification of *cis*-acting sequences that control *nanos* RNA localization. *Dev. Biol.* **176**, 36-50.

Gurdon, J. B. (1992). The generation of diversity and pattern in animal development. *Cell* **68**, 185-199.

Gururajan, R., Perry-O'Keefe, H., Melton, D. A. and Weeks, D. L. (1991). The *Xenopus* localized messenger RNA An3 may encode an ATP-dependent RNA helicase. *Nature* **349**, 717-719.

Harland, R. M. (1991). In situ hybridization: An improved whole-mount method for *Xenopus* embryos. In *Xenopus laevis: Practical Uses in Cell and Molecular Biology*. **36**, (eds. B. K. Kay and H. B. Peng), pp. 685-695. San Diego: Academic Press.

Jamrich, M., Mahon, J., Gavis, E. and Gall, J. (1984). Histone RNA in amphibian oocytes visualized by in situ hybridization to methacrylate-embedded tissues. *EMBO J.* **3**, 1939-1943.

Kay, B. K. (1991). Injection of oocytes and embryos. In *Xenopus Laevis: Practical Uses in Cell and Molecular Biology*. (eds B. K. Kay and H. B. Peng), **36**, 663-669. San Diego: Academic Press.

Kessler, D. S. and Melton, D. A. (1994). Vertebrate embryonic induction: Mesodermal and neural patterning. *Science* **266**, 596-604.

Kessler, D. S. and Melton, D. A. (1995). Induction of dorsal mesoderm by soluble, mature Vg1 protein. *Development* **121**, 2155-2164.

Kim-Ha, J., Webster, P. J., Smith, J. L. and Macdonald, P. M. (1993). Multiple regulatory elements mediate distinct steps in localization of *oskar* mRNA. *Development* **119**, 169-178.

King, M. L. and Barklis, E. (1985). Regional distribution of maternal messenger RNA in the amphibian oocyte. *Dev. Biol.* **112**, 203-212.

Kislauskis, E. H., Zhu, X. and Singer, R. H. (1994). Sequences responsible for intracellular localization of β-actin messenger RNA also affect cell phenotype. *J. Cell Biol.* **127**, 441-451.

Kloc, M. and Etkin, L. (1995). Two distinct pathways for the localization of RNAs at the vegetal cortex in *Xenopus* oocytes. *Development* **121**, 287-297.

Kloc, M., Spohr, G. and Etkin, L. D. (1993). Translocation of repetitive RNA sequences with the germ plasm in *Xenopus* oocytes. *Science* **262**, 1712-1714.

Krieg, P. A. and Melton, D. A. (1984). Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs. *Nucl. Acids Res.* **12**, 7057-7070.

Krieg, P. A. and Melton, D. A. (1987). In vitro RNA synthesis with SP6 RNA polymerase. *Meth. Enzymol.* **155**, 397-415.

Ku, M. and Melton, D. A. (1993). Xwnt-11: A novel maternally expressed *Xenopus* wnt gene. *Development* **119**, 1161-1173.

Lantz, V. and Schedl, P. (1994). Multiple *cis*-acting targeting sequences are required for *orb* mRNA localization during *Drosophila* oogenesis. *Mol. Cell. Biol.* **14**, 2235-2242.

Linnen, J. M., Bailey, C. P. and Weeks, D. L. (1993). Two related localized mRNAs from *Xenopus laevis* encode ubiquitin-like fusion proteins. *Gene* **128**, 181-188.

Macdonald, P. and Struhl, G. (1988). *Cis*-acting sequences responsible for localizing *bicoid* mRNA at the anterior pole of *Drosophila* embryos. *Nature* **336**, 595-598.

Macdonald, P. M. (1990). *Bicoid* mRNA localization signal: Phylogenetic conservation of function and RNA secondary structure. *Development* **110**, 161-171.

Macdonald, P. M., Kerr, K., Smith, J. L. and Leask, A. (1993). RNA regulatory element BLE1 directs the early steps of *bicoid* mRNA localization. *Development* **118**, 1233-1243.

Macdonald, P. M., Leask, A. and Kerr, K. (1995). exl protein specifically binds BLE1, a *bicoid* mRNA localization element, and is required for one phase of its activity. *Proc. Natl. Acad. Sci. USA* **92**, 10787-10791.

Melton, D. A. (1987). Translocation of a localized maternal mRNA to the vegetal pole of *Xenopus* oocytes. *Nature* **328**, 80-82.

Mosquera, L., Forristall, C., Zhou, Y. and King, M. L. (1993). A mRNA localized to the vegetal cortex of *Xenopus* oocytes encodes a protein with a *nanos*-like zinc finger. *Development* **117**, 377-386.

Mowry, K. L. (1996). Complex formation between stage-specific oocyte factors and a *Xenopus* mRNA localization element. *Proc. Natl. Acad. Sci. USA* **93**, 14908-14613.

Mowry, K. L. and Melton, D. A. (1992). Vegetal messenger RNA localization directed by a 340-nt sequence element in *Xenopus* oocytes. *Science* **255**, 991-994.

Neuman-Silberberg, F. S. and Schüpbach, T. (1993). The *Drosophila* dorsoventral patterning gene *gurken* produces a dorsally localized RNA and encodes a TGFα-like protein. *Cell* **75**, 165-174.

Nieuwkoop, P. D. (1969). The formation of mesoderm in urodelean amphibians. I. Induction by the endoderm. *Roux's Arch. EntwMech. Org.* **162**, 341-373.

Nüsslein-Volhard, C., Frohnhöfer, H. G. and Lehmann, R. (1987). Determination of anteroposterior polarity in *Drosophila*. *Science* **238**, 1675-1681.

Phillips, C. R. (1982). The regional distribution of poly(A) and total RNA concentrations during early *Xenopus* development. *J. Exp. Zool.* **223**, 265-275.

Pondel, M. D. and King, M. L. (1988). Localized maternal mRNA related to transforming growth factor β mRNA is concentrated in a cytokeratin-enriched fraction from *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA* **85**, 7612-7616.

Rebagliati, M. R., Weeks, D. L., Harvey, R. P. and Melton, D. A. (1985). Identification and cloning of localized maternal mRNAs from *Xenopus* eggs. *Cell* **42**, 769-777.

Sagata, N., Okuyama, K. and Yamana, K. (1981). Localization and segregation of maternal RNAs during early cleavage of *Xenopus laevis* embryos. *Devel. Growth Differ.* **23**, 23-32.

Seeger, M. A. and Kaufman, T. C. (1990). Molecular analysis of the *bicoid* gene from *Drosophila pseudoobscura*: Identification of conserved domains within coding and noncoding regions of the *bicoid* mRNA. *EMBO J.* **9**, 2977-2987.

Serano, T. L. and Cohen, R. S. (1995). A small predicted stem-loop structure mediates oocyte localization of *Drosophila K10* mRNA. *Development* **121**, 3809-3818.

St Johnston, D. (1995). The intracellular localization of messenger RNAs. *Cell* **81**, 161-170.

Steward, O. and Banker, G. A. (1992). Getting the message from the gene to the synapse: Sorting and intracellular transport of RNA in neurons. *Trends Neurosci.* **15**(5), 180-186.

Sundell, C. L. and Singer, R. H. (1991). Requirement of microfilaments in sorting of actin messenger RNA. *Science* **253**, 1275-1277.

Tannahill, D. and Melton, D. A. (1989). Localized synthesis of the Vg1 protein during early *Xenopus* development. *Development* **106**, 775-785.

Thomsen, G. H. and Melton, D. A. (1993). Processed Vg1 protein is an axial mesoderm inducer in *Xenopus*. *Cell* **74**, 433-441.

Wallace, R. A. and Misulovin, Z. (1978). Long-term growth and differentiation of *Xenopus* oocytes in a defined medium. *Proc. Natl. Acad. Sci. USA* **75**, 5534-5538.

Wang, C. and Lehmann, R. (1991). *Nanos* is the localized posterior determinant in *Drosophila*. *Cell* **66**, 637-647.

Weeks, D. L. and Melton, D. A. (1987a). A maternal mRNA localized to the animal pole of *Xenopus* eggs encodes a subunit of mitochondrial ATPase. *Proc. Natl. Acad. Sci. USA* **84**, 2798-2802.

Weeks, D. L. and Melton, D. A. (1987b). A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for a growth factor related to TGF-β. *Cell* **51**, 861-867.

Yisraeli, J. and Melton, D. A. (1988). The maternal mRNA Vg1 is correctly localized following injection into *Xenopus* oocytes. *Nature* **336**, 592-595.

Yisraeli, J., Sokol, S. and Melton, D. A. (1990). 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 RNA. *Development* **108**, 289-298.

Zhou, Y. and King, M. L. (1996). RNA transport to the vegetal cortex of *Xenopus* oocytes. *Dev. Biol.* **179**, 173-183.

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