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A 69-kDa RNA-binding protein from *Xenopus* oocytes recognizes a common motif in two vegetally localized maternal mRNAs

(cytoplasmic mRNA localization/UV crosslinking/Vg1 mRNA/oocyte)

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ABSTRACT **Vg1 mRNA, a maternal message encoding a member of the transforming growth factor β superfamily, undergoes localization to the vegetal cortex of *Xenopus laevis* oocytes during a narrow period of oogenesis. A 340-nucleotide sequence has been identified in Vg1 RNA that directs its vegetal localization [Mowry, K. L. & Melton, D. A. (1992) *Science* 255, 991–994]. To understand how cis- and trans-acting factors are involved in Vg1 mRNA localization, we have looked for specific interactions *in vitro* between oocyte proteins and Vg1 mRNA. S100 extracts of late-stage oocytes contain a protein-binding activity that protects specific regions of labeled Vg1 mRNA from degradation by RNase T1. The use of different regions of Vg1 RNA in competition reactions reveals two binding sites, both in the first half of the 3′ untranslated region of Vg1 message. UV crosslinking predominantly labels a 69-kDa protein; saturation analysis and competitor studies indicate that this protein binds with a high affinity to the downstream site, which corresponds to the 340-nucleotide vegetal localization sequence. Binding to this region is inhibited by another vegetally localized message, transforming growth factor β5 but is not inhibited by an animally localized RNA, An2. These data indicate that vegetally localized mRNAs share a binding motif that helps them achieve their intracellular distribution through specific RNA–protein interactions.**

The polarity of *Xenopus* oocytes is evident from very early stages and is perhaps presaged by an asymmetry detectable in primordial germ cells (1, 2). As oogenesis proceeds, this polarity is elaborated by a series of morphological events that occur along the animal–vegetal axis, such as asymmetric distribution of yolk platelets, migration of pigment granules to the animal hemisphere, accumulation of polar granules at the vegetal pole, and the displacement of the germinal vesicle to the animal hemisphere (3, 4). On a molecular level, this organization is reflected in a small class of RNA molecules that are localized to particular regions of the oocyte cytoplasm (5, 6). The heterogeneity of the single-cell egg is then translated into cellular differences in the developing embryo by early cleavage planes, helping to create the cell–cell interactions that contribute to producing the body axis. Despite the importance of polarity in oocytes, little is understood about its generation, molecular nature, or interpretation.

Vg1 mRNA, a member of the transforming growth factor β (TGFβ) superfamily, is localized to the vegetal cortex of late-stage oocytes and remains in the vegetal hemisphere of early embryos until after gastrulation (5, 7). Synthesized early during oogenesis and distributed homogeneously throughout stage I and II oocytes, Vg1 message accumulates along the vegetal cortex of oocytes during a short period in the middle of oogenesis, where it remains until maturation (8–10). Localization of Vg1 mRNA appears to occur in at least two steps, the first being translocation of the message to the vegetal hemisphere and the second being anchoring of the message along the cortex (10). Both microtubules and microfilaments have been implicated in this process, with microtubule inhibitors preventing translocation but not interfering with already localized message, and microfilament inhibitors causing a release of localized message without inhibiting its translocation (10). By covalently linking different regions of Vg1 RNA to reporter globin sequences, Mowry and Melton (11) have recently identified a 340-nucleotide (nt) sequence that specifies vegetal localization when injected into stage III oocytes.

Other intracellularly localized messages have been identified in a number of different cell types, including oocytes and embryos (12–17), hippocampal and photoreceptor neurons (18, 19), and cells in culture (20). In several cases, localized RNA is associated with cytoskeletal elements (21–24). The nature of this interaction and how specific RNAs become associated with rather ubiquitous structures are not clear. One possibility is that RNA-binding factors that recognize particular messages might mediate their connection with the cytoskeleton. The regions recognized by these factors might specify the destination of a particular RNA by determining which factor or factors are bound.

To dissect the process of mRNA localization in oocytes, we have begun to analyze the cis-acting signals and trans-acting factors involved in the localization of Vg1 mRNA during oogenesis. Use of a modified RNase-protection assay (25) has identified a soluble, Vg1 mRNA-specific binding activity in oocytes. Competition experiments, using unlabeled Vg1 RNA fragments, reveal two distinct protein-binding regions in the 3′ untranslated region (UTR) of Vg1 mRNA. The more downstream region was previously identified by microinjection studies as the vegetal localization signal (11). A 69-kDa protein binds to this region with high affinity, and the presence of this binding motif in other RNAs is correlated with vegetal localization. Localization of RNA in oocytes thus appears to be regulated both by information encoded in the 3′ UTR of the localized message and by cellular factors that specifically recognize these signals.

MATERIALS AND METHODS

Isolation of Oocytes. *Xenopus laevis* frogs were obtained from Xenopus I, Ann Arbor, MI. Oocytes were defolliculated by incubation at room temperature for 1–2 hr in 0.3% collagenase (Sigma) in phosphate-buffered saline (PBS) initially warmed to 37°C.

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Abbreviations: UTR, untranslated region; nt, nucleotide(s); TGFβ, transforming growth factor β.

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Plasmid Constructs and Labeling of RNAs. The plasmid pVg1 was generated by inserting the *Hin*dIII/*Eco*RI, Vg1-containing fragment from Vg1 plasmid B (D. Melton, Harvard University) into pBS+ (Stratagene) at the *Sma* I site. pVg1ΔHindIII/BsmI was constructed by deletion of the *Hin*dIII–*Bsm* I fragment from pVg1. pVg1MscI/SpeI was produced by inserting the 297-base-pair (bp) *Msc* I–*Spe* I fragment of pVg1 into *Xba* I/*Sma* I-cleaved pBS+. pVg1ΔBstEII/SspI$_2$ was constructed by deletion of the 262-bp *Bst*EII–*Ssp* I$_2$ fragment from pVg1. pVg1SspI$_2$/BsmI (domain B) was constructed by cutting pVg1 with *Ssp* I$_2$/*Bsm* I and blunt-end ligating the 370-bp fragment to pBS+ at the *Sma* I site. An2 and TGFβ-5 RNAs were transcribed from plasmids provided by D. Melton.

RNAs were synthesized from linearized DNA templates with T3 or T7 RNA polymerase as described (26) with the following exceptions. RNA probes were transcribed in a 5-μl reaction in the presence of 50 μCi (= 12.5 μM) of [α-^{32}P]UTP (Amersham, 800 Ci/mmol; 1 Ci = 37 GBq), 10 μM unlabeled UTP, and 500 μM of the remaining three unlabeled nucleotide triphosphates. Samples were incubated for 1 hr at 4°C (27) and purified on Sephadex G-50 columns. The competitor RNA transcripts were synthesized for 1 hr at 37°C, in the presence of 2.5 mM each of the four nucleotide triphosphates and 10 μCi (= 0.5 μM) of uridine 5′-[α-[^{35}S]thio]triphosphate (Amersham, >1000 Ci/mmol). For the UV crosslinking assays, RNA probes were transcribed as above, except that 20% of the unlabeled UTP (2 μM) was bromo-UTP (Sigma).

The competitor RNA transcripts diagrammed in Fig. 2 were synthesized as follows: (*i*) full length, 2.4 kb, Vg1 RNA transcript from pVg1 linearized with *Eco*RI; (*ii*) 1.17-kb transcript containing the entire Vg1 coding region from *Bst*EII-digested pVg1; (*iii*) 648-bp transcript containing the distal end of the 3′ UTR from *Eco*RI-digested pVg1ΔHindIII/BsmI; (*iv*) 1.8-kb transcript from *Bsm* I-digested pVg1; (*v*) 1.36-kb transcript from *Ssp* I-digested pVg1; (*vi*) 1.24-kb transcript from *Spe* I-digested pVg1; (*vii*) 297-bp transcript from pVg1MscI/SpeI linearized with *Hin*dIII; (*viii*) 1.5-kb transcript from *Bsm* I-cleaved pVg1ΔBstEII/SspI$_2$; (*ix*) 370-bp transcript from pVg1SspI$_2$/BsmI linearized with *Bsm* I.

Modified RNase-Protection Reactions. S100 extracts were prepared in TGKED buffer (50 mM Tris, pH 7.5/25% glycerol/50 mM KCl/0.1 mM EDTA/0.5 mM dithiothreitol/1 mM phenylmethanylsulfonyl fluoride) with modifications of described methods (28). Collagenase-treated oocytes of the desired stage were homogenized in 2 vol of TGKED buffer on ice. The extracts were cleared of most of the yolk and cortical debris by centrifugation in a microcentrifuge for 10 min at 4°C and then centrifuged in a Beckman type TLS 55 rotor for 60 min at 55,000 rpm. Aliquots of the extracts were stored at −80°C. Protein concentrations were then determined by using a Bio-Rad protein assay kit.

Binding reactions were done essentially as described (29). [^{32}P]UTP-labeled Vg1 probe (0.15 fmol) was incubated with 5 μg of S100 extract in a final volume of 20 μl for 30 min at room temperature. Subsequently, unprotected RNA was digested for 10 min with 200 units of RNase T1 (Sigma), and heparin (5 mg/ml) was then added for an additional 10 min. For competition experiments, ^{35}S-labeled competitor RNA was added to the extract before adding the ^{32}P-labeled probe. RNA–protein complexes were electrophoresed as described (29, 30). Quantitative analysis of the competition experiments was done by scanning the autoradiograms with a digitometer, calculating the fraction protected after competition, and normalizing the percentage of competition to the competition of full-length Vg1 RNA.

UV Crosslinking Assays. Six fmoles of RNA probe, either in the presence or absence of competitor RNA, was bound to 15 μg of S100 extract in a final volume of 20 μl, as described above for the modified RNase-protection experiments. After 30 min at room temperature, heparin was added to 5 mg/ml (31), and the samples were irradiated at 2.5 J/cm^2 with a UV light source of 312 nm. After a 15-min incubation at 37°C in RNase A at 1 mg/ml, the samples were denatured at 65°C and resolved on a discontinuous SDS/10% polyacrylamide gel. Quantitative analysis was done by scanning the autoradiograms on a flatbed scanner and analyzing the bands by using digital imaging software (IMAGE1.41, National Institutes of Health). Dissociation constants were calculated as described (31).

RESULTS

Sequence Specificity of RNA Binding. To determine whether oocytes contain a binding activity that specifically recognizes Vg1 RNA, we analyzed RNA–protein complexes by electrophoresis on nondenaturing polyacrylamide gels (30). S100 extracts prepared from oocytes were incubated with full-length Vg1 RNA (see Fig. 2, number 1) synthesized at a high specific activity. Unprotected RNA was then digested with RNase T1, and nonspecific RNA–protein complexes were eliminated by incubation with heparin (25). Under these conditions, only RNA sequences tightly associated with protein are protected and separated by electrophoresis from the rest of the digested RNA (29, 31).

Fig. 1 shows that stage VI oocytes contain a Vg1 RNA-binding activity that protects a well-defined band from RNase

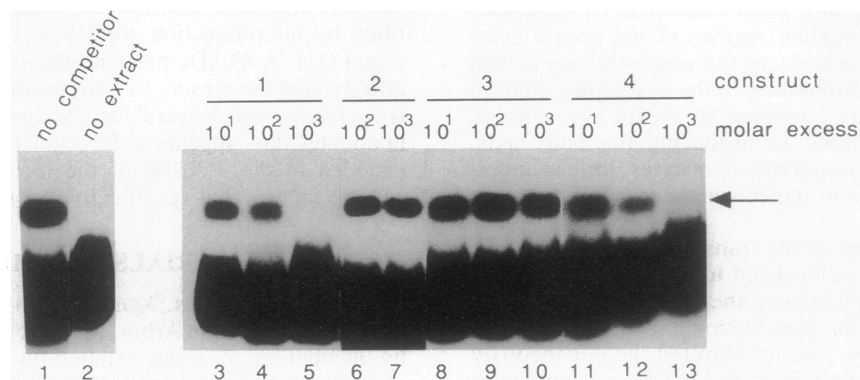

FIG. 1. Modified RNase protection of full-length Vg1 RNA probe. Vg1 RNA-binding activity in S100 extracts of stage V/VI oocytes was assayed in the presence (lanes 3–13) or absence (lane 1) of competitor RNA by RNase protection of a full-length Vg1 transcript. For convenience, the competitors are listed as constructs 1–4 (see Fig. 2) and are as described in text. The arrow at right indicates location of the protected band, which is absent when no extract is added (lane 2). The ratio of moles of competitor RNA in the reaction to moles of labeled, Vg1 RNA probe (0.15 fmol) is indicated above each lane as molar excess.

digestion. No Vgl RNA is protected when extract is absent, predigested with proteinase K, or heat-denaturated (Fig. 1, lane 2 and data not shown), indicating the involvement of proteins in protecting this fragment. The inclusion of unlabeled RNA as competitors in the binding reaction shows the sequence specificity of this RNA–protein interaction. At the extract concentration used (0.25 $\mu g/\mu l$), full-length Vgl mRNA completely abolishes RNA protection when present in a 1000-fold molar excess (Fig. 1, lane 5). Vgl transcripts lacking the 3′ UTR, however, do not compete at the same molar excess (lane 7). Within the 3′ UTR, the distal end is not involved in binding: transcripts lacking this region (lane 13) retain full ability to compete, whereas the region alone does not compete at all (lane 10). *Xenopus* β globin, total chicken RNA, and poly(A)$^+$ RNA from oocytes also did not compete at the same or even higher concentrations (data not shown). These experiments indicate not only how sequence-specific the binding activity is but also that the sequences capable of competing for binding reside in the first 600 nt of the 3′ UTR.

The beginning of the 3′ UTR was further analyzed by using smaller RNA competitors and deletions. The level of competition for each RNA, normalized to that with full-length Vgl RNA, is shown in Fig. 2. Truncations up to the *Spe* I site at the beginning of the 3′ UTR show a high level of competition (Fig. 2, numbers 4–6, respectively). In accordance with these data, protection is largely diminished when a fragment containing the coding region–3′ UTR junction, termed domain A, is present as competitor (Fig. 2, number 7). Deletion of this region from the start of the noncoding region reveals a second domain capable of competition (Fig. 2, number 8). This downstream region, termed domain B, which contains the vegetal localization signal identified by Mowry and Melton (11), also competes well by itself (Fig. 2, number 9). Thus,

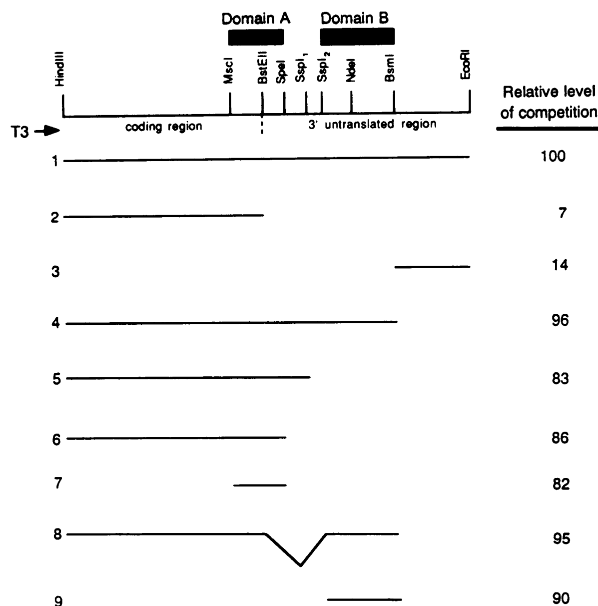

FIG. 2. Vgl RNA sequences involved in protein binding. Summary of the competition results from Fig. 1 and other experiments is shown schematically. The Vgl restriction map at top indicates the restriction enzyme sites used to generate various constructs; relative competition levels were calculated as described. Transcription of sense RNA is toward the right, with the vertical dotted line separating the coding region (at left) from the 3′ UTR (at right). Location of the T3 promoter used to synthesize the various RNAs shown here and mentioned in text is indicated. Positions of domains A and B are shown at top as black rectangles. In each construct, a horizontal line indicates the presence of the corresponding RNA sequence; diagonal lines indicate deleted sequences.

two independent binding domains, separated by 78 nt, are present at the beginning of the 3′ UTR.

A 69-kDa Protein Specifically Binds Vgl RNA Sequences. To more fully clarify the RNA–protein interactions, the proteins interacting with these domains were labeled by using a UV crosslinking technique. RNA was synthesized at a high specific activity in the presence of bromo-UTP and covalently crosslinked to bound proteins by UV irradiation. After RNase digestion, labeled proteins were electrophoresed in SDS/polyacrylamide gels and visualized by autoradiography. As in the RNase-protection assays above, high levels of heparin were included in the initial binding reaction to prevent nonspecific RNA–protein interactions (31). When full-length Vgl RNA is used as a probe, three bands, one at 69 kDa and two at ≈54 kDa, are labeled (Fig. 3*A*). The radioactive nucleotide used in the RNA synthesis has no effect on this pattern (data not shown). Domain B predominantly labels only the 69-kDa band (Fig. 3*A*); the same-sized band is also labeled by domain A RNA but is labeled at a much lower efficiency (data not shown). Construct 3 RNA, consisting of Vgl sequences that do not compete in the RNase-protection assay (Figs. 1 and 2), or β globin RNA, which does not undergo localization upon microinjection into stage III oocytes (9), do not significantly label any proteins of similar size (Fig. 3*A* and data not shown).

By using increased concentrations of domain B probe in the UV crosslinking assay, we determined the saturation binding curve for the 69-kDa protein. The dissociation constant (K_d), which is equal to RNA concentration at 50% saturation, is ≈0.34 nM (Fig. 4). This figure represents a maximum value, given the presence of other, minor binding proteins in the extract.

Domain B Contains a Binding Motif Found in Vegetally Localized RNA. The specificity of the interaction between domain B RNA and the 69-kDa protein was further demonstrated by UV crosslinking in the presence of competitor RNAs. When a large excess of either β globin or chicken RNA is included in the binding reactions, almost no reduction in the labeling of the 69-kDa protein is detected (Fig. 5*A*). A similar excess of domain B RNA virtually eliminates all labeling. These experiments clearly show that the 69-kDa protein is not a general RNA-binding protein but is recognizing particular sequences present in Vgl RNA.

Additional RNAs were used in competition studies to help identify the types of RNAs recognized by the 69-kDa protein. TGFβ-5 RNA is a maternal mRNA isolated from a *Xenopus* cDNA library that encodes a member of the TGFβ superfamily (32); like Vgl RNA, TGFβ-5 RNA is tightly localized along the vegetal cortex of middle- and late-stage oocytes

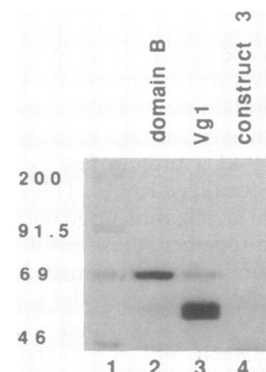

FIG. 3. UV crosslinking of S100 oocyte extracts to Vgl mRNA. Fifteen micrograms of S100 oocyte extracts was UV crosslinked to ^{32}P-labeled RNA probes as described. Lanes: 1, molecular size markers (sizes indicated in kDa at left); 2, domain B RNA (Fig. 2, construct 9); 3, full-length Vgl RNA; and 4, construct 3 (Fig. 2).

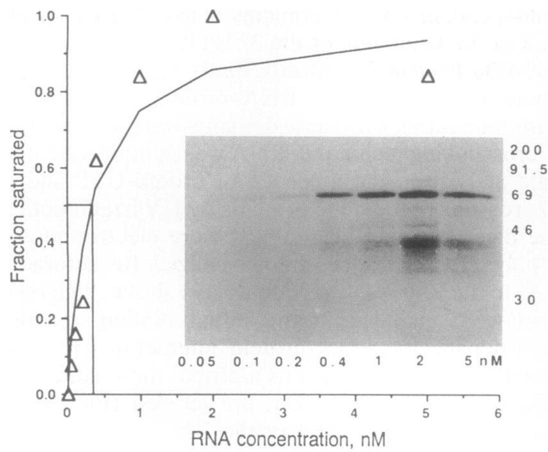

FIG. 4. Saturation binding curve of the 69-kDa-binding protein. Fifteen micrograms of extract was UV crosslinked with increased amounts of domain B probe. The fraction saturated is defined here as the ratio of 69-kDa-crosslinked product at each concentration to maximum crosslinked signal and measures the fractional saturation of binding sites on the protein. The curve is determined by the best-fit of the equation: fraction saturated = [RNA]/(K_d + [RNA]). The calculated K_d for the curve is 0.34 nM. Fifty percent fraction saturation is indicated. (*Inset*) Example of a saturation binding gel. Molecular size markers are indicated at right in kDa; the amount of domain B probe used per lane is indicated below.

(33). An2 RNA is a maternal message that undergoes localization to the animal hemisphere during the same period that Vgl RNA migrates to the vegetal cortex (5, 33). Like domain B sequences, TGFβ-5 RNA demonstrates a striking ability to compete for binding of the 69-kDa protein (Fig. 5*B*). An2 RNA, however, competes only slightly better than β globin RNA for binding. These differences in ability to compete can be quantified by determining the relative competition efficiency of each competitor, which is a reflection of the ratio of dissociation constants of the competitor and the probe. Table 1 shows the relative competition efficiencies for the competitors shown in Fig. 5*B*. TGFβ-5 RNA is >20-fold more efficient at binding the 69-kDa protein than is An2 RNA. In fact, when used as probes in the UV crosslinking assay (at the same molar concentration), only TGFβ-5 RNA, and not An2 RNA, labels the 69-kDa protein (data not shown). Thus, a binding motif that is recognized by the 69-kDa protein appears to be present in at least two vegetally localized RNAs and absent from an animally localized RNA.

DISCUSSION

The ability of binding proteins to protect Vgl RNA from RNase digestion has been used to identify sequence-specific RNA–protein interactions. Competition reactions revealed only two regions, both in the 3′ UTR, capable of preventing protection. The downstream region, domain B, binds with high affinity to a 69-kDa protein and contains a motif restricted to vegetally localized RNAs. This is the same domain that specifies vegetal localization upon injection into stage III oocytes (11). Taken together, these results strongly suggest that the 69-kDa protein that recognizes domain B plays a direct role in localizing messages to the vegetal cortex. Such sequence-specific RNA-binding proteins are likely to mediate interactions between Vgl RNA and the cytoskeletal proteins known to be involved in the localization process.

In *Xenopus* oocytes, not only are there many ubiquitous RNA-binding proteins (34), but also a number of oocyte-specific ribonucleoprotein complexes have been identified (35). Despite the wide range of RNAs that these proteins recognize, we presume that the relatively high levels of

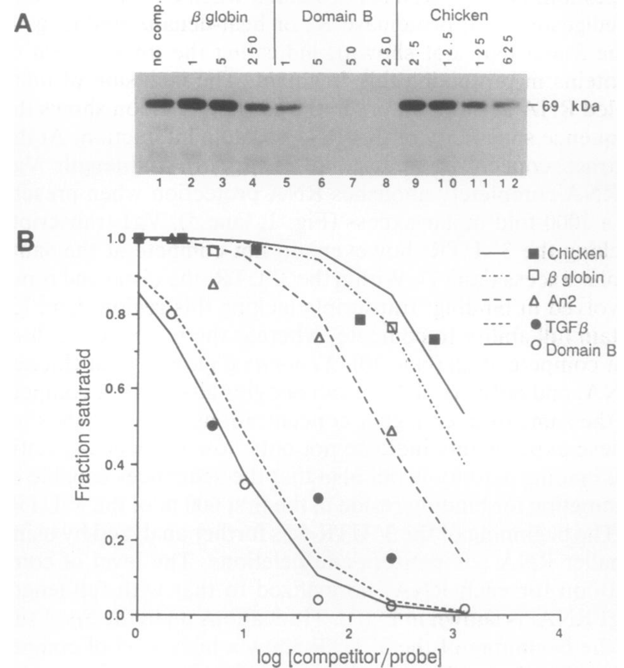

FIG. 5. Competition analysis of 69-kDa protein-binding specificity. Domain B probe (0.34 nM) was incubated with increased amounts of competitor RNAs and then UV crosslinked to 15 μg of S100 extract. (*A*) Competition with nonmaternal RNAs. β globin (lanes 2–4), domain B (lanes 5–8), and chicken (lanes 9–12) RNAs were used as competitors in the UV crosslinking assay. For β globin and domain B RNAs, the numbers above gels indicate the molar excess of competitor over probe; for chicken RNA, weight excess is indicated. Lane 1 shows the UV crosslinked product without any competitor. (*B*) Comparison of maternal and nonmaternal competitor RNAs. Fraction saturated is defined here as amount of crosslinked product produced with competitor RNA normalized to amount of crosslinked product without any added competitor. ■, Chicken RNA; □, β globin RNA; △, An2 RNA; ●, TGFβ-5 RNA; and ○, domain B RNA. Concentration ratio graphed for chicken RNA is the weight ratio of RNAs; the other graphs all represent molar ratios. Curves are drawn to the best fit of data to the function: fraction saturated = $1/[1 + (K_d^p/K_d^c)([c]/[p])]$, where p is probe and c is competitor RNA.

heparin, coupled with the low K_d of the 69-kDa protein, account for the virtual absence of such proteins in the UV crosslinking experiments with domain B RNA as probe. The two other bands seen when full-length Vgl RNA is used as a probe are labeled to various levels by a number of different RNAs as well (data not shown) and, thus, appear to be more general RNA-binding proteins. In particular, these proteins may well be the p54/p56 cytoplasmic mRNA-binding protein

Table 1. Relative competition efficiencies of different RNAs

RNA	Relative competition efficiency
Domain B	1.00
TGFβ-5	0.65
An2	0.03
β globin	0.01
Chicken	<0.01

Competition efficiency is defined as the ratio of dissociation binding constant for domain B (Fig. 4) to dissociation binding constant for competitor (as determined from the best-fit curve shown in Fig. 5). These figures have been normalized to the competition efficiency derived from Fig. 5 for domain B and, thus, represent the ability of each competitor to compete for binding relative to that of domain B.

previously identified as a major component of *Xenopus* oocyte messenger ribonucleoproteins (36, 37). Although we detect labeling of the 69-kDa protein with domain A RNA as a probe, we have been unable to obtain an estimate of its K_d because of the low efficiency of binding and the significant presence of other binding proteins recognizing it in the crude extract (data not shown). The precise interaction of the 69-kDa protein with Vg1 RNA remains to be determined, however, and may well be affected by the interaction of these other lower-affinity, binding proteins.

So far, only two messages, Vg1 and TGFβ-5, have been reported as localized to the vegetal cortex of *Xenopus* oocytes. Like Vg1, TGFβ-5 is a member of the TGFβ superfamily, was isolated from a *Xenopus* oocyte cDNA library, and undergoes localization during the same window of oogenesis and in an identical fashion (32, 33). At the amino acid level, the two genes are only 35% homologous, and the 3′ UTRs show no obvious stretches of homology. TGFβ-5 sequences that compete with domain B for binding also appear to reside in the 3′ UTR (Z.E., unpublished data) and may consist primarily of a common secondary structure, as has been suggested for the anterior localization signal present in the 3′ UTR of bicoid RNA (38, 39). This shared motif appears to act as a vegetal "address," recognized by RNA-binding proteins alone or in complexes, which mediate the association of specific messages to general cytoskeletal structures and potentially direct them to the proper location.

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