

# ***fatvg* encodes a new localized RNA that uses a 25-nucleotide element (FVLE1) to localize to the vegetal cortex of *Xenopus* oocytes**

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Accepted 8 September; published on WWW 21 October 1999

## **SUMMARY**

**Vegetally localized transcripts have been implicated in a number of important biological functions, including cell fate determination and embryonic patterning. We have isolated a cDNA, *fatvg*, which encodes a localized maternal transcript that exhibits a localization pattern reminiscent of Vg1 mRNA. *fatvg* is the homologue of a mammalian gene expressed in adipose tissues. The *fatvg* transcript, unlike Vg1 which localizes strictly through the Late pathway, also associates with the mitochondrial cloud that is characteristic of the METRO or Early pathway. This suggests that *fatvg* mRNA may utilize both the METRO and Late pathways to localize to the vegetal cortex during oogenesis. We have dissected the cis-acting localization elements of *fatvg* mRNA and compared these elements with**

**Vg1 mRNA. Our results indicate that, like most localized RNAs, in a variety of systems, transcripts of *fatvg* contain localization elements in the 3'UTR. The 3'UTR of *fatvg* mRNA contains multiple elements that are able to function independently; however, it functions most efficiently when all of the elements are present. We have defined a short 25-nucleotide element that can direct vegetal localization as a single copy. This element differs in sequence from previously described Vg1 localization elements, suggesting that different localization elements are involved in the localization of RNAs through the Late pathway.**

Key words: *Xenopus*, Oocyte, RNA localization, *fatvg*

## **INTRODUCTION**

Asymmetric distribution of gene products within subcellular regions has been widely observed. In theory, localized gene products can be stored as proteins or RNAs. The localization of RNAs rather than proteins is a comparatively economical way for a cell to attain highly concentrated amounts of localized gene products within specific regions.

Localized RNAs have been implicated in specialized functions in a variety of eukaryotic cells ranging from yeast to somatic cells. In budding yeast, the unequal distribution of *ASH1* mRNA to the distal end of the daughter cell regulates mating type switching (Long et al., 1997; Takizawa et al., 1997). In chicken embryo fibroblasts,  $\beta$ -actin mRNA plays a role in cell motility and localizes to the lamellipodia at the leading edge of the cells (Kislauskis et al., 1997; Lawrence and Singer, 1986). A recent finding also correlates localized  $\beta$ -actin mRNA with the metastatic potential of tumor cell lines (Shestakova et al., 1999).

The setting up of polarities by localized RNAs in oocytes is critical in the subsequent establishment of embryonic patterns. A search for localized RNAs in ascidian eggs has led to the discovery of RNAs that localize to the myoplasm or the ectoplasm specifically (Swalla and Jeffery, 1996a,b). Localized RNAs have also been recently reported in zebrafish oocytes (Maegawa et al., 1999; Bally-Cuif et al., 1998). The antero-

posterior identity of *Drosophila* oocytes is determined by differential RNAs localization to opposite poles (Lasko, 1999). Bicoid mRNA localizes to the anterior margin of the oocyte (Berleth et al., 1988; Frigerio et al., 1986) and the Bicoid protein forms a morphogen gradient in embryos to specify anterior cell fates such as head and thorax (Driever et al., 1990). Both *nanos* and *oskar* mRNAs localize to the posterior pole region (Ephrussi et al., 1991; Kim-Ha et al., 1991; Wang and Lehmann, 1991). The protein products of *nanos* are required for posterior development such as the abdomen (Wang and Lehmann, 1991). *oskar* is involved in the assembly of the pole plasm, which is to form primordial germ cells (Ephrussi and Lehmann, 1992; Ephrussi et al., 1991). The polarity of *Xenopus* oocytes is exemplified by localized RNAs along the animal-vegetal axis (Schnapp et al., 1997). For example, a number of localized RNAs such as An1 (Linnen et al., 1993), An2 (Weeks and Melton, 1987a), An3 (Gururajan et al., 1991; for An1-3, see Rebagliati et al., 1985), Xlan4 (Reddy et al., 1992), An4a (Hudson et al., 1996) and PABP (Schroeder and Yost, 1996) are enriched in the animal hemisphere, whereas Vg1 (Weeks and Melton, 1987b; Rebagliati et al., 1985), VegT (Horb and Thomsen, 1997; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996), Xwnt-11 (Ku and Melton, 1993), Xcat2 (Mosquera et al., 1993), Xlsirts (Kloc et al., 1993), Xdazl (Houston et al., 1998) and Xpat (Hudson and Woodland, 1998) are localized to the vegetal region.

In *Xenopus*, RNAs localize to the vegetal cortex through two pathways: the METRO (MEssage TRansport Organizer) and the Late pathway (Kloc and Etkin, 1995). In stage I and II oocytes, METRO RNAs (e.g. Xcat2, Xwnt-11 and Xlsirts) are localized to the mitochondrial cloud, which also contains lipid, mitochondria and electron-dense materials, as shown by electron microscopy (Heasman et al., 1984). Between stages I and II, the mitochondrial cloud migrates, together with the METRO RNAs, from a position next to the germinal vesicle to the vegetal cortex. During the course of migration, the mitochondrial cloud disperses into islands and the METRO RNAs are translocated to the vegetal cortex by stage II-III. Vg1 mRNA utilizes the Late pathway and is distributed throughout the cytoplasm in stage I and II oocytes. At late stage II to early stage III, Vg1 mRNA accumulates into a wedge-shaped structure and later translocates to the vegetal cortex (Kloc and Etkin, 1995). In comparison with the METRO RNAs, localized Vg1 mRNA occupies a broader region of the vegetal cortex.

The 3'UTRs of a number of localized RNAs have been characterized in detail (reviewed by Bashirullah et al., 1998). Some of these studies have provided fundamental knowledge of RNA localization signals concerning sequence elements as well as structural requirements. It has been shown that the oskar 3'UTR contains multiple localization signals 100-200 nucleotides (nt) in length, which are required for distinct steps in the localization process (Kim-Ha et al., 1993). A 53-nt region in the 3'UTR of the bicoid transcript (BLE1) is required for an early event in bicoid localization (Macdonald et al., 1993). In *Xenopus*, localization signals have been reported in Vg1 (Mowry and Melton, 1992), Xcat2 (Zhou and King, 1996a,b) and Xlsirts (Kloc et al., 1993) RNAs. For example, within the 340-nt Vg1 localization element (Mowry and Melton, 1992), four reiterative elements (E1, E2, E3 and E4) (Deshler et al., 1997) and a repeated VM1 subelement/hexanucleotide element have been identified independently (Havin et al., 1998; Gautreau et al., 1997).

The vegetal cortical regions of *Xenopus* oocytes contain localized determinants important for axis specification and lineage determination. Cytoplasmic transfer studies have demonstrated the existence of a dorsal determinant in the vegetal cortical region (Holowacz and Elinson, 1995). The localized mRNA Vg1 encodes a TGF- $\beta$  related protein and is involved in embryonic induction and patterning (Hyatt and Yost, 1998; Kessler and Melton, 1995). With the aim of isolating novel genes that encode localized maternal determinants, we have screened a *Xenopus* oocyte library enriched for vegetally localized transcripts by random in situ hybridization. Our cloning of *fatvg* has prompted us to ask if *fatvg* mRNA utilizes the same localization signals as Vg1 mRNA to achieve vegetal localization. In the present study, we have determined whether Vg1 localization elements are required for the localization of *fatvg* mRNA. By characterizing the localization signals of *fatvg* mRNA, we have discovered a short 25-nt localization signal, FVLE1. Unlike the other Vg1 localization elements VM1 and E2, both of which only localize when present in multiple copies, a single copy of FVLE1 can direct vegetal localization.

## MATERIALS AND METHODS

### Poly(A)<sup>+</sup>RNA isolation

Mitochondrial cloud materials were isolated according to a procedure used by Christine Dreyer (Max Plank Institute, Germany).

Manipulations of oocytes were carried out according to Kloc and Etkin (1999). Ovaries were dissected from 200 froglets (body length 4-6 cm), *Xenopus laevis* (Xenopus Express), which contain a majority of stage I-III oocytes (Dumont, 1972). After collagenase treatment, oocytes were transferred to a 50 ml tube. An equal volume of TES buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 0.25 M sucrose) was added and the oocytes were homogenized on ice through a syringe and an 18-gauge needle. A 3 ml sample of the oocyte lysate was placed on top of a 35 ml centrifuge tube (Beckman 344058) containing a step gradient of 8 ml each of 60%, 20%, 10% and 5% Percoll (Sigma) in TES buffer. Centrifugation was carried out for 45 minutes at 110,000 *g* and 4°C. After fractionation, a layer of greyish 'fluffy' material obtained near the middle layer was the mitochondrial cloud layer. The mitochondrial cloud layer was phenol-chloroform extracted and ethanol-precipitated. Poly(A)<sup>+</sup>RNA was extracted using magnetic beads coated with oligo(dT) (DyNAL, Norway). 5  $\mu$ g poly(A)<sup>+</sup>RNA was used for the construction of a  $\lambda$ ZAP-XR11 directional cDNA library (Stratagene).

### In situ hybridization

Whole-mount in situ hybridization was carried out according to Kloc and Etkin (1999) and Harland (1991). In situ hybridization on sectioned materials was performed according to Lemaire et al. (1995).

### Construction of 3'UTR mutants

The vector pBluescript-KS (Stratagene) was used for all subcloning experiments. Deletion constructs were constructed by PCR. Site-directed mutagenesis within the 3'UTR were carried out using complementary primers containing the desired mutation and the QuikChange kit (Stratagene). In constructs fv3'(UTR/M) and fv3'(UTR/D), the three E2 sequences (UUCAC) were mutated to CCUGU and deleted, respectively. The DNA sequences of all constructs were confirmed by sequencing performed by the DNA core sequencing facility.

### Synthesis, injection and detection of RNA transcripts

Synthesis of RNA transcripts, oocyte injection and detection of injected RNAs were carried out according to Kloc and Etkin (1999). Ovaries were obtained from albino female *Xenopus laevis* (Xenopus Express). Stage III and early stage IV oocytes were obtained by manual defolliculation. Injection was performed using a Drummond Nanoject Injector. For each transcript tested, a total of at least 60-150 oocytes each were injected and assayed for localization. Localizations of transcripts resembling wild-type 3'UTR injection were scored as ++. Transcripts that were localized to the vegetal cortex but not the 'crescent' region next to the germinal vesicle were scored as +. Transcripts that did not show detectable localization were scored as -.

## RESULTS

### *fatvg* is a member of a new protein family

Specific RNAs are localized to the mitochondrial cloud which, together with the localized RNAs, is translocated to the vegetal cortical region during oogenesis (Kloc and Etkin, 1995; Kloc et al., 1993). To identify novel localized RNAs, a *Xenopus* oocyte cDNA library enriched for vegetally localized transcripts was constructed. We showed that the cDNA library was representative of vegetally localized RNAs by performing plaque hybridizations using known localized RNAs including Vg1, Xcat2 and Xlsirts as probes. Positive plaques corresponded to cDNA clones that contain Vg1, Xcat2 and Xlsirts sequences were detected at a frequency of 0.01%, 0.1% and 0.02%, respectively, out of  $2.5 \times 10^5$  clones analyzed (data not shown).

Individual clones were isolated and antisense RNA probes were generated for in situ hybridization using different stages of

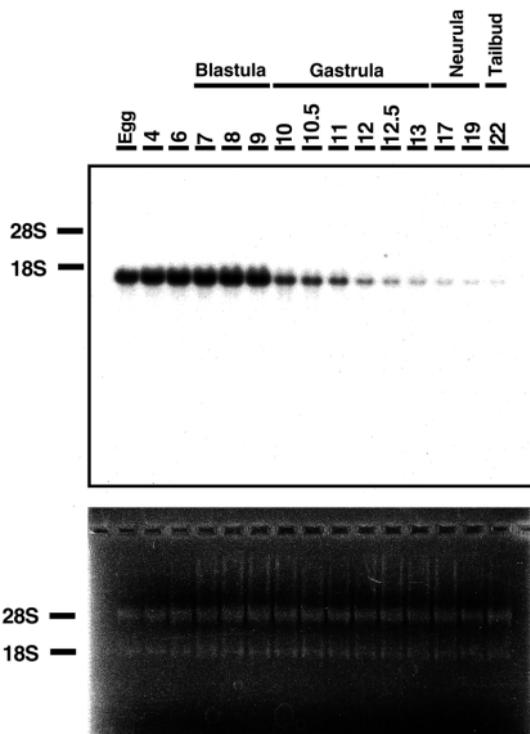


and adipophilin. For perilipin, the region of homology was limited to the N-terminal region (100 aa) (Greenberg et al., 1993). A short region of ADRP was also found to resemble a 33-aa repeat motif in S3-12 (Scherer et al., 1998). Interestingly, *fatvg* also showed a significant similarity to a protein independently named TIP47 and PP17b. TIP47 is a cytosolic cargo selection protein involved in intracellular trafficking from endosomes to the trans-Golgi network (Diaz and Pfeffer, 1998), whereas PP17b is a placental tissue protein that is detected at an elevated level in cervical carcinoma (Than et al., 1998). Although ADRP, TIP47/PP17b and perilipin appeared to play completely distinct roles, the similarities among the amino acid sequences suggest that they may comprise a new protein family.

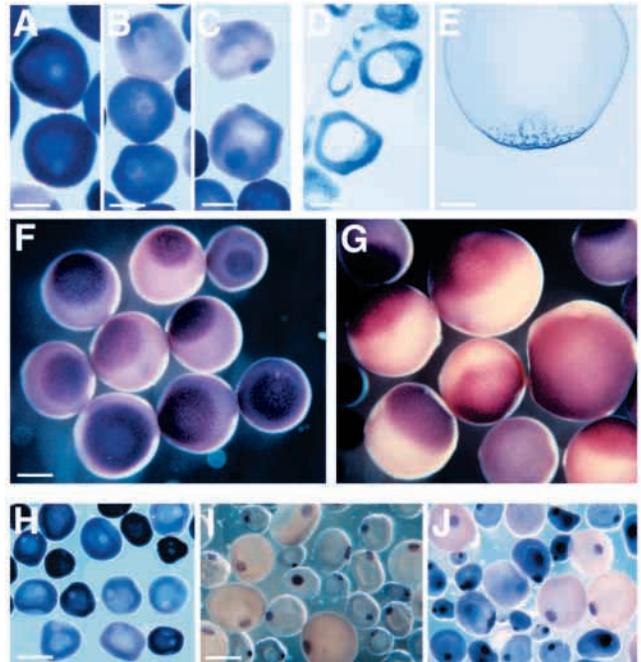
We conclude that *fatvg*, which has been isolated from a mitochondrial cloud-enriched *Xenopus* oocyte library, is the frog homologue of ADRP and is related to other lipid droplet associated proteins.

### *fatvg* encodes a vegetally localized transcript

In stage I oocytes, *fatvg* mRNA was distributed throughout the cytoplasm and was excluded from the mitochondrial cloud as shown by in situ hybridization (Fig. 3A). Localization began at stage II when the RNA was first detected in the center of the mitochondrial cloud (Fig. 3B). After the mitochondrial cloud reached the vegetal cortex, *fatvg* mRNA was detected in the entire mitochondrial cloud (Fig. 3C). Association with the mitochondrial cloud is a characteristic of RNAs that utilize the METRO pathway such as *Xcat2* and *Xlsirts*. Late pathway RNAs such as *Vg1* have never been found associated with the



**Fig. 2.** Temporal expression of *fatvg* mRNA during early development. In a northern analysis (upper panel), a probe complementary to the coding region and the 3'UTR of *fatvg* was hybridized to total RNA at various stages of development. Ethidium bromide staining (lower panel) showed the amount of ribosomal RNAs and served as a loading control.



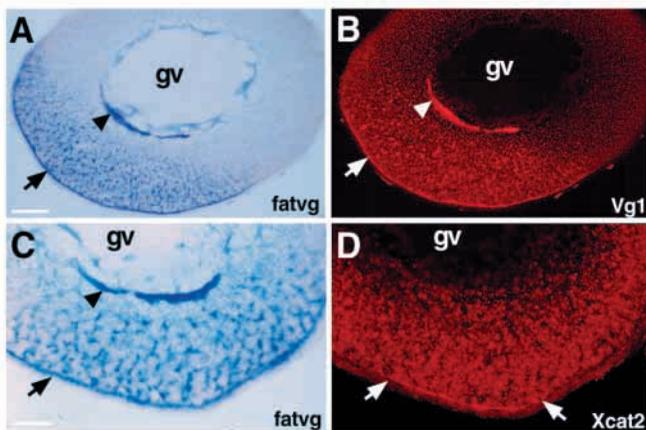
**Fig. 3.** *fatvg* encoded a vegetally localized RNA in oocytes. Whole-mount in situ hybridization was carried out using albino oocytes. (A) In stage I oocytes, *fatvg* mRNA was distributed throughout the cytoplasm but was excluded from the mitochondrial cloud. Bar, 50  $\mu$ m. (B) In stage II oocytes, *fatvg* mRNA was first detected in the center of the mitochondrial cloud. The majority of the RNA was detected in the cytoplasm. Bar, 75  $\mu$ m. (C) *fatvg* mRNA was detected in the entire mitochondrial cloud which was at the vegetal cortex. Bar, 100  $\mu$ m. (D,E) In situ hybridization performed on sectioned stage I and stage III oocytes. (D) No staining was observed in the mitochondrial cloud in stage I oocytes. Bar, 60  $\mu$ m. (E) A 'wedge'-shaped localization pattern of *fatvg* mRNA was observed in stage III oocytes. Bar, 120  $\mu$ m. (F) In stage III-IV oocytes, *fatvg* mRNA was detected in the vegetal pole region. (G) *fatvg* mRNA occupied almost the entire vegetal hemisphere in stage V oocytes. Bar, 400  $\mu$ m in F,G. (H-J) For comparison, whole-mount in situ hybridization is also shown for the localization of *Vg1* (H), *Xcat2* (I) and *Xlsirts* (J) in stage I and II oocytes. Bars, 250  $\mu$ m (H); 300  $\mu$ m (I,J).

mitochondrial cloud. For comparison, the localizations of *Vg1*, *Xcat2* and *Xlsirts* RNAs in stage I and II oocytes are also shown (Fig. 3H-J). As had been reported in earlier studies (Forristall et al., 1995; Kloc and Etkin, 1995), *Vg1* was not detected in the mitochondrial cloud (Fig. 3H), whereas *Xcat2* (Fig. 3I) and *Xlsirts* (Fig. 3J) localized to the entire mitochondrial cloud as early as stage I. In stage III oocytes, *fatvg* mRNA was detected towards the apex of the vegetal hemisphere (Fig. 3F). As oogenesis proceeded, the hybridization pattern broadened equatorially from the vegetal pole region and occupied almost the entire vegetal hemisphere by stage V (Fig. 3G). In situ hybridization was also performed on sectioned oocytes. Fig. 3D shows clearly that *fatvg* mRNA was not detected in the mitochondrial cloud in sectioned stage I oocytes. Fig. 3E shows that *fatvg* mRNA was distributed in a 'wedge'-shaped localization pattern in sectioned stage III oocytes, as was described for *Vg1* (Deshler et al., 1997; Kloc and Etkin, 1995). Therefore, our results indicate that the majority of *fatvg* mRNA utilizes the Late pathway and part of the RNA may follow the METRO pathway.

Double in situ hybridization was performed on sectioned stage III oocytes to compare the localization patterns of *fatvg* with representative examples of RNAs that belong to the METRO and Late pathways. By examining oocyte sections that were hybridized to digoxigenin-labelled *fatvg* and fluorescein-labelled *Vg1* antisense RNA probes, we showed that *fatvg* and *Vg1* mRNAs colocalized to three areas: the vegetal cytoplasm, the vegetal cortical region and a 'crescent'-shaped structure in close proximity to the vegetal side of the germinal vesicle (Fig. 4A,B). The localization patterns of *fatvg* and *Xcat2* were also compared using antisense *fatvg* and *Xcat2* probes (Fig. 4C,D). We showed that *Xcat2* mRNA localized to a more restricted vegetal cortical region, as demonstrated in earlier studies (Fig. 4D) (Forristall et al., 1995; Kloc and Etkin, 1995). Additionally, we showed that *Xcat2* mRNA was not detected in the 'crescent' region (Fig. 4D). Based on our observations, we conclude that only RNAs that follow the Late pathway localize to the 'crescent' structure next to the germinal vesicle. The function or the identity of the 'crescent' may provide insight into the mechanism of the Late pathway.

We also determined the temporal expression of *fatvg* during early development. A northern blot was performed using RNA obtained from unfertilized eggs and different stages of embryos (Fig. 2). A single transcript of approximately 1.6 kb was detected. The high level of expression between unfertilized eggs and late blastula stage embryos (stage 9) reflected the abundant maternal content of the transcript. The expression level of *fatvg* mRNA declined gradually beginning from the gastrula stage. A low level of mRNA persisted until the tailbud stage.

In summary, *fatvg* encodes a maternally localized RNA



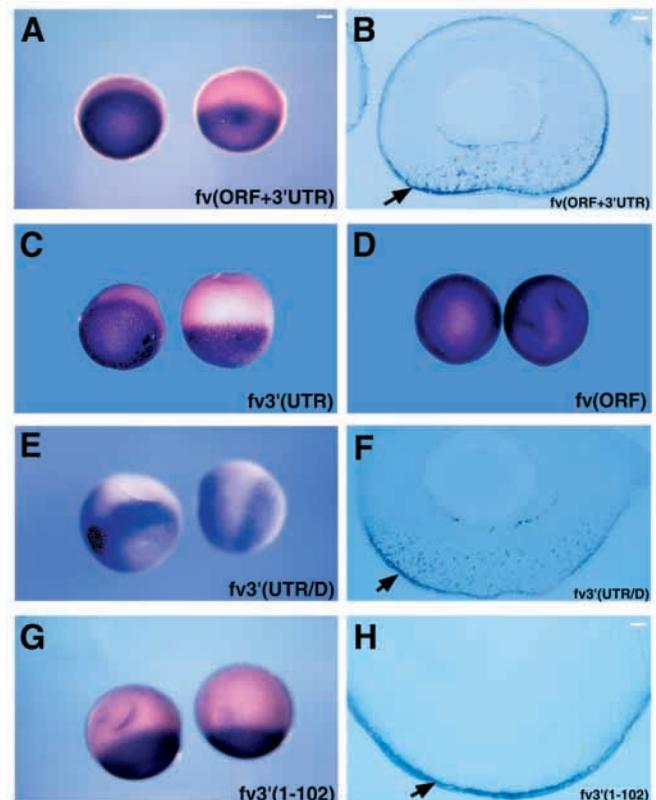
**Fig. 4.** Localization pattern of *fatvg* mRNA was identical to *Vg1* mRNA in stage III oocytes. Double in situ hybridization was performed on sectioned albino oocytes. (A,B) An oocyte section hybridized to *fatvg* and *Vg1* antisense RNA probes. (A) *fatvg* mRNA was visualized using chromogenic substrate BCIP/NBT, which gave a purple colour. *fatvg* mRNA was present in three regions: the 'crescent' area next to the germinal vesicle, the vegetal cytoplasm and the vegetal cortex. (B) *Vg1* mRNA was visualized on the same section as in A using Vector Red substrate. *Vg1* mRNA localized to the same regions as *fatvg* mRNA at this stage of oogenesis. Bar, 80  $\mu$ m in A,B. (C,D) An oocyte section hybridized to *fatvg* and *Xcat2* antisense RNA probes. (C) *fatvg* mRNA showed the same pattern of localization as described in A. (D) *Xcat2* mRNA localized to a restricted region at the vegetal cortex (delimited by arrows) but was not detected in the 'crescent' area. Bar, 40  $\mu$ m in C,D. gv, germinal vesicle; arrowhead, 'crescent' area; arrow, vegetal cortex

transcript and is present throughout early development. The majority of *fatvg* mRNA follows the Late pathway to translocate to the vegetal cortical region. With the exception of an association with the mitochondrial cloud during stage II of oogenesis, the overall localization pattern of *fatvg* in oocytes resembles that of *Vg1* mRNA.

### *fatvg* 3'UTR directs vegetal localization

*Vg1* is the first localized mRNA reported to utilize the Late pathway. Our result shows that *fatvg* utilizes both the METRO and Late pathways and localizes to the vegetal cortex of oocytes in a pattern similar to *Vg1*. In the present study, we focused on the identification of sequence elements involved in the localization of *fatvg* mRNA through the Late pathway in order to compare these elements to those of *Vg1* localization.

To determine if a given transcript has the ability to localize in oocytes using the Late pathway, we used an injection assay in which digoxigenin-labelled RNA was injected into stage III oocytes. Localization was determined directly by detecting the injected digoxigenin-labelled RNAs in oocytes after culture. We first showed that localization of injected *fv(ORF+3'UTR)* transcript was identical to the endogenous distribution of *fatvg*



**Fig. 5.** Visualization of digoxigenin-labelled RNAs after injection of wild-type and mutant *fatvg* 3'UTR transcripts into stage III albino oocytes. Localization was observed in oocytes injected with transcripts *fv(ORF+3'UTR)* (A), *fv3'(UTR)* (C), *fv3'(UTR-D)* (E) and *fv3'(1-102)* (G) transcripts. No localization was detected in oocytes injected with the *fv(ORF)* transcript (D). Bar, 100  $\mu$ m (A,C-E,G). Sections of injected oocytes are also shown for transcripts *fv(ORF+3'UTR)* (B), *fv3'(UTR-D)* (F) and *fv3'(1-102)* (H). Arrows indicate vegetal cortical localization of injected transcripts. Note that injected RNAs are only detected in the 'crescent' area for transcripts that contain a full-length 3'UTR (B,F). Bar, 50  $\mu$ m (B,F); 25  $\mu$ m (H).

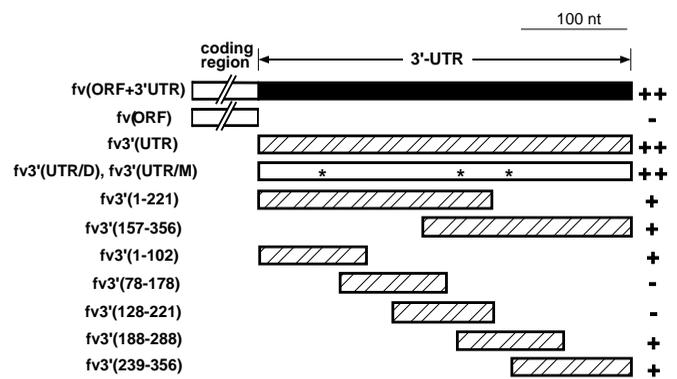
mRNA (Fig. 5A). Next, we asked which region of the *fatvg* mRNA contained the signal for localization. Since localization signals have been found to reside in the 3'UTR in almost all of the localized transcripts studied, we determined the ability of transcripts *fv*(ORF) and *fv*3'(UTR) to localize in injected oocytes. Fig. 5C shows that the *fv*3'(UTR) transcript localized efficiently to the vegetal cortex. The *fv*(ORF) transcript did not show any detectable sign of localization (Fig. 5D). Similar to the *fv*(ORF+3'UTR) transcript (Fig. 5B), the *fv*3'(UTR) transcript also localized to the crescent region (data not shown) indicating that the 3'UTR of *fatvg* contained all of the information required for vegetal cortical localization. We conclude that exogenous *fatvg* mRNA is able to recapitulate vegetal localization when injected into oocytes and that the localization signal of *fatvg* mRNA is located in the 3'UTR.

### Removal of E2 elements in *fatvg* 3'UTR has no effect on localization

In order to characterize the localization elements in the 3'UTR of *fatvg* mRNA, we analyzed the sequence of *fatvg* 3'UTR to detect any known localization elements. Based on sequence comparison, three E2 elements (UUCAC) were identified in the 3'UTR of *fatvg* mRNA (Fig. 1A). None of the Vg1 localization elements, including E1, E3, E4 and VM1/hexanucleotide element, were found. It was shown that the E2 localization element was a critical element responsible for Vg1 localization since deletion of all E2 elements abolished the ability of injected Vg1 mRNA to localize (Deshler et al., 1997). We therefore tested the function of E2 elements in *fatvg* 3'UTR by deletion and mutation. Fig. 5E shows that localization of the *fatvg* 3'UTR deletion mutant *fv*3'(UTR/D), which had all three E2 elements deleted, was completely unaffected. The same result was obtained for *fv*3'(UTR/M) in which all E2 sequences were mutated (data not shown). The localization patterns of these mutants were identical to injected wild-type *fv*3'(UTR) and *fv*(ORF+3'UTR) transcripts. In fact, the 3'UTR mutant transcripts were also detected in the 'crescent' region next to the germinal vesicle, in the vegetal cytoplasm and at the vegetal cortex (Fig. 5F). Therefore, the E2 elements in the 3'UTR of *fatvg* mRNA are not required for the correct localization of the transcript since removal of these elements does not alter the ability of injected *fatvg* 3'UTR to localize.

### *fatvg* 3'UTR contains multiple localization signals

In addition to sequence analysis, an alternative approach to identify localization signals within *fatvg* 3'UTR was to assay different regions of the 3'UTR for vegetal localization. We divided the 3'UTR into overlapping fragments to minimize the potential destruction of localization signals at the junctions of adjoining fragments. Injection of transcripts containing sequences between 1-221 nt and 157-356 nt resulted in vegetal localization (Fig. 6). This result suggested either that a localization signal is present within the overlapping region or multiple localization signals are located within the 3'UTR. To distinguish between these possibilities, the 3'UTR was divided into five overlapping regions, which contained nucleotide sequences between 1-102 nt, 78-178 nt, 128-221 nt, 188-288 nt and 239-356 nt, respectively. Fig. 6 shows a summary of the constructs tested. Vegetal cortical localization was observed in transcripts that contained the 5' region [construct *fv*3'(1-102)] (Fig. 5G) or the 3'-region



**Fig. 6.** Constructs used for the mapping of *fatvg* 3'UTR. Transcripts containing a full-length *fatvg* 3'UTR [*fv*(ORF+3'UTR), *fv*3'(UTR), *fv*3'(UTR-D) and *fv*3'(UTR-M)] showed strong localization and were scored as ++. Transcripts containing short regions of *fatvg* 3'UTR [*fv*3'(1-221), *fv*3'(157-356), *fv*3'(1-102), *fv*3'(188-288) and *fv*3'(239-356)] showed a weaker localization and were scored as +. No localization was observed for transcripts that contain the coding region of *fatvg* [*fv*(ORF)] and two short regions of the 3'UTR [*fv*3'(78-178), *fv*3'(128-221)] and these transcripts were scored as -. \*Sites of E2 elements that were deleted or mutated in *fv*3'(UTR-D) and *fv*3'(UTR-M), respectively.

[*fv*3'(188-288) and *fv*3'(239-356)], although these injected transcripts were not detected in the 'crescent' region (Fig. 5H). Localization was abolished in transcripts that only contain the middle region of the 3'UTR [*fv*3'(78-178) and *fv*3'(128-221)]. The fact that both the 5' and 3' regions of *fatvg* 3'UTR contained localization signals suggested that multiple signals responsible for vegetal cortical localization were present in *fatvg* 3'UTR. Our results also indicate that although these multiple localization signals can function independently of each other, the entire 3'UTR sequence is required to achieve efficient localization.

### Characterization of a localization signal in the 5' region of *fatvg* 3'UTR

Further characterization was carried out to analyze the localization signal within the 1-102 nt region at the 5' end of *fatvg* 3'UTR. In a search for sequences that are potentially required for localization, we identified a stretch of 10 uracil residues and an E2 element (UUCAC) in the 1-102 nt region. We asked if these sequences were necessary for localization in the context of the 1-102 nt region by deletions [*fv*3'(1-102/D1) and *fv*3'(1-102/D2)] and mutations [*fv*3'(1-102/M1), *fv*3'(1-102/M2) and *fv*3'(1-102/M3)]. Our results showed that alteration of these two sequence elements did not affect localization (summarized in Fig. 7A). We also asked if the localization of the 1-102 nt region was dependent on its position or orientation when joined to the *fatvg* coding region. Fig. 7B shows a group of four constructs with the 1-102 nt region in both forward (constructs ORF-3F and ORF-5F) and reverse orientations (constructs ORF-3R and ORF-5R), attached to either ends of the coding region. We showed that localization occurred when the 1-102 nt region was presented in the forward orientation. This result suggested that if any secondary RNA structure such as a stem-loop was present for the recognition of a localization factor, the stem-loop structure was only functional when the 1-102 nt region was in the normal

forward orientation. The same stem-loop structure formed in the reverse orientation, though with the same base-pairing in the stem region, will not be functional.

**A short 25-nt sequence element in *fatvg* 3'UTR directs vegetal cortical localization**

In order to identify localization elements within the first 102 nucleotides of *fatvg* 3'UTR, we carried out a detailed mapping of the corresponding region. Fig. 7C shows a summary of the mapping constructs tested. All transcripts used contained the 1.3 kb ORF of the *fatvg* transcript to avoid a drastic change in transcript length during progressive deletions. We demonstrated previously that a transcript that contained the ORF region did not localize after injection (Fig. 5D). In this mapping study, we showed that localization was detected for transcript *fv3'*(28-102) but not *fv3'*(1-28). Construct *fv3'*(28-102) was further divided to generate two shorter constructs, *fv3'*(28-58) and *fv3'*(59-102). Transcript *fv3'*(28-58) retained the ability to localize in injected oocytes. Localization was detected in oocytes injected with transcripts *fv3'*(28-102) or *fv3'*(28-58) (Fig. 8A,B). Additional regions containing the 31-nt sequence in construct *fv3'*(28-58) were also assayed for localization. Transcripts *fv3'*(1-58) and *fv3'*(28-72) also demonstrated vegetal localization. These results are in keeping with the function of the 31-nt sequence in construct *fv3'*(28-58) as a localization element.

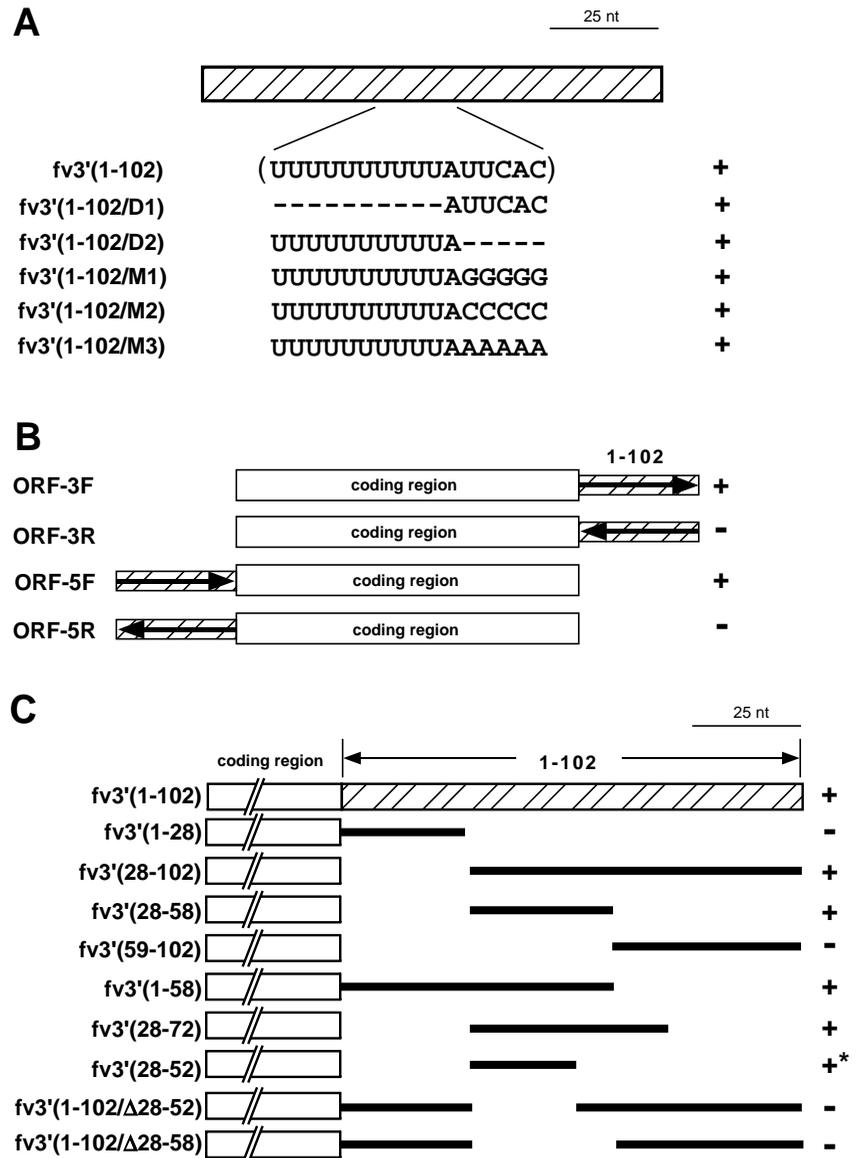
Interestingly, an E2 element was located at the 3' end of the 31-nt element. We have already shown by deletion [*fv3'*(1-102/D2) in Fig. 7A] that such an E2 element was not required for the localization of the first 102 nt of *fatvg* 3'UTR. To substantiate this finding, the 31-nt element was further reduced to a 25-nt element by removing the E2 element from the 3' end. Indeed, the 25-nt element can still localize to the vegetal cortex, as shown in whole-mount and sectioned oocytes in Fig. 8E,F, respectively, although localization was less efficient.

Finally, in order to provide evidence that FVLE1 is required for vegetal localization, we used the 1-102 nt region to generate constructs in which the FVLE1 sequence was removed by deletion. Fig. 7C shows that the transcripts *fv3'*(1-102/Δ28-52) and *fv3'*(1-102/Δ28-58) did not show any sign of localization. Therefore, deletion of the FVLE1 sequence abolished the ability of the 1-102 nt region of *fatvg* 3'UTR to localize.

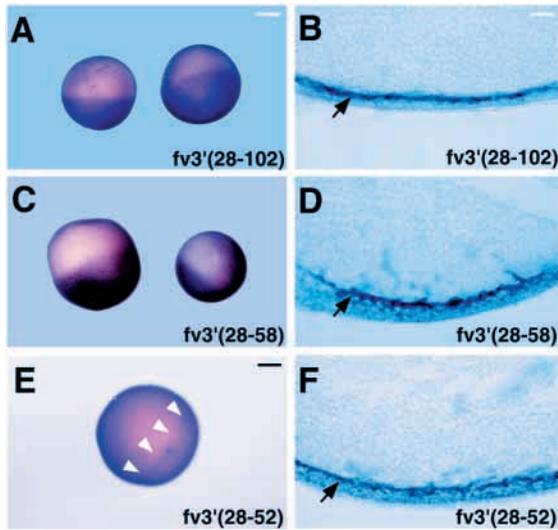
In summary, the 1-102 nt of *fatvg* 3'UTR contains a short element, FVLE1, which can direct vegetal cortical localization. FVLE1 is also required to mediate localization by the 5' region of *fatvg* 3'UTR through the Late pathway.

**DISCUSSION**

We have isolated from a cDNA library a novel gene, *fatvg*, by random in situ hybridization. *fatvg* encodes a maternal transcript, which localizes to the vegetal cortical region of oocytes by using both the METRO and Late pathways. The 3'UTR of *fatvg* mRNA contains multiple localization signals in the 5' and 3' regions. Detailed mapping of the first 102-nt sequence at the 5' end of the 3'UTR gives rise to a short 25-nt localization element, FVLE1, which can direct vegetal cortical localization as a single copy. The sequence



**Fig. 7.** Analyses of 1-102 nt of *fatvg* 3'UTR. (A) The requirements for a U-rich region and an E2 element in transcript *fv3'*(1-102) were tested. All transcripts showed localization when injected into oocytes. (B) *fv3'*(1-102) transcript was able to function when placed at the 5' or the 3' end of the coding region but only localized when presented in the forward orientation. (C) Mapping of *fv3'*(1-102) by deletion. By injecting transcripts containing different regions of *fv3'*(1-102), *fv3'*(28-58) was found to retain the ability to localize. A shorter transcript *fv3'*(28-52) was also tested, which was devoid of an E2 element at the 3' end of *fv3'*(28-58). \*Transcript *fv3'*(28-58) was able to localize, albeit more weakly.



**Fig. 8.** Visualization of injected digoxigenin-labelled RNA after injection of deletion constructs of 1-102 into stage III albino oocytes. Localization of injected transcripts are shown as whole mount for *fv3'(28-102)* (A), *fv3'(28-58)* (C) and *fv3'(28-52)* (E). Arrowheads indicate the boundary of weak localization in E. Bar, 200  $\mu\text{m}$  (A,C); 150  $\mu\text{m}$  (E), in whole-mount oocytes. Sections of injected oocytes are also shown for transcripts *fv3'(28-102)* (B), *fv3'(28-58)* (D) and *fv3'(28-52)* (F). Bar, 15  $\mu\text{m}$  for all sections.

of *FVLE1* is different from any *Vg1* localization elements reported.

ADRP, the mouse counterpart of *fatvg*, has been shown to locate on the membrane surface of lipid droplets (Brasaemle et al., 1997). A recent study shows that ADRP increases long chain fatty acid uptake when transfected into cultured cells and may function in the formation or stabilization of lipid droplets in adipocytes (Gao and Serrero, 1999). Perilipins share some homology to ADRP and have been suggested to play a role in both lipid deposition and mobilization (Londos et al., 1995). We postulate that *fatvg* is involved in the formation and/or utilization of the major energy source yolk platelets, which is also a membrane-bound structure, in the course of *Xenopus* development. Vegetal localization of *fatvg* may ensure efficient formation or assembly of yolk platelets in the vegetal region and therefore contributes to the asymmetric distribution of yolk along the animal-vegetal axis. The action of *fatvg* could function in concert with the downward movement of yolk particles from the animal region away from the germinal vesicle, which has been shown to result in a densely packed yolk mass in the vegetal region (Danilchik and Gerhart, 1987). Alternatively, *fatvg* could be strategically localized to the vegetal region, which differentiates into the gut endoderm in embryos, for the assimilation of yolk for growth and development during embryogenesis. Determination of the protein expression level of *fatvg* will help to discriminate between the above possibilities and provide further information for the functional roles of *fatvg*. Additionally, our preliminary data showed that *fatvg* mRNA is associated with the germ plasm in cleavage stage embryos (A. P. C., M. K. and L. D. E., unpublished observations). To date, only METRO RNAs have been found associated with the germ plasm in embryos. We do not rule out the possibility that vegetal localization of *fatvg*

mRNA represents an additional pathway of distributing maternal transcripts to the germ plasm for the specification of germ cells. The possible functions of *fatvg* involved in the formation and development of germ cells are under study. Although no localized RNAs have been reported in mouse oocytes, it will also be interesting to determine if ADRP mRNA does localize to specific regions in mouse oocytes.

RNAs that belong to the METRO and Late pathways are classified according to their distribution during oogenesis (Forristall et al., 1995; Kloc and Etkin, 1995). In stage I oocytes, METRO RNAs (e.g. *Xcat2* and *Xlsirts*) are found only in the mitochondrial cloud and Late pathway RNAs (e.g. *Vg1*) are present in the cytoplasm but not detected in the mitochondrial cloud. Similar to *Vg1*, *fatvg* mRNA is not detected in the mitochondrial cloud in stage I oocytes. In stage III oocytes, a unique characteristic of *Vg1* mRNA is a 'wedge'-shaped distribution in the vegetal region. *fatvg* also shows the same pattern during stage III of oogenesis. However, part of the *fatvg* mRNA is found associated with the mitochondrial cloud in stage II oocytes immediately before the Late pathway functions. Based on these criteria, we have concluded that *fatvg* mRNA demonstrates the overall characteristic of a Late pathway RNA but with a variation as shown by the association with the mitochondrial cloud. Such a result indicates a possible continuity between the METRO and Late pathway, as has been suggested previously (Kloc and Etkin, 1998). Further information from novel Late pathway RNAs will help to define additional characteristics for different localization pathways and possible interactions between the pathways.

It has been shown that in stage III oocytes, *Vg1* mRNA is detected in a 'wedge'-shaped structure prior to a complete cortical localization during late oogenesis. In the present study, we have observed that *Vg1* and *fatvg* mRNAs colocalize in a 'crescent' next to the germinal vesicle. The successful detection of such a 'crescent' structure is probably a result of increased sensitivity and resolution due to the use of sectioned oocytes for non-radioactive in situ hybridization. Injected *fatvg* mRNA containing a full-length 3'-UTR also localizes to the 'crescent'. The crescent therefore contains RNAs, both endogenous or injected, destined to migrate to the vegetal cortex. Additionally, we have observed that injected transcripts containing small regions of *fatvg* 3'-UTR, although being translocated to the vegetal cortex, are never present in the 'crescent'. A possible explanation is that the localization of a Late Pathway RNA involves three steps: first accumulation in the 'crescent', then translocation through the vegetal cytoplasm and, finally, anchoring at the vegetal cortex. All these steps require a single specific localization signal in the RNA to be recognized by the translocation machinery. A full-length *fatvg* 3'-UTR transcript may contain sequences that can stabilize the interaction between the localization signal and the translocation machinery. Full-length 3'-UTR transcripts are therefore localized to the vegetal pole efficiently. On the other hand, small regions of the 3'-UTR that contain only the localization signal, but not the rest of the sequence that has a stabilizing effect, are still able to localize; however, at a reduced efficiency. Although such small RNA transcripts can follow the normal pathway, a reduced efficiency of interaction with the localization factors may therefore result in a diminished level of RNA both in the 'crescent' region and the vegetal cortex. Preliminary results using immunostaining show

that regions around the 'crescent' structure contain endoplasmic reticulum (ER) (M. K. and L. D. E., unpublished observations). The involvement of the ER in RNA localization has been suggested in previous studies (Deshler et al., 1997). Our identification of the 'crescent' structure may add a new component to the Late pathway and provide insight into the mechanism of localization by the Late pathway RNAs.

We have shown that multiple localization signals are present in fatvg 3'UTR. Injected RNA transcripts that possess the 5' or 3' regions of fatvg mRNA are able to function independently and direct vegetal localization. Multiple localization signals have been reported in the 3'UTR of localized RNAs in oocytes such as Vg1 in *Xenopus* and oskar in *Drosophila* (Bashirullah et al., 1998). In Vg1 (Deshler et al., 1997; Gautreau et al., 1997) and fatvg mRNAs, localization elements are all dedicated to vegetal cortical localization. In oskar 3'-UTR, different regions are responsible for intermediate steps of the localization process, including transport from the nurse cells to the oocytes, transient association with the anterior margin of the oocytes and final localization to the posterior region (Kim-Ha et al., 1993). Such multiple intermediate steps of RNA localization are probably related to the merostic nature of insect oocytes. On the contrary, in *Xenopus* oocytes, maternal transcripts are exclusively provided by the germinal vesicle in the absence of nurse cells. The final step of RNA localization to the posterior pole of a *Drosophila* oocyte could be functionally analogous to vegetal localization of RNAs in *Xenopus* oocytes. The multiple localization signals in fatvg and Vg1 3'UTR may therefore have evolved to serve one single purpose: to ensure efficient localization of RNAs to the vegetal cortical region to associate with specific structures that subsequently determine different cell fates during embryogenesis.

Our mapping of the 5' region of fatvg 3'UTR has resulted in the identification of a short 25-nt localization element, FVLE1. FVLE1 has a unique property when compared to previously identified localization elements: one single copy of FVLE1 can direct vegetal cortical localization. BLE1, a 53-nt region identified in stem-loop V of bicoid 3'UTR, has the ability to localize only when present as two copies or if accompanied by the flanking region stem-loop IV (Macdonald and Kerr, 1998). A similar situation has been reported in Vg1 3'UTR. A 85-nt 5' subelement, which contains two VM1 localization elements, directs localization only as two tandem repeats or in the presence of a VM1-containing 3' region of the Vg1 localization element (Gautreau et al., 1997). Furthermore, the E2 element, when present as single or tandem copies, is not sufficient to direct localization (Deshler et al., 1998). Therefore, the localization mediated by a single copy of the 25-nt FVLE1 suggests that the element contains all of the necessary recognition sites for the binding of factors for localization, albeit not functionally as efficient as the intact 3'UTR unit.

Within the FVLE1 sequence, we have noticed a potential UA-rich cytoplasmic polyadenylation signal (CPE) UUUUUUAU (McGrew and Richter, 1990; Paris and Richter, 1990) near the 3' end of FVLE1 and flanking sequences. However, it is unlikely that the vegetal localization of FVLE1-containing transcripts is related to the cytoplasmic polyadenylation pathway. First, one of the constructs tested contains a deletion of 10 uracil residues that overlaps with the potential CPE sequence. We have already shown that this

mutant transcript fv3'(1-102/D1) does localize and its localization resembles that of the wild-type transcript fv3'(1-102). Therefore, removal of the potential CPE by the deletion of uracil residues has no effect on localization. Second, it has been shown that the nuclear polyadenylation signal AAUAAA is necessary for CPEs to function (Vassalli et al., 1989). Many of the FVLE1-containing transcripts tested such as fv3'(1-102) and fv3'(28-52) lack the AAUAAA signal and, nevertheless are able to localize to the vegetal cortex. Therefore, we believe that the pathway responsible for the vegetal localization of FVLE1 is different from that of cytoplasmic polyadenylation.

The significance of the E2 element immediately 3' to FVLE1 is not clear. We have shown that removal of all E2 elements from the entire fatvg 3'UTR or the single E2 element from within the 5' region of fatvg 3'UTR have no effect on localization. E2 elements as defined by the sequence UUCAC therefore are not required for proper localization of fatvg 3'UTR. However, deletion of the E2 element 3' to the FVLE1 reduces the efficiency of localization. A possible explanation is that the E2 element is involved in the interaction of FVLE1 with the translocation machinery such as localization factors. Analysis of the BLE1 sequence in bicoid 3'UTR has shown that BLE1 is made up of a recognition domain and a flanking RNA helix (Macdonald and Kerr, 1998). It is postulated that the helix region promotes the correct folding of the recognition domain, which as a consequence interacts with a localization factor. Since FVLE1 can direct localization, the sequence that interacts with the localization factor probably remains intact. Similar to the helix region in BLE1, the E2 sequence 3' to FVLE1 may affect the structural stability of FVLE1 and therefore reduces the interaction of localization factors with the FVLE1 sequence.

We have shown that the localization of fatvg mRNA does not require the Vg1 localization element E2. Our initial identification of key localization elements within the fatvg mRNA transcripts allows subsequent isolation of localization factors using sequence information from within a confined region. The search for RNA-binding proteins is likely to redefine novel functions for cellular proteins and, more importantly, improves our current knowledge of mechanisms of RNA localization.

This work is supported by NIH grant GM50221 to L. D. E. The DNA sequencing and analysis facility in MDACC is supported by NCI grant CA-16672.

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