ManLev and then decorated with biotin hydrazide (Fig. 4B). The toxicity of the conjugate was dependent on the expression of ketones: Cells expressing high levels of ketones (>700,000 ketones per cell as estimated by flow cytometry analysis) were sensitive to the conjugate with lethal doses (LD_{50}) in the range of 1 to 10 nM. In contrast, the conjugate showed no toxicity against cells expressing fewer ketones (<50,000 ketones per cell). These results indicate that cell surfaces can be metabolically engineered to support selective drug delivery, and that the sensitivity of target cells can be controlled by modulating the expression level of the unique targeting epitope.

Variations of this strategy can be envisioned, such as the direct targeting of cell surface ketones with hydrazide-conjugated drugs or probes and the use of other mutually reactive organic functional group pairs. The chemoselective formation of hydrazone linkages among small molecule pro-drugs has been accomplished in whole animals and human subjects (*4*), setting the precedent for the application of cell surface engineering in vivo. Other potential applications of cell surface remodeling include engineering new determinants for immunological recognition, tissue-specific cell trafficking, and cell adhesion to synthetic substrates.

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31. We thank A. Fischer, P. Schow, and P. Giblin for stimulating discussions and technical advice. Supported by an ARCS Foundation graduate fellowship (L.K.M.) and by grants from the Camille and Henry Dreyfus Foundation, the Pew Scholars Program, the Alfred P. Sloan Foundation, the Exxon Education Foundation, the W. M. Keck Foundation, and the Laboratory-Directed Research and Development Program of Lawrence Berkeley National Laboratory under the Department of Energy, contract number DE-AC03-76SF00098 (C.R.B.).

12 December 1996; accepted 14 April 1997

Localization of *Xenopus* Vg1 mRNA by Vera Protein and the Endoplasmic Reticulum

James O. Deshler,* Martin I. Highett,* Bruce J. Schnapp†

In many organisms, pattern formation in the embryo develops from the polarized distributions of messenger RNAs (mRNAs) in the egg. In *Xenopus*, the mRNA encoding Vg1, a growth factor involved in mesoderm induction, is localized to the vegetal cortex of oocytes. A protein named Vera was shown to be involved in Vg1 mRNA localization. Vera cofractionates with endoplasmic reticulum (ER) membranes, and endogenous Vg1 mRNA is associated with a subcompartment of the ER. Vera may promote mRNA localization in *Xenopus* oocytes by mediating an interaction between the Vg1 3′ untranslated region and the ER subcompartment.

One function of mRNA localization is to restrict translation of specific mRNAs to particular domains of early embryos (*1*, *2*), thereby conferring the beginnings of pattern formation. Vg1 mRNA encodes a transforming growth factor–β involved in mesoderm induction (*3*) and is localized to the vegetal blastomeres of early *Xenopus* embryos (*4*). Localization of Vg1 mRNA begins in late stage II oocytes where Vg1 mRNA accumulates in a wedge-shaped region of the vegetal hemisphere (*5*) before being transported to the vegetal cortex of stage III oocytes by a microtubule-dependent process (*5*, *6*). Localization is directed

Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed.

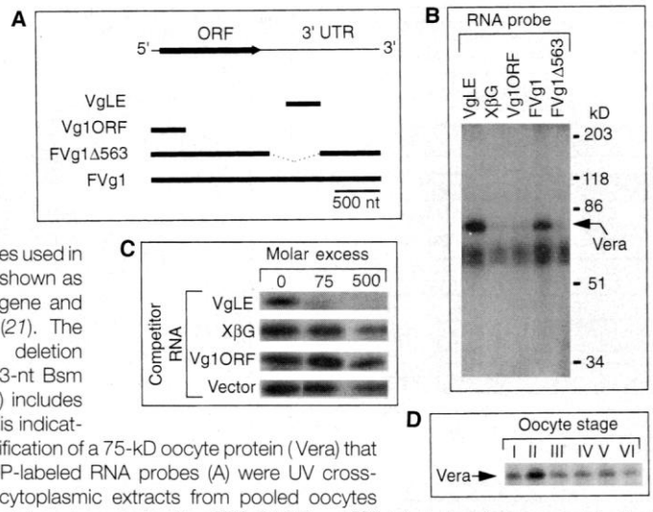

Fig. 1. A 75-kD protein, Vera, specifically binds the VgLE and is expressed at the time of Vg1 mRNA localization. (**A**) Vg1 RNA probes used in the cross-linking assay. At the top is the Vg1 gene with the open reading frame (ORF) and 3′ UTR indicated. RNA probes used in the cross-linking assay are shown as thick lines below the Vg1 gene and were transcribed in vitro (*21*). The 563-nucleotide (nt) deletion (FVg1Δ563, lacking the 563-nt Bsm I–Spe I Vg1 DNA fragment) includes the entire 366-nt VgLE and is indicated by a dotted line. (**B**) Identification of a 75-kD oocyte protein (Vera) that cross-links to the VgLE. ^{32}P-labeled RNA probes (A) were UV cross-linked in the presence of cytoplasmic extracts from pooled oocytes (stage IV through VI) (*22*). Proteins were resolved by SDS-PAGE and ^{32}P-labeled proteins were detected by autoradiography. XβG represents an RNA probe including nts 148 through 596 of the *Xenopus* β-globin gene. (**C**) Vera binding to the VgLE is specific. Gels show competitive inhibition of Vera cross-linking to a ^{32}P-labeled VgLE probe by unlabeled competitor RNAs including an arbitrarily chosen fragment of a plasmid vector. The molar excess of unlabeled RNA to the ^{32}P-labeled VgLE probe is indicated above the gel lanes. (**D**) Vera cross-linking activity is present throughout oogenesis. Gels show UV cross-linking to a ^{32}P-labeled VgLE RNA, using whole-cell extracts from staged oocytes. Each cross-linking reaction contained equivalent amounts of oocyte protein.

by a 366-nucleotide element in the Vg1 3′ untranslated region (UTR): the Vg1 localization element (VgLE) (*7*).

We used an ultraviolet (UV) cross-linking assay (Fig. 1) to identify cytoplasmic proteins in *Xenopus* oocytes that specifically bind the VgLE. All ^{32}P-labeled RNA probes cross-linked with similar efficiency to a pair of polypeptides at ~60 kD, which suggests that this interaction is nonspecific. However, only probes containing the VgLE labeled a 75-kD polypeptide.

If labeling of the 75-kD protein by the VgLE-containing probes (Fig. 1B) were due to a specific RNA-protein interaction, then in competition experiments, unlabeled VgLE should inhibit cross-linking more efficiently than other unlabeled RNAs. Unlabeled RNAs not containing the VgLE barely competed at a 500-fold molar excess, whereas a 75-fold molar excess of unlabeled VgLE inhibited cross-linking (Fig. 1C). Thus, VgLE binding to the 75-kD polypeptide is specific. We have named this protein "Vera" to reflect "VgLE binding and ER association," as described below (*8*). Vera cross-linking activity is present during all stages of oogenesis and peaks slightly at stage II just before localization begins (Fig. 1D).

Examination of the VgLE nucleotide sequence revealed four repeated sequence elements: E1, E2, E3, and E4 (Fig. 2A). Except for an additional E2 and E4 located upstream of the VgLE, all of the repeated elements are confined to the VgLE. In a previous study, deletions that removed one or more of the repeats (Fig. 2A) impaired localization (*7*). We used these repeated elements to analyze RNA localization and Vera binding.

Mutant VgLEs lacking all copies of E1, E2, E3, or E4 were constructed and their

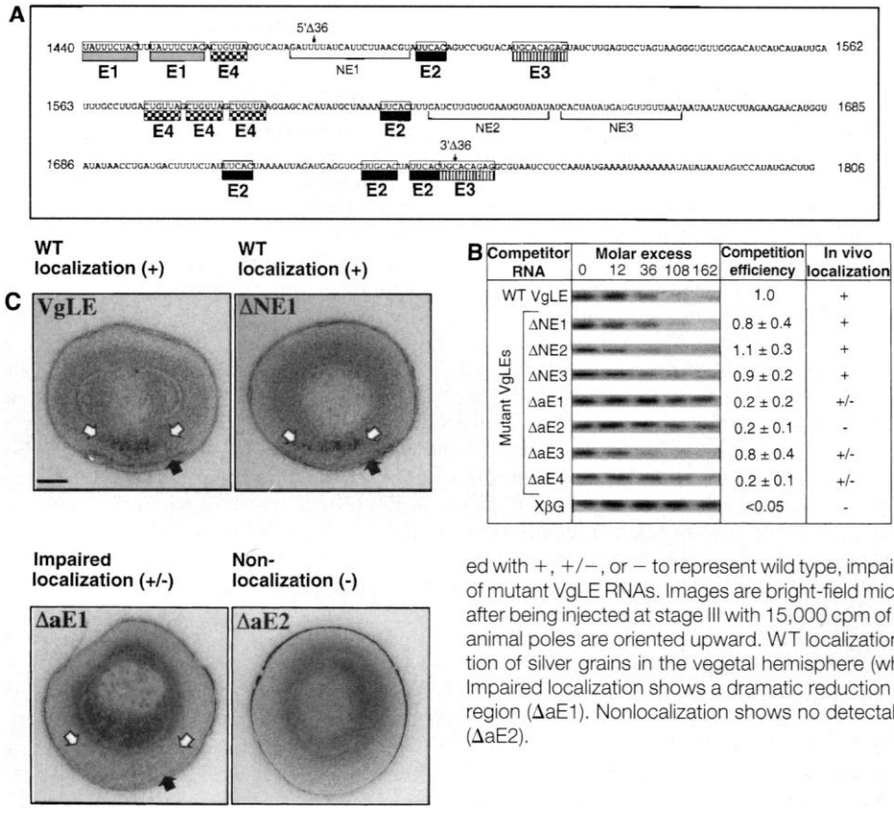

Fig. 2. Repeated sequences within the VgLE are involved in Vera binding and localization. (**A**) Four repeated sequence elements—E1, E2, E3, and E4—are concentrated within the 366-nt VgLE. There are two copies of E1 (UAUUUCUAC), four copies of E2 (UUCAC) and one E2-like element (UUGCAC), two copies of E3 (UGCACAGAG), and three copies of E4 (CUGUUA). Mutations in which all copies of one repeat were deleted were made by site-directed or polymerase chain reaction mutagenesis and are referred to as ΔaE1, ΔaE2, ΔaE3, and ΔaE4. The positions of the three non-element deletions (ΔNE1, ΔNE2, and ΔNE3) constructed for this study are indicated. Also indicated are 5′ (5′Δ36) and 3′ (3′Δ36) deletions, which impaired localization in a previous study (*7*). (**B**) The affinity of Vera for wild-type and mutant VgLEs was measured by competitive inhibition of Vera cross-linking to wild-type (WT) ^{32}P-labeled VgLE probe. Competition efficiencies (*22*) reflect the relative affinities of the mutant RNAs for Vera. The in vivo localization phenotypes of mutant VgLEs based on the morphological assay (C) are indicated with +, +/−, or − to represent wild type, impaired, or nonlocalization, respectively. (**C**) Microinjection of mutant VgLE RNAs. Images are bright-field micrographs of sections from oocytes cultured for 5 days after being injected at stage III with 15,000 cpm of the indicated ^{32}P-labeled RNA (2×10^8 cpm/μg) (*23*); animal poles are oriented upward. WT localization (VgLE and ΔNE1) is characterized by an accumulation of silver grains in the vegetal hemisphere (white arrows) and at the vegetal cortex (black arrows). Impaired localization shows a dramatic reduction of silver grains (white and black arrows) in the vegetal region (ΔaE1). Nonlocalization shows no detectable accumulation of silver grains in the vegetal region (ΔaE2).

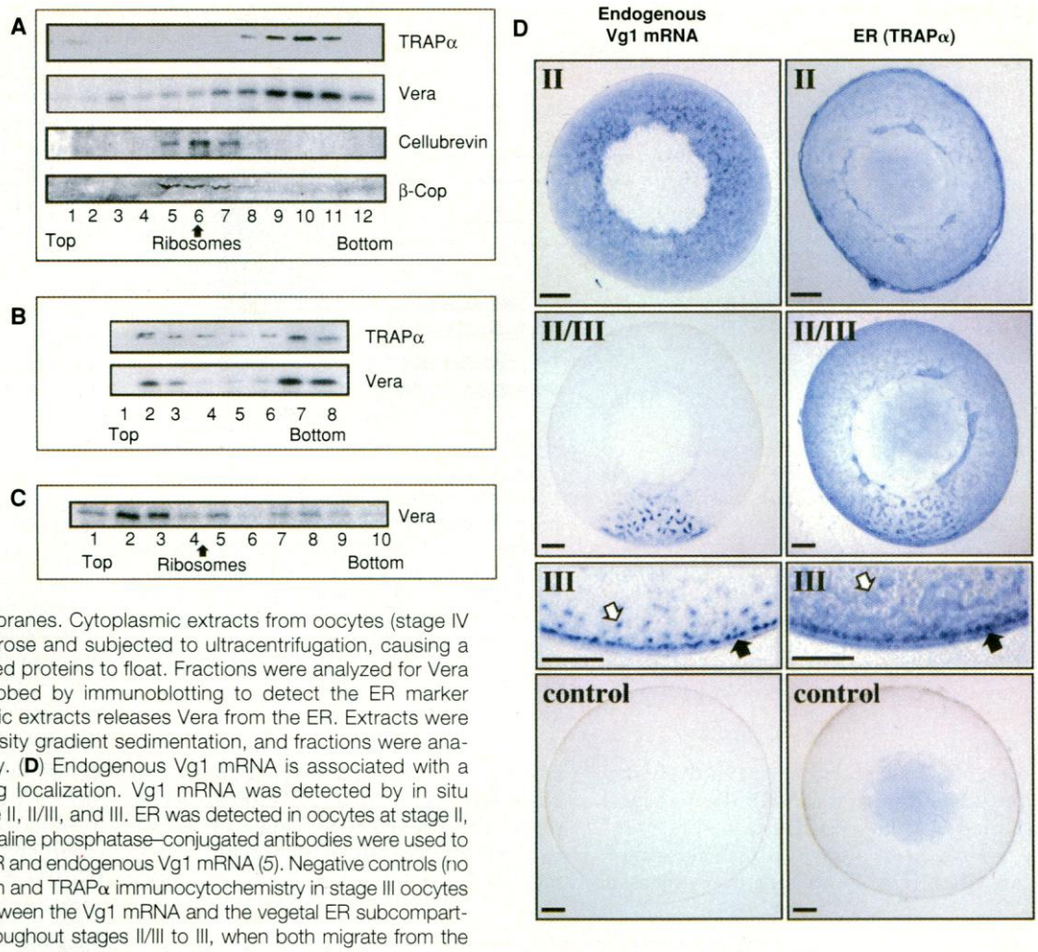

Fig. 3. Vg1 mRNA localization involves an interaction with the ER. (**A**) Vera cross-linking activity cosediments with ER membranes. Cytoplasmic extracts from pooled oocytes (stage IV through VI) were fractionated by sucrose density gradient sedimentation. Fractions were analyzed for Vera cross-linking activity (Vera) or probed by immunoblotting to detect an integral membrane protein of the rough ER (TRAPα), secretory vesicles (cellubrevin), or the β-Cop protein of the Golgi complex (β-Cop). Primary antibodies were visualized with alkaline phosphatase–conjugated secondary antibodies. The ribosomal RNA peak was determined by measuring absorption at 254 nm. Fractions collected from the top or bottom of the gradient are indicated. (**B**) Flotation of Vera cross-linking activity with ER membranes. Cytoplasmic extracts from oocytes (stage IV through VI) were overlaid with sucrose and subjected to ultracentrifugation, causing a portion of the membrane-associated proteins to float. Fractions were analyzed for Vera cross-linking activity (Vera) or probed by immunoblotting to detect the ER marker (TRAPα). (**C**) Dilution of cytoplasmic extracts releases Vera from the ER. Extracts were diluted 10-fold before sucrose density gradient sedimentation, and fractions were analyzed for Vera cross-linking activity. (**D**) Endogenous Vg1 mRNA is associated with a subcompartment of the ER during localization. Vg1 mRNA was detected by in situ hybridization (*5*) in oocytes at stage II, II/III, and III. ER was detected in oocytes at stage II, II/III, and III by TRAPα antibodies. Alkaline phosphatase–conjugated antibodies were used to detect primary probes for both the ER and endogenous Vg1 mRNA (*5*). Negative controls (no primary probe) for in situ hybridization and TRAPα immunocytochemistry in stage III oocytes are also shown. The association between the Vg1 mRNA and the vegetal ER subcompartment appears to be maintained throughout stages II/III to III, when both migrate from the wedge-shaped region to the vegetal cortex (black arrow), with only a few globular structures remaining above the cortex (white arrow).

relative affinities for Vera were determined by competitive inhibition of Vera cross-linking to a [^{32}P]-labeled VgLE probe. Three similarly sized nonelement deletions (ΔNE1, ΔNE2, and ΔNE3) (Fig. 2A) were also tested. Deleting all copies of E1, E2, or E4 diminished binding to Vera, whereas deletion of E3 or of nonelement regions had no effect (Fig. 2B). The affinities of ΔaE1, ΔaE2, and ΔaE4 were approximately one-fifth that of wild-type VgLE (Fig. 2B). Thus, three of the four repeated elements—E1, E2, and E4—are involved in Vera binding.

To determine whether the mutant RNAs with reduced affinities for Vera were impaired for localization in vivo, ^{32}P-labeled RNAs were microinjected into stage III oocytes (Fig. 2C). All three of the mutant RNAs with reduced affinities for Vera (ΔaE1, ΔaE2, and ΔaE4) failed to localize efficiently, whereas the three nonement deletions localized as well as wild-type VgLE (Fig. 2, B and C). The mutant RNA ΔaE3 had wild-type affinity for Vera but localized poorly, which suggests that E3 might be required for a step of the localization process that does not involve Vera binding. Among the element deletion mutants, two localization patterns could be distinguished. Nonlocalization was observed in oocytes injected with ΔaE2, and impaired localization was observed in oocytes injected with ΔaE1, ΔaE3, or ΔaE4 (Fig. 2, B and C). These localization patterns were not due to differential stabilities of the RNAs, as similar amounts of all full-length ^{32}P-labeled RNAs could be extracted from oocytes after microinjection and culture for 5 days (*9*).

Because all three mutations that disrupt binding to Vera also impair (ΔaE1 and ΔaE4) or abolish (ΔaE2) localization, we conclude that Vera and its interaction with the VgLE are involved in Vg1 mRNA localization. These results also suggest that all four of the repeated elements (E1 through E4) are involved in Vg1 mRNA localization.

Certain mRNAs are transported in large particles (*10*). To ascertain whether Vera is associated with a large particle, we determined the sedimentation characteristics of Vera cross-linking activity in cytoplasmic extracts. Vera cross-linking activity sediments faster than do endogenous ribosomes, which suggests that Vera is associated with a large particle or organelle (Fig. 3A). The Vera cross-linking activity co-sediments with TRAPα, an integral membrane protein associated with the protein translocation machinery of the endoplasmic reticulum (ER) (*11*). This is observed in gradients with different compositions and spin durations (*9*) and in flotation experiments (Fig. 3B). Vera is not an integral membrane protein, because Vera cross-linking activity remains in the soluble fractions when the extract is diluted before sedimentation (Fig. 3C).

The association of Vera with the ER raised the question of whether endogenous Vg1 mRNA is associated with the ER during localization. Endogenous Vg1 mRNA is distributed throughout the cytoplasm of early stage II oocytes (Fig. 3D), but at the transition between stages II and III (II/III) of oogenesis, Vg1 mRNA accumulates in a wedge-shaped region between the nucleus and vegetal cortex (Fig. 3D)

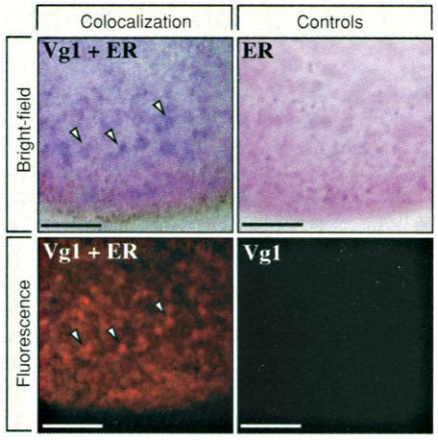

Fig. 4. Colocalization of Vg1 mRNA and the ER to globular structures of the vegetal wedge-shaped region in a stage II/III oocyte. Vg1 mRNA was detected by in situ hybridization (*5*), which produces a blue color, followed by immunolocalization of ER in the same oocytes with Vector Red (Vector Laboratories), which produces a red color. This double labeling results in a purple color at regions of overlap of the blue (Vg1) and red (ER) signals (upper left) when observed with bright-field illumination. It can be seen that the purple globular structures (arrowheads) correspond to ER compartments when the same specimen is visualized by fluorescence of Vector Red (bottom left), which exclusively reveals the ER but not Vg1 mRNA (bottom right; only Vg1 mRNA is labeled). Specimens in which only the ER is labeled (upper right) show purely red globular structures with bright-field illumination.

(*5*). As oogenesis proceeds to stage III, Vg1 mRNA labeling accumulates at the vegetal cortex (Fig. 3D) (*5*, *12*, *13*).

In stage II/III oocytes, TRAPα labeling reveals an ER subcompartment that coincides with the distribution of Vg1 mRNA (Fig. 3D), both in the shape of the wedge region and in the globular substructure. At stage III, a layer of ER is found tightly associated with the vegetal cortex (Fig. 3D), which is similar to the pattern of Vg1 mRNA. Double-labeling experiments (Fig. 4) show that endogenous Vg1 mRNA and ER colocalize to the same globular substructures in the wedge-shaped region, which indicates that Vg1 mRNA is associated with the ER during localization. Before localization, Vg1 mRNA and the ER are probably not associated, because in stage II oocytes the Vg1 mRNA distribution is punctate and the ER is reticular (Fig. 3D).

Because Vg1 translation appears to be repressed during localization (*2*), Vg1 mRNA is presumably localized by a translation-independent mechanism. Although Vg1 mRNA might associate with the ER via the signal recognition particle at the time of translation, the present study provides evidence for a distinct mRNA-ER targeting mechanism that involves signals in the 3′ UTR and discriminates between distinct compartments of the ER. Our studies also imply a link between Vg1 mRNA localization and microtubule-based organelle transport. The observation that Vg1 mRNA localization to the vegetal cortex during stage III is inhibited by microtubule-depolymerizing drugs (*5*, *6*) may be explained by the fact that Vg1 mRNA is attached to the ER, and the ER is transported on microtubules (*14*). Although a specific role for Vera has not been resolved, a possible function might be to link Vg1 mRNA to the vegetal ER subcompartment. Other mRNAs encoding membrane (*15*) or secreted proteins (*16*) have polarized distributions in somatic cells, which suggests that the ER may play a central role in the spatial organization of eukaryotic gene expression.

Note added in proof: While this paper was in review, a 78-kD protein likely to be Vera, was independently indentified by UV cross-linking to the VgLE (*24*).

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8. Our methods differ from those used to identify a 69-kD VgLE binding protein (*17*) in the use of thio–uridine triphosphate (UTP) rather than bromo-UTP. When the bromo-UTP procedure was used with our extracts, a 69-kD polypeptide distinct from Vera was detected. The 69-kD protein observed in our extracts was not associated with the ER, was not specific for the VgLE, and increased dramatically during stages V and VI of oogenesis (*9*). It is still possible that the 69-kD protein observed previously (*17*) is a different protein that we did not observe.
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22. Whole-cell extracts were prepared from manually defolliculated oocytes of each stage (I through VI) (*18*), homogenized in TGKED buffer [50 mM tris (pH 7.5), 25% glycerol, 50 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, and 1 mM phenylmethanylsulfonyl fluoride] (4 μl per oocyte) (*17*), and microfuged for 2 min. Concentrated cytoplasmic extract (*19*) from collagenase-treated (0.2%) oocytes (stage IV through VI) was fractionated in discontinuous sucrose gradients (12 to 60% w/w) by centrifugation for 2 hours at 32,000 rpm in an SW 55 Ti rotor at 4°C. For cross-linking, 10,000 cpm (10^8 cpm/μg) of a [^{32}P]/thio-UTP–labeled RNA probe (*20*) was added to a 5-μl reaction containing 10 mM Hepes (pH 7.6); 3 mM $MgCl_2$; 125 mM KCl; 5% glycerol; 1 mM dithiothreitol; *Escherichia coli* tRNA (20 μg/μl); and 0.02 μl of concentrated cytoplasmic extract, 0.1 μl of a sucrose gradient fraction (0.5 μl for dilution experiments), 0.25 μl of a fraction from flotation experiments, or 1 μg of stage-specific whole-cell extract. The mixture was then incubated at 25°C for 30 min, heparin was added to 5 mg/ml, and the reaction was exposed to UV light for 10 min. Ribonuclease A was added (1 mg/ml), followed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. For competition experiments, unlabeled RNAs were added 10 min before probe addition. Fraction saturation was calculated by dividing the amount of ^{32}P-labeled protein (Vera) in the presence of unlabeled competitor by the amount of ^{32}P-labeled Vera in the absence of competitor. The affinity of each competitor RNA relative to the probe (K_d^{comp}/K_d^{probe}; K_d, dissociation constant) was determined by fitting the fraction saturation data to the following function: fraction saturation = $[1 + (K_d^{probe}/K_d^{comp})([comp]/[probe])]^{-1}$. The K_d^{probe}/K_d^{comp} was determined for each RNA in at least three independent experiments, and competition efficiencies were calculated by dividing the relative affinities for Vera of the mutant RNAs by that of the wild-type VgLE RNA.
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25. We thank M. Kirschner for use of his frogs and oocyte injection facilities; T. Rapoport for antibodies and helpful advice on the manuscript; D. Melton for a Vg1 cDNA clone; E. Arn for suggesting the use of thio-UTP as a cross-linking reagent; V. Muresan, C. Batchelder, and R. Maike-Lye for comments on the manuscript; and A. Lyass for technical assistance. J.O.D. was supported by NIH fellowship GM16114-03. M.I.H. was supported by a postdoctoral fellowship from the Science and Engineering Research Council/North Atlantic Treaty Organization. This work was supported by grants from NIH (NS-26846), the March of Dimes (1 FY94-0478 01), and the Council for Tobacco Research (3802R1) to B.J.S.

21 November 1996; accepted 21 March 1997