Complex formation between stage-specific oocyte factors and a *Xenopus* mRNA localization element

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ABSTRACT It is a long-standing proposal that localization of maternal factors in eggs can provide the basis for pattern formation in the early embryo. The localized information can be stored as RNA, one example being Vg1 RNA, which is localized exclusively to the vegetal hemisphere of *Xenopus* oocytes and eggs. Localization of Vg1 mRNA is directed by a 340-nt sequence element contained within its 3' untranslated region. To understand the mechanism of localization, I have tested whether factors from the oocyte interact specifically with the RNA localization sequence. Results presented here show that a set of oocyte proteins form complexes with the localization element both *in vitro* and *in vivo*. These proteins are specifically enriched in the stages of oogenesis during which localization occurs and recognize sub-elements of the RNA localization element that are essential for localization *in vivo*. These data suggest that formation of a localization-specific RNA–protein complex may be the first step in directing Vg1 mRNA to its subcellular destination.

For many organisms, patterning in the embryo appears to be an expansion of the initial polarity of the oocyte. During oogenesis, maternal components become regionally localized in the oocyte, and are differentially distributed among the early blastomeres of the embryo (reviewed in ref. 1). Localized information can in principle be stored as RNA or protein, and whereas localized maternal mRNA has been described in a number of organisms (reviewed in ref. 2), the molecular mechanisms underlying the localization process are only now being unraveled.

In eggs of the frog, *Xenopus laevis*, developmental polarity along the animal/vegetal (A/V) axis is coincident with unequal distribution of specific maternal mRNAs (3–6). Along this axis of developmental potential, the animal pole gives rise to ectodermal cell types, the vegetal pole contains prospective endoderm, and mesoderm results from an inductive signal from the vegetal blastomeres that is received by the overlying cells (7). Localized to the vegetal pole is Vg1 mRNA, which encodes a member of the transforming growth factor β family (8) and has been implicated in mesoderm induction (9, 10). *In situ* hybridization studies have shown that Vg1 RNA is evenly distributed in young oocytes (stages I–II) and is localized later during the middle stages (III–IV) of oogenesis to the vegetal hemisphere, where it remains in fully grown oocytes (stage VI) and eggs (11). Vegetal localization of Vg1 RNA is mediated by a 340-nt cis-acting localization signal contained within the 3' untranslated region (UTR) of the mRNA (12). An ever increasing number of localized RNAs have been shown to contain such cis-acting localization elements within their 3' UTRs (reviewed in ref. 2). But these elements are generally large and complex, and a major unanswered question is how do these signals interact with the localization machinery?

The identities of transacting protein factors have remained largely elusive, but certain RNA binding proteins have been shown to interact either genetically or biochemically with localized RNAs and are thus suggested to have roles in the localization process. In *Drosophila*, mutations in certain genes encoding RNA binding proteins have been shown to have profound effects on RNA localization (13–19), but direct biochemical interactions between such gene products and their putative target RNAs have not yet been demonstrated. Biochemical studies aimed at identification of proteins that interact directly with localized RNAs may uncover other RNA binding proteins with potential roles in localization. Indeed, a 69-kDa protein that binds *in vitro* to Vg1 RNA has been described (20), and this protein has been suggested to have a role in mediating an association between Vg1 RNA and the cytoskeleton (21). In *Drosophila*, the exl protein, which was identified by *in vitro* binding to a *bicoid* RNA localization element, has been implicated in *bicoid* localization (22).

As a step towards unraveling the molecular machinery responsible for localization of RNA, I have tested whether the 340-nt localization sequence of Vg1 RNA can be specifically recognized by factors from the oocyte. Results presented here identify a set of stage-specific RNA binding proteins that form a complex with the localization element and specifically recognize essential cis-sequences within the Vg1 RNA localization signal.

MATERIALS AND METHODS

RNA Transcripts. RNA was transcribed from constructs containing the Vg1 sequences from chimeric β-globin/Vg1–3' UTR constructs that were assayed previously for localization *in vivo* (12). The Vg1 localization transcript (loc. txt.) was transcribed from either pSP73-370 or pSP73-340 (derived, respectively, from pXβG-366 and pXβG-340/3'), giving identical results in all assays. Vg1 deletion transcripts, 5'Δ36, 3'Δ35, and 3'Δ88, were transcribed from pSP73-5'Δ36, pSP73-3'Δ35, and pSP73-3'Δ88 (which were derived, respectively, from pXβG-330/5', pXβG-304/3', and pXβG-251/3'). The XβG transcript was transcribed from pSP73-Xβ5', which contains 323-bp of *Xenopus* β-globin coding sequence (23). *In vitro* transcription reactions (24) contained 0.5 mM each of CTP and ATP, 50 μm GTP, 0.5 mM diguanosine triphosphate, and 50 μCi of [α-^{32}P]UTP (800 Ci/mmol, 1 Ci = 37 GBq; DuPont/NEN).

Oocyte S100 Extracts. *Xenopus laevis* oocytes were defolliculated by incubation in 2 mg/ml type I collagenase (Sigma). Oocytes were homogenized at 0°C in one volume of 50 mM Tris·HCl (pH 9), 50 mM KCl, 0.1 mM EDTA, 25% (vol/vol) glycerol, and the supernatant obtained after centrifugation at 1900 $\times$ *g* for 15 min was centrifuged for 2 hr at 100,000 $\times$ *g*, as in ref. 25. Oocytes of all stages were used for total extract, and for staged oocyte extracts oocytes were sorted manually

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into pools of differing stages (26) as follows: the stage I–II pool contained oocytes of ≤450 μm in diameter, the stage III–IV pool contained oocytes of 450–1000 μm in diameter, and the stage V–VI pool contained oocytes of ≥1000 μm in diameter. The S100 extracts were typically 10–20 mg/ml in total protein, and equal amounts of total protein were used for comparison of staged extracts.

In Vitro Binding Assays. *In vitro* binding reactions contained 5 mg/ml heparin, 1% glycerol, 50 mM KCl, 10 mM DTT, 5.2 mM Hepes (pH 7.9), 1.5 mM ATP, 1 mM MgCl$_2$, 0.1 mM EDTA, 40 μg/ml yeast tRNA, 40 μg/ml competitor RNA, and 3–10% S100 extract in a volume of 10 μl. The percent of extract used was titered such that for 1 ng input RNA transcript, maximum binding observed in the presence of 400-fold excess nonspecific competitor RNA [either the XβG transcript or total oocyte RNA (≈95% rRNA)], was fully competable by a 400-fold excess of sequence-specific competitor RNA. The *in vitro* binding reactions were preincubated for 10 min at 25°C, ≈1 ng ^{32}P-labeled RNA transcript was added and incubated for 10 min. For RNA gel shift, after addition of 3 μl of 50% glycerol, reactions were loaded directly onto a nondenaturing 4% polyacrylamide gel (27) and run for 5 hr. For UV crosslinking, binding reactions were crosslinked for 10 min in a Stratalinker (Stratagene). RNase A (Sigma) was added (1 mg/ml), incubated for 15 min at 37°C, and the crosslinked proteins were separated by SDS/PAGE. For RNase footprinting, RNase T1 (Pharmacia) was added (0.5 unit/μl) after binding, the reactions were incubated for 5 min at 25°C, and loaded directly onto a nondenaturing gel. Individual bands were cut from the gel, eluted as described in (28), and resolved on a 15% polyacrylamide/8 M urea gel (1× TBE). The bands were cut from the gel, eluted as above, and each fragment was analyzed by separation on a 20% polyacrylamide/8 M urea gel after complete digestion with RNases T1 and U2 (Pharmacia).

Microinjection. Stage III–IV oocytes were microinjected with ≈5 nl of *in vitro* transcribed RNA at ≈6 × 10^6 cpm/μl. After culture (29) for 2 days, oocytes were opened with forceps and immediately UV irradiated. The oocytes (≈10 per sample) were homogenized in S100 buffer, centrifuged at 16,000 × g for 10 min at 4°C, and the supernatant was subjected to SDS/PAGE.

RESULTS

Factors from Specific Stages of Oogenesis Bind *in Vitro* to the Localization Element. Experiments to map a localization sequence on Vg1 RNA have defined a 340-nt sequence present within the 3′ UTR as sufficient to direct localization of a chimeric reporter RNA (12). Because the localization element alone also localizes to the vegetal pole after injection into *Xenopus* oocytes (data not shown), this sequence element itself must be capable of associating with factors necessary for localization.

To probe interactions between oocyte factors and the RNA localization sequence, a gel-shift assay was employed. In the experiment shown in Fig. 1*A*, radiolabeled RNA transcribed from either the localization sequence (lanes 4–6) or a nonlocalized sequence (lanes 1–3) was incubated with oocyte extract. The *in vitro* binding reactions were then loaded directly onto a non-denaturing gel. During electrophoresis, the binding of factors to the Vg1 localization transcript (lane 5) causes a shift in mobility such that the complex migrates more slowly than does the free RNA (lane 4). By contrast, the mobility of the nonlocalized transcript, *Xenopus* β-globin, does not shift significantly under these conditions (lanes 1–3). To test specificity of the Vg1 RNP complex, oocyte total RNA was used as a nonspecific competitor (lane 5), and the localization element itself was used as a sequence-specific competitor (lane 6). As expected for formation of a sequence-specific complex, the low mobility complex obtained with the localization element tran-
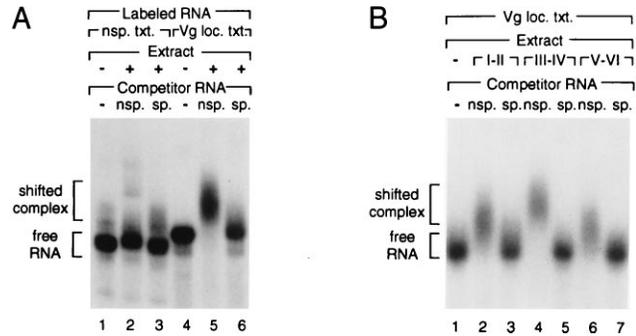

FIG. 1.   *In vitro* binding of oocyte factors to the localization element. (*A*) *In vitro* binding reactions contained ^{32}P-labeled RNA [Vg1 localization transcript (Vg loc. txt., lanes 4–6) or XβG transcript (nsp. txt., lanes 1–3)], unlabeled competitor RNA (oocyte total RNA (nsp., lanes 2 and 5) or Vg1 localization transcript (sp., lanes 3 and 6)] and S100 extract (+, lanes 2, 3, 5, and 6). An autoradiogram of a nondenaturing gel is shown, with the positions of the shifted complex and free RNA (lanes 1 and 4) indicated at left. (*B*) S100 extracts, prepared from stage I–II (lanes 2 and 3), stage III–IV (lanes 4 and 5), and stage V–VI (lanes 6 and 7) oocytes were incubated with ^{32}P-labeled Vg1 localization element transcripts (Vg loc. txt.), and unlabeled competitor RNA [nonspecific (nsp., lanes 2, 4, and 6) or Vg1 localization transcript (sp., lanes 3, 5, and 7)]. An autoradiogram of a nondenaturing gel is shown, with the positions of the shifted complexes and free RNA (lane 1) indicated at the left.

script (lane 5) is unaffected by competition with a nonspecific RNA sequence. The bound factors are competed away only when the localization transcript is used as competitor (lane 6), causing the labeled RNA to migrate with a mobility similar to that of free RNA. The shifted complex obtained with the localization element transcript is sensitive to treatment with protease (not shown), suggesting that one or more proteins contribute to formation of the complex with the RNA. These results indicate that a factor or factors from the oocyte are capable of specifically recognizing the localization sequence.

Because Vg1 RNA is localized during a defined period of oogenesis, staged oocyte extracts were next tested for their ability to form specific complexes with the localization sequence (Fig. 1*B*). The staged S100 extracts were normalized to one another on the basis of protein concentration and were prepared from oocytes of the following stages: stages I–II, before localization of Vg1 has begun (lanes 2 and 3); stages III–IV, during which time Vg1 is localized (lanes 4 and 5); and stages V–VI, after localization is complete and the oocytes are not competent to localize the RNA (lanes 6 and 7). The stage III–IV extract generates the most marked shift in mobility (lane 4), whereas the extracts from early (lane 2) and late (lane 6) stage oocytes give only partial mobility shifts. In each case, complex formation could be competed by the addition of unlabeled localization transcript (lanes 3, 5, and 7). These results show that the mobilities obtained for the shifted complexes vary during oogenesis. One or more factors interacting with the localization element appear to be preferentially present or available for binding during the middle stages of oogenesis. Thus, the timing of Vg1 localization is correlated with the ability to form a specific ribonucleoprotein (RNP) complex.

Because the gel shift results suggested that the complexes could contain several factors in association with the localization transcript, UV crosslinking was used to test whether multiple proteins were interacting with the RNA localization sequence. In the experiment shown in Fig. 2*A*, radiolabeled RNA transcribed from the localization sequence was incubated in the presence of staged oocyte extracts (as in Fig. 1*B*). The *in vitro* binding reactions were irradiated with UV light, and the resulting covalent protein–RNA complexes were treated with RNase to leave short-labeled oligoribonucleotides
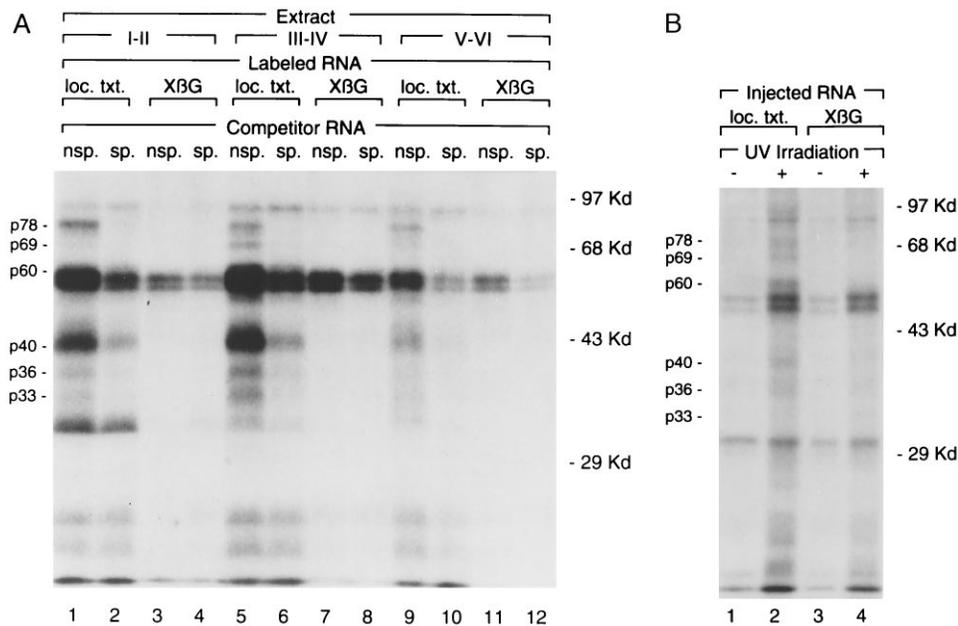
FIG. 2.   UV crosslinking of proteins to the localization element. (*A*) *In vitro* binding reactions containing S100 extracts, prepared from stage I–II (lanes 1–4), stage III–IV (lanes 5–8), and stage V–VI (lanes 9–12) oocytes, ^{32}P-labeled Vg1 localization transcripts (loc. txt., lanes 1, 2, 5, 6, 9, and 10) or XβG transcripts (lanes 3, 4, 7, 8, 11, and 12) and unlabeled competitor RNA [nonspecific (nsp., lanes 1, 3, 5, 7, 9, and 11) or Vg1 localization transcript (sp., lanes 2, 4, 6, 8, 10, and 12)] were crosslinked by UV irradiation. An autoradiogram of an SDS/10% polyacrylamide gel is shown, with the sizes of molecular weight markers indicated at right and the positions of crosslinked proteins of interest shown at left. (*B*) ^{32}P-labeled Vg1 localization transcripts (loc. txt., lanes 1 and 2) or XβG transcripts (lanes 3 and 4) were microinjected into stage III–IV oocytes, which were cultured and UV irradiated (+, lanes 2 and 4). An autoradiogram of an SDS/10% polyacrylamide gel is shown. Indicated at right are the sizes of molecular weight markers, and the positions of crosslinked proteins of interest are shown at left.

crosslinked to the proteins. Fig. 2*A* shows an autoradiogram of the products resolved on an SDS/polyacrylamide gel. Indicated at the left are six proteins that crosslink to the localization element in the presence of nonspecific competitor (lanes 1, 5, and 9), and are competed by the sequence-specific (localization transcript) competitor (lanes 2, 6, and 10). By contrast, the ≈54–56 kDa doublet is clearly nonspecific; it is not competable, and is bound by the nonlocalized transcript, *Xenopus* β-globin (lanes 3, 4, 7, 8, 11, and 12). Based on their approximate molecular weights, the binding proteins will be referred to as p78, p69, p60, p40, p36, and p33. (On this particular gel p60 is incompletely resolved from p56. However, it is evident on lighter exposures of this gel, and is better resolved in Fig. 4*B*.) These results identify a set of proteins that are capable of sequence-specific binding to the localization transcript. As in the gel-shift assay, the highest yield of binding proteins is observed using the middle stage extract, whereas the late stage extract has minimal binding activity. It is notable that p69 is detected in only the middle stage extracts, with p33, p36, p40, and p60 being more abundant in stages III–IV as well. In agreement with the results shown in Fig. 1*B*, the UV crosslinking results (Fig. 2*A*) indicate that certain binding proteins are either present or available for binding to the localization element preferentially during the period of oogenesis during which localization occurs.

Specific Proteins Associate with the Localization Sequence During Localization *in Vivo*. The *in vitro* binding data presented above indicate that a set of oocyte proteins are capable of forming specific complexes with the localization sequence. Next, it was important to ask whether these same proteins are present on RNA that is in the process of localization *in vivo*. To address this, UV crosslinking was performed on microinjected RNAs. Localization of Vg1 RNA can be mimicked in culture: when middle stage oocytes are cultured under appropriate conditions, endogenous or microinjected synthetic Vg1 RNA becomes vegetally localized (30). In this experiment, radiolabeled localization transcript or β-globin RNA were

microinjected into middle stage oocytes. The oocytes were cultured to allow time for the RNA to interact with the localization machinery, opened and quickly irradiated with UV light. By opening the oocytes with forceps immediately prior to irradiation, without dilution of cytoplasm, associations formed *in vivo* between oocyte proteins and the injected RNA are likely to be maintained and, more importantly, binding to additional proteins is unlikely. Thus, the injected RNA is expected crosslink to proteins bound *in vivo*. The crosslinked proteins were fractionated on an SDS/polyacrylamide gel, and the results are shown in Fig. 2*B*. At the left are indicated the mobilities of the six proteins that form sequence-specific complexes with the localization transcript *in vitro*. It is notable that proteins with similar mobilities bind to the localization transcript during localization *in vivo* (lane 2). These include proteins of ≈78 kDa, 69 kDa, 60 kDa, 40 kDa, and less distinctly proteins of ≈36 kDa and 33 kDa. None of these proteins are bound by the nonlocalized β-globin RNA, which binds only to the non-sequence-specific 54–56 kDa proteins and a protein of ≈30 kDa. It apparent from these results that the proteins that bind to the localization element *in vivo* have mobilities similar to those proteins bound *in vivo*.

Oocyte Factors Bind to Discrete Sites on the Localization Element. Because the results shown above indicated that oocyte proteins could bind specifically to the localization element, an RNase footprinting approach was used to define factor binding sites on the RNA localization element. Binding reactions were performed as in Fig. 1, except that the reactions were treated with RNase prior to electrophoresis. The expectation was that bound factors might protect the RNA from degradation, and that smaller complexes (perhaps with single proteins) could be detected by RNA gel shift. Indeed, four RNase-resistant RNP complexes (A–D) were obtained after treatment with T1 ribonuclease, as shown in Fig. 3*A* (lane 1). Complexes A–D are sensitive to protease treatment (not shown), suggesting protein binding. Specificity was tested by competition with a nonspecific competitor (lane 1) or the

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localization element (lane 2), and the bound factors were competed only by the sequence-specific competitor. RNA was isolated from each complex and run on a denaturing gel, as shown in Fig. 3*B*. Each complex yielded a single major RNA fragment (A = 18 nt, B = 18 nt, C = 24 nt, D = 29 nt). These bands (Fig. 3*B*, lanes A–D) were also cut from the gel and RNA was eluted from each slice. RNA from each sample was redigested with base-specific ribonucleases (not shown), allowing these RNA fragments to be mapped on the localization element, as depicted in Fig. 4*A*. The RNase-resistant complexes map to several sites on the localization element: complexes A and B each contain the same RNA fragment from the middle of the element, and complexes C and D map at or near the 3′ and 5′ ends, respectively. The positions of deletions that disrupt localization (12) are indicated, relative to the binding sites (Fig. 4*A*). Of these, the smallest 5′ deletion (5′Δ36) lacks site D, and an internal deletion (not shown) lacks site A/B. The smallest 3′ deletion (3′Δ35) still contains site C, but a larger 3′ deletion (3′Δ88) that abolishes localization eliminates site C. The RNase footprinting results indicate that proteins bind *in vitro* to cis-acting sequences that are crucial for localization *in vivo*.

Deletion Mutations That Abolish or Impair Localization *in Vivo* Are Defective in Protein Binding. To test for potential roles in localization for the proteins forming specific complexes with the localization sequence, *in vitro* RNA binding was next assayed using localization transcripts bearing mutations that disrupt localization *in vivo*. UV crosslinking was used to compare the binding profiles for mutant localization transcripts (Fig. 4*B*, lanes 3–8) with that of the complete localization transcript (lane 1). When 36 nt are removed from the 5′ end of the localization sequence, overall protein binding is dramatically altered (5′Δ36, lane 3). With this deletion, which disrupts localization *in vivo*, binding of five of the six binding proteins is lost; only p69 is capable of binding to this mutant transcript. The effects of deletions at the 3′ end of the localization sequence are more subtle. Deletions of 35 nt and 88 nt, which either impair or destroy localization *in vivo*, show defects in p69 binding. Binding of p69 is reduced twofold with the 3′Δ35 deletion that impairs localization (lane 7) and is diminished fivefold with the 3′Δ88 deletion that abolishes localization *in vivo* (lane 5). These data show that deletion

FIG. 3. RNA footprinting. (*A*) RNA–protein complexes, formed in the presence of ^{32}P-labeled Vg1 localization transcript and nonspecific (nsp., lane 1) or specific (sp., lane 2) competitor RNAs, were treated with T1 RNase. An autoradiogram of a nondenaturing gel is shown, and the RNase-resistant complexes are labeled A–D at the left. (*B*) The RNA fragments from complexes A–D were resolved on a 15% polyacrylamide gel. An autoradiogram is shown, with the sizes of RNA molecular weight markers (lane M) listed at the left. The sequences of the RNA fragments are: A and B = UUAAUAAUAAUAUCUUAG; C = ACUUUUCUAUUUCACUAAAAUUAG; and D = auccccA-UUUCUACUUUAUUUCUACACUG. The first six nt of fragment D are polylinker sequences (lowercase letters), whereas the remaining 23 nt are the 5′ end of the Vg1 localization element.

from the 5′ end of the element abolishes binding of p78, p60, p40, p36, and p30, whereas deletion from the 3′ end decreases binding of p69. The *in vitro* binding results are summarized in Fig. 4*C*, and the *in vivo* phenotypes of each deletion are indicated in Fig. 4*A*. These results indicate that binding of each of the proteins that complex with the Vg1 localization element *in vitro* can be correlated with deletions that are critical for function of the element *in vivo*.

DISCUSSION

In a model for RNA localization in which interaction between the localization signal and transacting factors is the first step in the localization pathway, formation of a localization-specific RNP complex is a key event. However, questions as to the identities of such transacting factors and whether multiple factors assemble with the localization element to form a localization-specific RNP complex have remained largely unanswered. In this work, a set of six oocyte proteins have been identified that bind directly to the Vg1 localization element to form a specific RNP complex. One or more of the RNA binding proteins identified in this study may serve a role in RNA recognition, since RNAs destined for localization must be recognized from amid the vast array of nonlocalized RNAs. This recognition event presumably relies not only upon the localization elements that generally reside in the 3′ UTRs of localized RNAs, but also upon oocyte RNA binding proteins capable of recognizing such localization elements. A second role for RNA binding proteins in the localization process may be in mediating interactions with the cytoskeleton to achieve translocation. Recently, such a role has been reported for a 69-kDa Vg1 RNA binding protein (VgRBP) (21), and VgRBP may in fact be identical to the 69-kDa Vg1 binding protein (p69) described here. However, this possibility must await further investigation, since there is no indication as to where VgRBP might bind within the localization element or whether this protein is preferentially present during stages III–IV of oogenesis. Several lines of evidence indicate that the Vg1 RNA binding proteins identified in this study are relevant to the process of RNA localization *in vivo*. Crosslinking results with proteins bound *in vivo* suggest that Vg1 RNA molecules that are in the process of being localized associate the same (or similar) proteins identified by *in vitro* binding experiments using the localization sequence. The availability of sequence-specific binding proteins is correlated with the timing of Vg1 localization. Localization of Vg1 occurs during the middle stages of oogenesis, and extracts prepared from middle stage oocytes contain a unique pattern of localization element binding factors, compared with extracts prepared from oocytes that are not in the process of localizing Vg1 RNA. Additionally, RNase footprinting and *in vitro* binding experiments using deletion mutations that affect localization *in vivo* indicate that these binding proteins recognize critical cis-acting localization element sequences.

The *in vitro* UV crosslinking experiments (Fig. 2*A*) identify a set of six proteins (p78, p69, p60, p40, p36, p33) that bind in a sequence-specific manner to the Vg1 localization sequence. One issue that is raised by this finding is whether these proteins can bind simultaneously to the localization sequence to form a complex, or whether each binds separately to a different RNA molecule. The former model is clearly favored by comparison of the data from the gel-shift (Fig. 1*B*) and UV crosslinking (Fig. 2*A*) experiments performed using staged oocyte extracts. The middle stage extract (III–IV) generates the largest mobility shift, indicating that a greater mass of protein is bound to the probe RNA with the middle stage extracts. The UV crosslinking data indicate that a larger number of proteins are capable of binding with the middle stage extract as well; all six proteins (p78, p69, p60, p40, p36, p33) are bound. By contrast, with the late stage extract, only
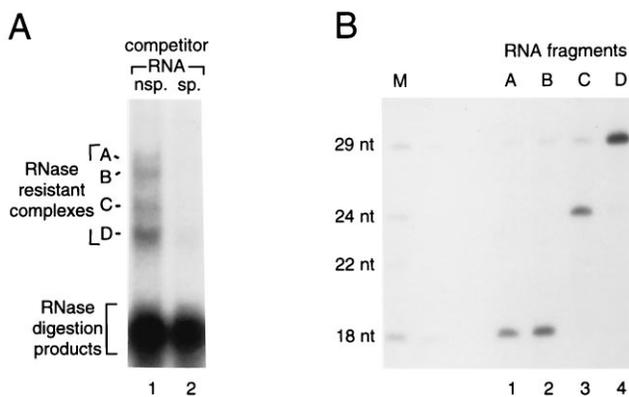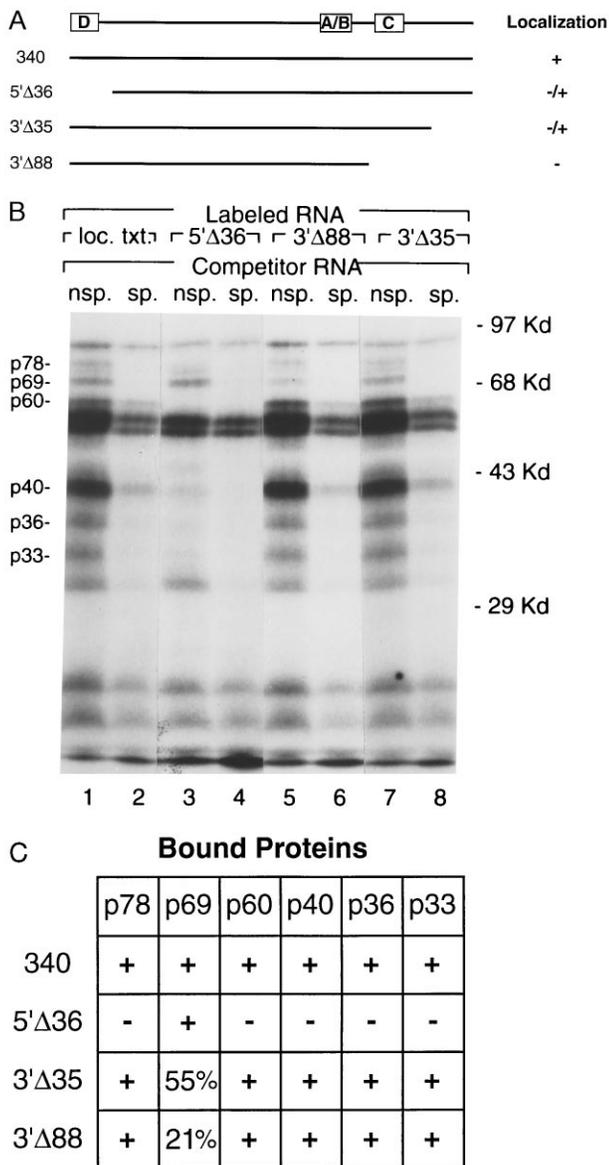
FIG. 4.   Analysis of deletion mutants. (*A*) A schematic of the minimal localization element is shown at the top, with the positions of the RNA fragments from complexes A–D (see Fig. 3) indicated by boxes. The deletion transcripts that abolish or impair localization are represented schematically by lines, drawn to scale below the minimal localization transcript. The activity of each transcript in localization assays (12) is indicated to the right: (+) normal vegetal localization, (−/+) impaired localization, and (−) no detectable localization. (*B*) The binding profiles for deletion transcripts 5′Δ36 (lanes 3 and 4), 3′Δ88 (lanes 5 and 6), and 3′Δ35 (lanes 7 and 8) are compared with that of the entire localization transcript (lanes 1 and 2). *In vitro* binding reactions contained ³²P-labeled RNA transcripts, stage III–IV oocyte extract, and unlabeled competitor RNA [nonspecific (nsp., lanes 1, 3, 5, and 7) or Vg1 localization transcript (sp., lanes 2, 4, and 6)]. Shown is an autoradiogram of a polyacrylamide/10% SDS gel, with the positions of molecular weight markers shown at right. Indicated at left are the positions of the six proteins that form sequence-specific complexes with the localization transcript. (*C*) The results of *in vitro* binding assays are summarized: (+) normal binding of the indicated protein, (−) absence of protein binding (below 10% of normal), and reduced levels of protein binding indicated by the percent binding detected relative to wild type. The proteins are indicated at top, and the RNA transcripts are listed at left. Levels of protein binding were determined by densitometry of the autoradiograms, with any loading differences compensated for by normalization to the nonspecific protein of ≈22 kDa.

p78 and p40 are detectable by UV crosslinking, and the gel-shift data indicate that the late stage extract is capable of generating only a very minimal mobility shift. The early stage extract generates an intermediate mobility shift, and the UV crosslinking data indicate that the yield of protein binding is intermediate as well. Binding of p78 is robust, whereas binding of p40 is diminished and some binding is detected for p33, p36, and p60. Because Vg1 RNA is localized during the middle stages of oogenesis, it is intriguing that it is the middle stage extract that shows the greatest binding: p69 binding is detected only in middle stage extracts, and binding by p33, p36, p40, and p60 is increased there as well. The enrichment of these proteins in the middle stage extracts could result from stage-specific solubility rather than increased abundance. However, because microtubules have been implicated in the localization process, the S100 extracts were prepared under conditions to depolymerize microtubules and release any microtubule-associated factors into the soluble fraction, rendering them available for binding. The UV crosslinking experiments using microinjected RNAs to form complexes *in vivo* (Fig. 2*B*), also indicate that multiple proteins, with mobilities that are distinctly similar to those seen *in vitro*, are bound to the localization element. In this experiment, at least 95% of the recoverable RNA is estimated to be accurately localized, suggesting that the proteins detected by UV crosslinking are present in complexes formed on RNAs that are in the process of being localized. Taken together, these results suggest that during the middle stages of oogenesis, the localization element of Vg1 RNA associates with p78, p69, p60, p40, p36, and p33 to form an RNP complex that is capable of undergoing translocation to the vegetal pole.

Roles in localization for these binding proteins are suggested by their interaction with sequences within the localization element that are critical for localization *in vivo*. The RNase footprinting experiments show that protein binding sites (see Fig. 4*A*) are positioned on the Vg1 localization element such that they are removed by deletions that abolish localization *in vivo*. These deletion mutations also show defects in protein binding *in vitro* (Fig. 4 *B* and *C*). It is at first glance surprising that the 36-nt deletion from the 5′ end (5′Δ36) abolishes binding of five of the six specifically bound proteins. It is likely that these five proteins cannot bind directly to the deleted region, though all must bind RNA directly to be detected by UV crosslinking. One possible explanation for this result is that the 36-nt deletion may dramatically alter the secondary structure of the RNA element, eliminating structural motifs elsewhere within the element that are recognized by the factors. An alternative explanation is that the 36-nt deletion eliminates a binding site for one or two of these five proteins, and that binding of this protein(s) is necessary for the other proteins to be recruited to the complex. The second explanation is appealing, as it is clear from the footprinting data (Fig. 3) that at least one factor is bound to site D, which is contained within the 36-nt deletion. Furthermore, only one or two proteins are likely bound to D, as evidenced by the modest mobility shift observed for D binding as compared with either C or A/B. The *in vitro* binding results using deletions from the 3′ end (Fig. 4*B*) suggest possible redundancy within the localization element, as binding of p69 is reduced twofold by deletion 3′Δ35 and fivefold by 3′Δ88. This decrease in p69 binding as the 3′ end of the element is deleted could be explained by the existence of more than one p69 binding site within the localization element. The data are consistent with one p69 binding site being removed by each 3′ end deletion; the residual binding seen with the 3′Δ88 transcript could indicate an additional site elsewhere within the element.

This work provides the first evidence for a localization-specific RNP complex, composed of multiple proteins bound to an RNA localization element. The results presented here identify a set of oocyte proteins that associate specifically with

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the Vg1 localization element. Roles for these binding proteins in localization is underscored by the enrichment of these proteins during the precise stages of oogenesis when Vg1 is localized, as well as the finding that they recognize specific regions of the Vg1 localization element that are critical for function *in vivo*. Formation of a localization-specific RNP complex may result from binding of both proteins that specifically recognize localization-specific sequences and proteins that can mediate interactions with the localization machinery. It may be formation of a localization-specific RNP complex that targets localized RNAs to their subcellular destinations.

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