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A *Xenopus* Protein Related to hnRNP I Has a Role in Cytoplasmic RNA Localization

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Summary

Cytoplasmic localization of mRNA molecules is a powerful mechanism for generating cell polarity. In vertebrates, one paradigm is localization of Vg1 RNA within the *Xenopus* oocyte, a process directed by recognition of a localization element within the Vg1 3′ UTR. We show that specific base changes within the localization element abolish both localization in vivo and binding in vitro by a single protein, VgRBP60. VgRBP60 is homologous to a human hnRNP protein, hnRNP I, and combined immunolocalization and in situ hybridization demonstrate striking colocalization of hnRNP I and Vg1 RNA within the vegetal cytoplasm of the *Xenopus* oocyte. These results implicate a novel role in cytoplasmic RNA transport for this family of nuclear RNA-binding proteins.

Introduction

Targeting of mRNA molecules to specific subcellular regions can be regarded as a fundamental mechanism for spatial regulation of gene expression. Asymmetric distribution of specific mRNAs underlies cell polarity in a variety of systems and cell types, including somatic cells and germ cells. In many diverse cell types, regional functional specialization and specification of cell fates can be provided through sorting of mRNAs to distinct cytoplasmic domains (reviewed in Bassell et al., 1999). In eggs and oocytes, localization of mRNAs can provide the basis for embryonic patterning (reviewed in Bashirullah et al., 1998). Prominent examples are found in *Drosophila*, where localized mRNAs underlie embryonic patterning (reviewed in Lasko, 1999), and in the frog, *Xenopus*, where developmental polarity along the animal–vegetal axis is coincident with the localization of a number of maternal mRNAs (reviewed in Mowry and Cote, 1999). Vg1 mRNA, encoding a member of the transforming growth factor β family, is localized during oogenesis to the vegetal hemisphere of the *Xenopus* oocyte (Weeks and Melton, 1987). After fertilization, restricted expression of Vg1 protein in the vegetal hemisphere of the egg appears to be critical for correct patterning of the embryo (Dale et al., 1993; Thomsen and Melton, 1993; Henry et al., 1996; Joseph and Melton, 1998; Zorn et al., 1999). Thus, it is important to understand the molecular events that elicit mRNA localization.

Vegetal localization of Vg1 RNA is directed by a 340-nucleotide (nt) sequence element residing within the 3′ UTR of Vg1 mRNA (Mowry and Melton, 1992). Targeting of RNA molecules to distinct subcellular destinations through sequence elements within their 3′ UTRs has proven to be a general mechanism (reviewed in Bashirullah et al., 1998). These localization elements can be relatively large, perhaps because these RNA sequences often serve to direct multiple steps in localization pathways. In some cases, discrete portions of a localization element can direct specific steps in a pathway (Macdonald et al., 1993), while in other cases, subregions of an element are functionally redundant to one another (Kislauskis et al., 1994), sometimes in an additive fashion (Lantz and Schedl, 1994). The multistep nature of many localization pathways and the sequence complexity of the *cis*-elements suggest the involvement of multiple *trans*-acting localization factors, but the identities of such localization factors have, until recently, remained elusive.

Transport of Vg1 mRNA is a multistep process that is believed to commence with recognition of the RNA localization sequence by protein components of the oocyte localization machinery (Melton, 1987; Yisraeli et al., 1990; Mowry, 1996). Vg1 RNA-binding proteins (VgRBPs) with potential roles in the localization process have been identified through their ability to bind specifically to the Vg1 RNA localization element and include Vg1RBP (Schwartz et al., 1992), VgRBPs −78, −69, −60, −40, −36, and −33 (Mowry, 1996), and vera (Deshler et al., 1997). Vg1RBP and vera are identical to one another (Deshler et al., 1998; Havin et al., 1998) and homologous to ZBP-1, an RNA-binding protein involved in localization of β-actin mRNA in chick fibroblasts (Ross et al., 1997). However, ZBP-1 is not suggested to function alone in actin mRNA targeting (Ross et al., 1997), nor can a single protein factor be sufficient to direct the Vg1 localization process. The Vg1 localization element is capable of forming an RNP complex containing multiple protein factors, and this complex forms preferentially in extracts prepared from oocytes during the period of active Vg1 localization (Mowry, 1996). Moreover, sequences have been identified within the Vg1 localization element (Gautreau et al., 1997) that are critical for localization and are distinct from vera recognition sequences (Deshler et al., 1997, 1998). Mutational analysis (Gautreau et al., 1997) of the Vg1 RNA localization element has identified an 85 nt subelement from the 5′ end of the 340 nt sequence, which, when duplicated, is sufficient to direct vegetal localization. Contained within this subelement is VM1 (Vg1 motif 1), a reiterated hexanucleotide motif (UUUCUA), which is critical for localization (Gautreau et al., 1997); point mutations within VM1 completely abolish localization of Vg1 mRNA in vivo. These same mutations also resulted in defects in protein binding in vitro, suggesting that VM1 acts as a recognition or binding site for an essential *trans*-acting localization

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factor. However, the identity of this factor or factors was unknown.

In this work, we show VgRBP60 to be an essential *trans*-acting localization factor that interacts directly with the VM1 motif. Purification and cloning of VgRBP60 reveals it to be highly homologous to a human hnRNP protein, hnRNP I. Supporting a role for this protein in Vg1 RNA localization, we show that base changes within VM1 that specifically block VgRBP60 binding also abolish Vg1 RNA localization in vivo and demonstrate that Vg1 RNA and VgRBP60/hnRNP I are colocalized within the vegetal cytoplasm. Because VgRBP60 is a member of the hnRNP family of RNA-binding proteins, our findings raise the possibility that the assembly of specific RNA-binding proteins onto a transcript in the nucleus could determine the cytoplasmic fate of the RNA.

Results

To demonstrate roles for Vg1 RNA-binding proteins (VgRBPs) in localization, it is necessary to create base changes within putative binding sites and to test for defects in both localization in vivo and protein binding in vitro. Here, we focus on the VM1 motif, as our previous results suggested that VM1 could act as a binding site for an essential *trans*-acting localization factor. For each VM1 site within the minimal functional localization element, a duplication of nt 1–85 (vg2×1–85; Figure 1A), we changed three of the U's to G's (UUUCUA→GUGCGA). The effects of mutating VM1 are quite striking (Figure 1B): vegetal localization directed in vivo by the wild-type element (wt, left) is completely abolished by the introduction of mutations within VM1 (MT, middle). The localization of the RNA containing mutated VM1 sites (MT, middle) is indistinguishable from that of β-globin (βG, right), which does not localize when injected into oocytes (Yisraeli and Melton, 1988). The mutant RNA is fully stable in the oocyte, as judged by RNase protection assays (Figure 1B, lanes 2), indicating that base changes within VM1 have no effect on RNA stability and instead specifically disrupt in vivo localization of the RNA.

VgRBP60 Binding Is Abolished by Mutation of VM1

To determine whether the binding of a VgRBP was affected by mutation of VM1, we compared the UV cross-linking profiles of vg2×1–85 RNAs containing wild-type or mutant VM1 sites. As shown in Figure 1C, the binding of 5 of the 6 VgRBPs (−33, −36, −40, −69, and −78) is similar between the wild-type (wt, lane 1) and mutant (MT, lane 2) RNAs. By contrast, the binding of VgRBP60 is disrupted by mutations within VM1. With crude extracts (lanes 1 and 2), it was unclear whether the mutations within VM1 completely abolished VgRBP60 binding, as this protein migrates near the sequence nonspecific mRNA masking proteins p54/p56 (Marello et al., 1991). To demonstrate definitively that mutations within VM1 alter VgRBP60 binding, we fractionated *Xenopus* ovarian S100 protein extracts by heparin–agarose chromatography (see below) and used partially purified VgRBP60 for UV cross-linking analysis. Indeed, VgRBP60 binding was abolished by mutations within VM1 (MT, lane 4). Our results are distinct from a recent study (Havin et al., 1998) in which the 69 kDa Vg1RBP

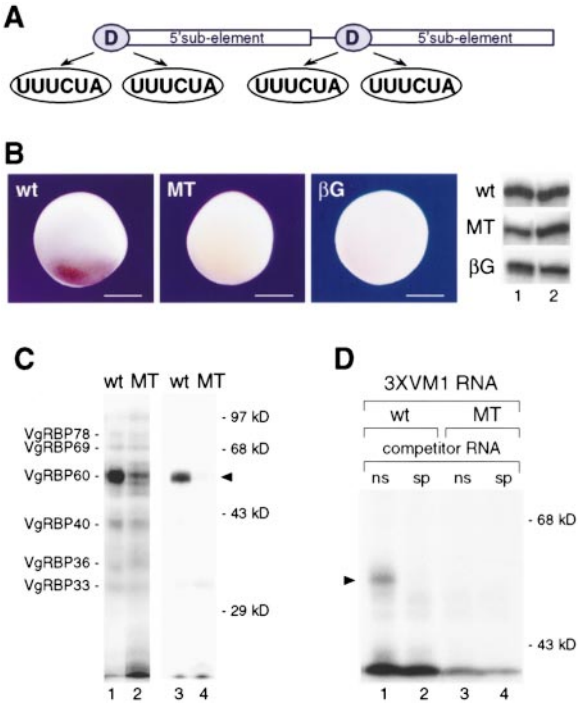

Figure 1. Site-Directed Mutagenesis of VM1

(A) A schematic of the vg2×1–85 transcript is shown, with RNA footprint site D shaded. The positions of the VM1 motifs are indicated below.

(B) For analysis of in vivo localization, oocytes were injected with βG/vg2×1–85 (wt, left), βG/vg2×1–85/MTvm1 (MT, middle), or β-globin (βG, right) RNA transcripts, and analyzed by whole-mount in situ hybridization. The vegetal poles are toward the bottom, and the scale bars represent 200 μm. At the far right are the results of RNase protection analyses for recovery of wild-type (wt, top), VM1 mutant (MT, middle), and β-globin (βG, bottom) RNA transcripts either upon injection (lanes 1) or at harvest (lanes 2).

(C) Protein binding was tested in vitro by UV cross-linking to ^{32}P-labeled vg2×1–85 RNA transcripts containing either wild-type (wt, lanes 1 and 3) or mutant (MT, lanes 2 and 4) VM1 sites. Binding reactions contained either oocyte S100 extract (lanes 1 and 2) or partially purified VgRBP60 (lanes 3 and 4). Cross-linked proteins were detected by autoradiography after SDS-PAGE. The positions of the VgRBPs and molecular weight markers are indicated; VgRBP60 is shown by an arrowhead.

(D) Direct binding to the VM1 motif was tested by UV cross-linking analysis performed with oocyte S100 extracts and ^{32}P-labeled 3×VM1 RNA transcripts containing either wild-type (wt, lanes 1 and 2) or mutated (MT, lanes 3 and 4) VM1 sequences, and either nonspecific (ns, lanes 1 and 3) or sequence-specific (sp, lanes 2 and 4) competitor RNA. Cross-linked proteins were detected by autoradiography after SDS-PAGE. The positions of VgRBP60 (arrowhead) and molecular weight markers are indicated at the right.

was suggested to interact with the VM1 motif because replacement of 20 nt regions containing VM1 reduced or abolished binding of the protein to the Vg1 localization element. However, with point mutations in VM1, we have found no apparent effect on VgRBP69 (Vg1RBP/vera) binding (Figure 1C, lane 2). To test explicitly whether VM1 represents the binding site for VgRBP60, we performed UV cross-linking analysis with S100 extracts and RNA transcripts consisting of three tandem copies of either the wild-type VM1 sequence or the mutated VM1 sequence. As shown in Figure 1D, VgRBP60 (but not

VgRBP69) is capable of binding directly to a VM1 multimer transcript. The binding is specific, as VgRBP60 binds to the wild-type VM1 sequence (wt; lane 1) but not the mutated VM1 sequence (MT; lane 3), and the binding to wild-type VM1 is competed by a molar excess of the wild-type sequence (lane 2). Together, these data demonstrate that a mutation within VM1 that abolishes localization in vivo also specifically disrupts the binding of VgRBP60 to the Vg1 RNA localization element. Thus, VgRBP60 is implicated with an essential role in Vg1 RNA localization.

Purification and Cloning of VgRBP60

To purify VgRBP60 for further characterization, we first fractionated S100 protein extracts by heparin–agarose chromatography. We assayed for the presence of VgRBP60 by virtue of its ability to UV cross-link to the vg2×1–85 wild-type RNA (Figure 2A). Fractions containing the highest VgRBP60 binding activity were pooled and applied to RNA affinity columns containing 5′ subelement RNA transcripts with either wild-type (wt) or mutant (MT) VM1 sites, and the eluates were subjected to UV cross-linking analysis. As shown in Figure 2B, the 60 kDa Vg1 RNA binding activity was observed only in the eluate from a column containing wild-type RNA (wt; lane 3). Moreover, we have observed by silver staining (Figure 2C) that a 60 kDa protein is the predominant protein obtained from the wild-type eluate (wt, lane 3) and that this 60 kDa protein is notably absent from the mutant eluate (MT, lane 2). The purified protein is capable of binding specifically to the wild-type VM1 multimer (Figure 2D; wt, lane 1), but not to the mutated VM1 multimer transcript (MT, lane 2). Together, these data indicate that the 60 kDa protein that we have purified corresponds to the VgRBP60 RNA binding activity.

The sequences of three tryptic peptides were determined from the purified VgRBP60 and used to design degenerate oligonucleotides for PCR. An ~760 bp PCR fragment was amplified and used to screen a *Xenopus* ovarian cDNA library. Two overlapping clones were obtained, which were revealed by sequence analysis to contain ~200 nt of 3′ UTR and to be truncated at the 5′ end. To analyze the 5′ end sequences, we used a RACE strategy and obtained five additional clones. Each contains a short 5′ UTR (25–33 nt) followed by an open reading frame (ORF) of ~1.6 kb. The complete ORF is 552 amino acids (Figure 3A), giving a predicted molecular mass of 61,272 daltons that correlates well with the apparent molecular mass of 60 kDa for VgRBP60. Moreover, the three peptide sequences obtained from the purified VgRBP60 are present in the cDNA sequence. Peptides 2 and 3 are perfect matches to the predicted sequence, and peptide 1 contains a conservative valine to isoleucine change at position 129; this discrepancy is likely to be a result of allelic variation, which is common in *Xenopus* (Graf and Kobel, 1991). Allelic variation is apparent at other positions as well. Among the cDNA clones, we have identified differences at several wobble base positions in addition to amino acid variations at positions 143 (D/N) and 219 (F/L).

VgRBP60 Is Related to hnRNP I

A database search revealed that VgRBP60 is highly related to the human RNA-binding proteins hnRNP I and

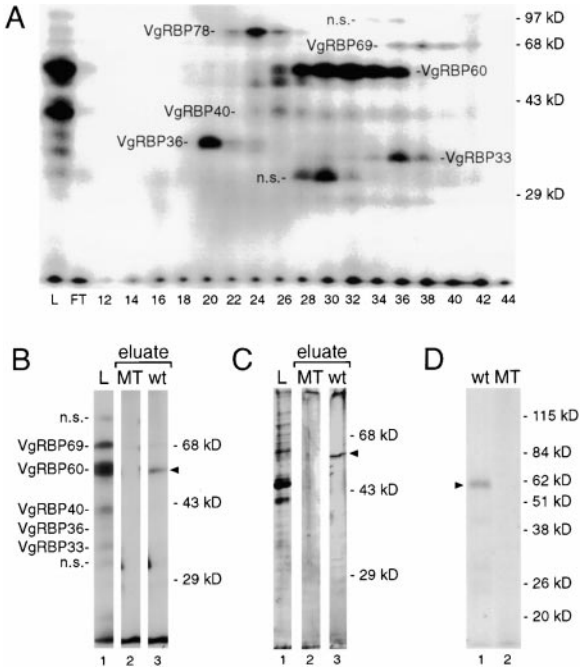

Figure 2. Purification of VgRBP60

(A) Fractions obtained after heparin–agarose chromatography were assayed for VgRBP60 binding activity by UV cross-linking to ^{32}P-labeled vg2×1–85 RNA. The fraction numbers are indicated below; L, load, FT, flowthrough. Each VgRBP is labeled, and the positions of molecular weight standards are indicated at the right. (ns, nonspecific).

(B) The VgRBP60 pool (lane 1, fractions 30–34 from [A]) was fractionated by RNA affinity chromatography. VgRBP60 binding activity was assayed in the eluates from VM1 mutant (MT, lane 2) and wild-type (wt, lane 3) RNA affinity columns by UV cross-linking to vg2×1–85 RNA. The positions of the VgRBPs are noted on the left, and molecular weight standards are shown on the right. VgRBP60 is indicated by the arrowhead.

(C) The VgRBP60 pool from heparin–agarose chromatography (lane 1) and the eluates from either VM1 mutant (MT, lane 2) or wild-type (wt, lane 3) RNA affinity columns were resolved by SDS-PAGE and silver stained. The positions of VgRBP60 (arrowhead) and molecular weight markers are indicated at the right.

(D) Purified VgRBP60 (as in [B] and [C] above, lanes 3) was assayed by UV cross-linking for the ability to bind either wild-type (wt, lane 1) or VM1 mutant (MT, lane 2) 3×VM1 RNA transcripts. Cross-linked proteins were resolved by SDS-PAGE and detected by autoradiography. VgRBP60 is indicated by an arrowhead; the positions of molecular weight standards are shown at the right.

PTB (polypyrimidine tract–binding protein), which are spliced isoforms of one another (Gil et al., 1991; Patton et al., 1991; Ghetti et al., 1992). These proteins are classified as members of the hnRNP family of nuclear RNA-binding proteins, which consists of ~20 different groups (e.g., A1, A2, I, etc.), within which spliced isoforms, or variants with specific amino acid changes, have been reported (Dreyfuss et al., 1993; Ashiya and Grabowski, 1997; Chan and Black, 1997). Amino acid sequence alignment (Figure 3A) reveals VgRBP60 to be most closely related (87% identical) to hnRNP I, as indicated by the presence of a sequence (amino acids 298–322, underlined) that distinguishes the human hnRNP I and PTB isoforms (Ghetti et al., 1992). In the *Xenopus* oocyte,

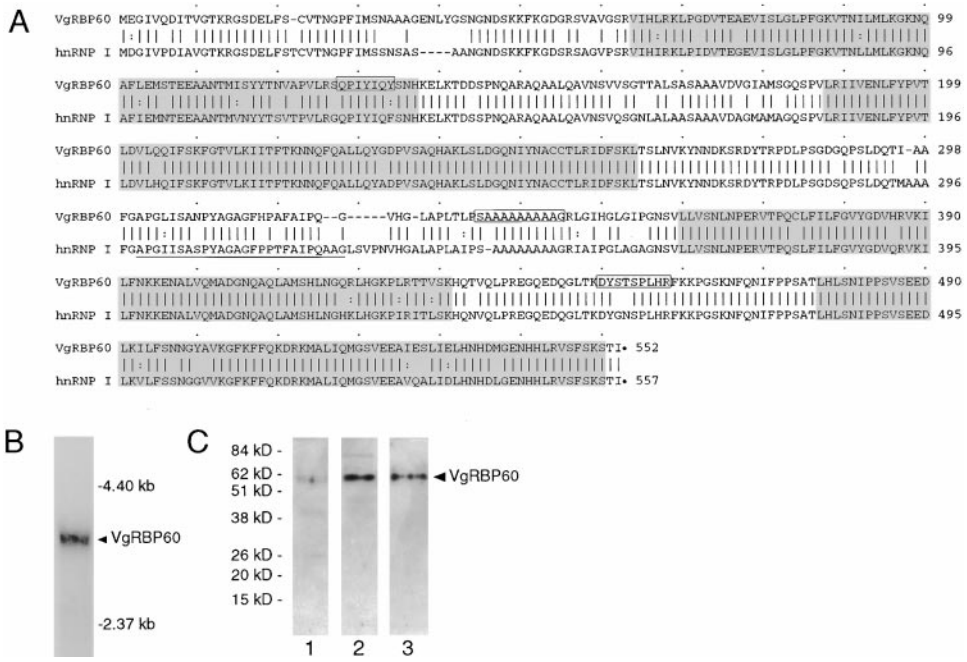

Figure 3. Identification of VgRBP60

(A) The predicted amino acid sequence of VgRBP60 is aligned with that of human hnRNP I (Ghetti et al., 1992). Identical and conserved amino acids are marked between the sequences by lines and dots, respectively. The four RRM domains are shaded, and the amino acid sequence that is present in the hnRNP I isoform and absent from the predominant PTB isoform is underlined. The positions of the sequenced peptides (peptide 1 at 127–133, peptide 2 at 335–345, and peptide 3 at 450–458) are indicated by boxes.

(B) Northern blot analysis reveals a transcript of ~3.5 kb for VgRBP60 in *Xenopus* oocytes. The positions of VgRBP60 mRNA and RNA size markers are indicated at the right.

(C) VgRBP60 is recognized by anti-peptide antibodies directed against human PTB/hnRNP I (a gift of D. Black). Protein samples containing 25 μg of oocyte S100 extract (lane 1), 25 μg of protein obtained after heparin–agarose chromatography (lane 2), or 10 ng of purified VgRBP60 were analyzed by Western blot. VgRBP60 is indicated by an arrowhead, and the positions of molecular weight standards are shown at the left.

we have detected expression of only the hnRNP I isoform, as all clones sequenced ($n = 14$) contain amino acids 298–322, and a single transcript of ~3.5 kb is observed by Northern blot analysis of oocyte RNA (Figure 3B).

VgRBP60 contains four putative RNA-binding domains (Figure 3A, shaded) that are noncanonical members of the RRM class of RNA-binding domains (reviewed in Burd and Dreyfuss, 1994). In human PTB/hnRNP I, RRM3 and RRM4 have been suggested to be the major RNA-binding domains whereas the N-terminal half of the protein is believed to be involved in protein–protein interactions (Perez et al., 1997; Oh et al., 1998). A nuclear localization signal has been mapped for hnRNP I/PTB within the N-terminal 60 amino acids (Ghetti et al., 1992; Perez et al., 1997; Romanelli et al., 1997), and this potential bipartite nuclear localization signal GTKRG (amino acids 11–15) KKFK (amino acids 49–52) is apparent in the VgRBP60 sequence as well.

VgRBP60 Protein and Vg1 RNA Are Colocalized within the Oocyte Cytoplasm

As hnRNP proteins have classically been described as nuclear proteins that bind nascent hnRNA transcripts, it was important to test whether VgRBP60 could be detected within the cytoplasm of *Xenopus* oocytes in a manner consistent with a role in Vg1 RNA localization. Anti-peptide antisera directed against the C terminus of human hnRNP I/PTB (a generous gift of D. Black) recognize a 60 kDa protein that copurifies with VgRBP60 (Figure 3C), and whole-mount immunocytochemistry of stage III oocytes using these antibodies demonstrates cytoplasmic localization of VgRBP60 (Figure 4). Significant localization of VgRBP60 is evident within the vegetal hemisphere cytoplasm (Figure 4B), while staining is diffuse and indistinct within the animal hemisphere (Figure 4A). Intriguingly, the distribution of VgRBP60 within the vegetal hemisphere (Figure 4B) is highly reminiscent of the pattern seen by in situ hybridization for Vg1 RNA during its localization (Figure 4C). To test explicitly whether VgRBP60 and Vg1 RNA are colocalized within the vegetal cortex, we performed in situ hybridization using a fluorescently labeled Vg1-specific probe, followed by immunocytochemistry with the anti-hnRNP I/PTB antibodies. These double-staining results (Figures 4D–4F) demonstrate striking colocalization of VgRBP60 and Vg1 RNA within the vegetal cortical cytoplasm of stage III oocytes. The distribution of VgRBP60 is shown in red (Figure 4D), Vg1 RNA is shown in green (Figure 4E), and the overlap is shown in yellow (Figure 4F). These data reveal that VgRBP60 is colocalized with Vg1 RNA in the vegetal cytoplasm during the stage of oogenesis when Vg1 RNA localization is ongoing. Moreover, the subcellular distribution of VgRBP60 supports a role in

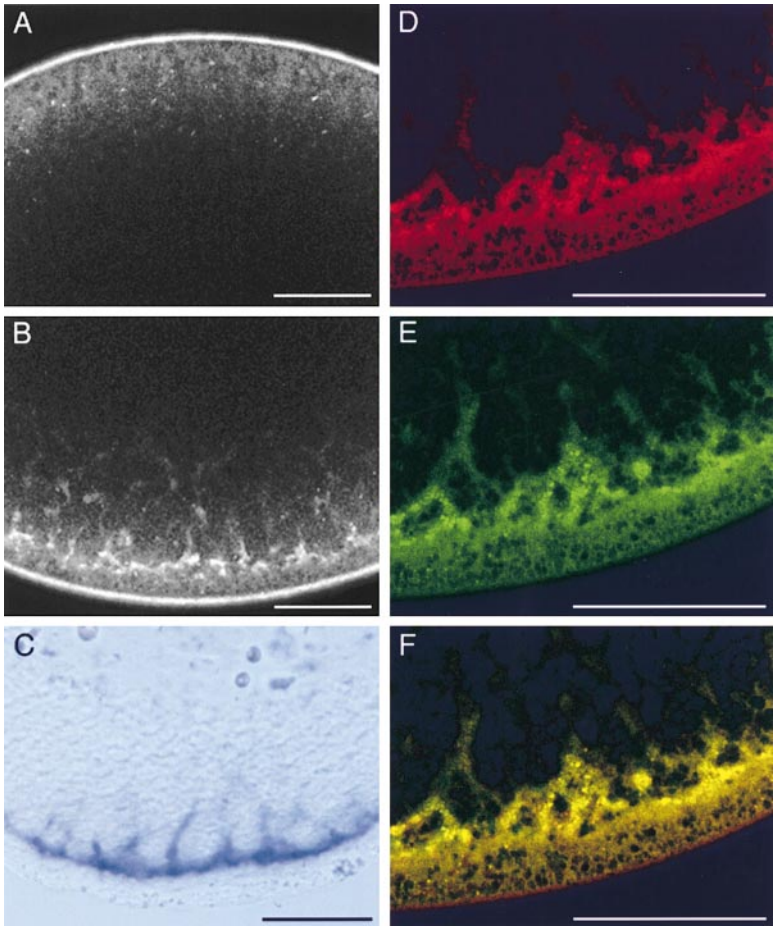

Figure 4. Colocalization of VgRBP60 Protein and Vg1 RNA

(A and B) The distribution of VgRBP60 within stage III oocytes was determined by immunocytochemistry using anti-peptide antibody directed against PTB/hnRNP I (courtesy of D. Black). Optical confocal sections of a stage III oocyte are shown: (A) animal hemisphere view; (B) vegetal hemisphere view. The images are oriented with the vegetal hemisphere toward the bottom.

(C) Vg1 mRNA was detected in stage III oocytes, by in situ hybridization using a digoxigenin-labeled probe. Shown is a paraffin section, vegetal pole toward the bottom.

(D–F) Stage III oocytes were subjected to in situ hybridization with a fluorescently labeled Vg1 RNA probe, followed by immunocytochemistry with anti-peptide PTB/hnRNP I antibody. Shown is an optical confocal section through the vegetal cortex, viewed in the red channel (D) to detect VgRBP60/hnRNP I and in the green channel (E) to detect Vg1 RNA; the overlap is shown in yellow (F). All scale bars (A–F) represent 50 μm.

cytoplasmic RNA localization for this hnRNP family member.

Discussion

We have studied the localization of Vg1 mRNA in the frog oocyte as a model to gain mechanistic insight into how RNA molecules can be targeted to specific regions of the cell cytoplasm, thus generating spatially restricted gene expression. A key step in the localization process is recognition of *cis*-acting sequences within the RNA by protein components of the RNA transport machinery. We had previously identified an essential sequence motif, VM1, that is reiterated within the Vg1 localization element (Gautreau et al., 1997). Base changes within VM1 selectively block localization in vivo; as shown in Figure 1B, the mutant RNA is stable yet is not vegetally localized. These same base changes block binding of only VgRBP60/hnRNP I; the other VgRBPs are capable of binding (Figure 1C), yet the RNA cannot localize. In order to examine whether base changes within VM1 might be affecting a site (or sites) elsewhere within the RNA that could instead represent primary binding sites for VgRBP60/hnRNP I, we also tested whether the protein could bind the VM1 sequence directly. Our results using multimerized VM1 sites for in vitro binding (Figure 1D) demonstrate that VM1 is indeed a direct binding site for VgRBP60/hnRNP I, but does this association occur in vivo? Two lines of evidence support an in vivo interaction. First, as discussed above, mutations within the VgRBP60/hnRNP I–binding site VM1 abolish localization in vivo (Figure 1B). Second, during the stage of oogenesis when Vg1 is being transported, Vg1 RNA and VgRBP60/hnRNP I are colocalized within the vegetal cytoplasm (Figure 4E). These data indicate that binding of VgRBP60/hnRNP I to the VM1 motif represents a specific RNA–protein interaction that is necessary for localization of Vg1 RNA.

Roles for hnRNPs in cytoplasmic RNA transport may prove to be a general phenomenon. Recent studies in both mammalian oligodendrocytes and *Drosophila* embryos have implicated hnRNP proteins in cytoplasmic RNA transport processes (Hoek et al., 1998; Lall et al., 1999), and our studies have revealed an key role for VgRBP60/hnRNP I in the cytoplasmic localization of Vg1 RNA. Taken together, these results suggest that hnRNP proteins may perform critical functions in the selective transport of RNAs within cells. In mammalian cells, the hnRNP I and PTB proteins have primarily been implicated in aspects of nuclear RNA biogenesis, including alternative mRNA splicing, transcriptional control, and intranuclear RNA transport (reviewed in Valcarcel and Gebauer, 1997). Certain hnRNPs have been shown to shuttle between the nucleus and cytoplasm, suggesting potential roles in nucleocytoplasmic transport (reviewed in Lee and Silver, 1997; Nakielny and Dreyfuss, 1997).

However, models for hnRNP involvement in nucleocytoplasmic transport have not included persistent binding to the RNA in the cytoplasm to regulate cytoplasmic RNA targeting. Thus, the relationship between VgRBP60 and hnRNP I presents intriguing mechanistic possibilities and suggests a link between cytoplasmic RNA localization and nuclear RNA processing or transport. Could the association of a specific complement of binding proteins with an RNA in the nucleus designate an RNA molecule for cytoplasmic localization? The binding of certain RNA-binding proteins in the nucleus could thus promote the ability of an RNA to find a specific destination in the cytoplasm. We speculate that significant cross-talk can occur between nuclear and cytoplasmic events in RNA biogenesis.

Experimental Procedures

Constructs and Mutagenesis

Mutated VM1 sites within nt 1–85 were engineered by PCR amplification of pSP73-340 (Mowry, 1996) using a primer specific for nt 1–32, containing the desired base changes, and a downstream primer specific for nt 65–85. The VM1 mutant 1–85 PCR fragments were cloned as tandem duplications, either directly into pSP73 (Promega), or downstream of a *Xenopus* β-globin (βG) coding sequence (Krieg and Melton, 1984). 3×VM1 multimer constructs were prepared by cloning oligonucleotides containing either wild-type (top strand, 5′-GATCCATTTCTACATTTCTACATTTCTACAGGCCTG-3′) or mutant (top strand, 5′-GATCCAGTGCGACAGTGCGACAGTGCGACAG GCCTG-3′) VM1 motifs into pSP73.

Microinjection and In Vivo Analysis of Localization

Transcription of chimeric (βG) RNAs, microinjection and culture of stage III oocytes, as well as whole-mount in situ hybridization, RNase protection analyses, and transcription of β-globin specific antisense probes were as described previously (Gautreau et al., 1997).

UV Cross-Linking

UV cross-linking was performed as in Mowry (1996), except that RNA–protein complexes were formed in 15 μl reactions containing 1 ng of ^{32}P-labeled RNA substrate, and 10 μl of oocyte protein (10–50 μg crude S100 extract, 1–10 μg heparin–agarose fractions, or 5–50 ng purified VgRBP60).

Purification and Cloning of VgRBP60

S100 protein extracts were prepared as in Mowry (1996) and applied to a heparin–agarose (BioSepra) column in 25 mM HEPES (pH 7.9), 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, plus inhibitors (0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml trypsin inhibitor, 1 μg/ml antipain; Sigma). After elution by a linear 40–600 mM KCl gradient, fractions enriched for VgRBP60 were pooled, and incubated with 5 mg/ml heparin, 23 mM KCl, 1% glycerol, 1.5 mM ATP, 1 mM $MgCl_2$, 10 mM DTT, 40 μg/ml yeast tRNA, 5.2 mM HEPES (pH 7.9), 0.1 mM EDTA, 0.1 mg/ml glycogen, 0.1 mg/ml BSA, 0.5 mM UTP, and 0.1 mg/ml *E. coli* rRNA. The pool was applied to RNA affinity columns, prepared as in Ruby et al. (1990) by immobilizing biotinylated RNA transcripts on streptavidin agarose (GIBCO/BRL). The columns were washed with buffer A (10% glycerol, 20 mM HEPES [pH 7.9], 1 mM $MgCl_2$, 0.1 mM EDTA) plus 100 mM KCl/1% NP-40, and buffer A plus 200 mM KCl, and eluted with buffer A plus 1 M KCl. Purified VgRBP60 was cut from an SDS-PAGE gel, and the sequences of three tryptic peptides were determined by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

Oligonucleotides 5′-GGAATTCCARCCIGTITAYATHCARTA-3′ and 5′-CGCCGAAAAGAATAAAGAGGCTTTGG-3′, designed from the determined peptide sequences and conserved PTB/hnRNP I cDNA sequences (Gil et al., 1991; Patton et al., 1991; Ghetti et al., 1992; Jansen-Durr et al., 1992) were used to amplify a fragment of 762 nt by PCR from *Xenopus* ovary cDNA. cDNA clones were obtained by screening a *Xenopus* ovary cDNA library (kindly provided by T. Komiya) with a PCR fragment probe. Additional clones were obtained by 5′ RACE (Marathon/Clonetech), using a primer (5′-CCCA TATCATGGTTGTGCAATTCAATCAATG-3′) corresponding to peptide LIELHNHDM. Wisconsin Package 9.1 (GCG) was used for sequence analysis, and database searches were performed using BLAST.

Immunoblotting and Immunocytochemistry

Western blotting was performed as described in Denegre et al. (1997), with detection by enhanced chemiluminescence (Genius 7, Boehringer). Primary antibody was rabbit polyclonal anti-peptide antibody directed against human PTB/hnRNP I (generously provided by D. Black), and peroxidase-conjugated goat anti-rabbit IgG (Sigma) was used as secondary antibody.

Immunocytochemistry was performed as in Denegre et al. (1997). Primary antibody was rabbit polyclonal anti-peptide antibody directed against the C terminus of PTB/hnRNP I (a gift of D. Black), and secondary antibody was AlexaTM 568–conjugated anti-rabbit IgG (Molecular Probes); each was applied at 1:100 dilution. Microscopy was performed on a Zeiss LSM 410 inverted confocal microscope. For combined immunocytochemistry/in situ hybridization, oocytes were processed for whole-mount in situ hybridization as in Gautreau et al. (1997), with an antisense Vg1 RNA probe labeled with BODIPY FL-14-UTP (Molecular Probes). After a final wash in PBS/1% Tween 20, immunocytochemistry followed immediately, as described above.

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