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Conserved and clustered RNA recognition sequences are a critical feature of signals directing RNA localization in *Xenopus* oocytes

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Abstract

Although it is widely regarded that the targeting of RNA molecules to subcellular destinations depends upon the recognition of *cis*-elements found within their 3′ untranslated regions (UTR), relatively little is known about the specific features of these *cis*-sequences that underlie their function. Interaction between specific repeated motifs within the 3′ UTR and RNA-binding proteins has been proposed as a critical step in the localization of Vg1 RNA to the vegetal pole of *Xenopus* oocytes. To understand the relative contributions of repeated localization element (LE) sequences, we used comparative functional analysis of Vg1 LEs from two frog species, *Xenopus laevis* and *Xenopus borealis*. We show that clusters of repeated VM1 and E2 motifs are required for efficient localization. However, groups of either site alone are not sufficient for localization. In addition, we present evidence that the *X. borealis* Vg1 LE is recognized by the same set of RNA-binding proteins as the *X. laevis* Vg1 LE and is capable of productive interactions with the *X. laevis* transport machinery as it is sufficient to direct vegetal localization in *X. laevis* oocytes. These results suggest that clustered sets of *cis*-acting sites within the LE direct vegetal transport through specific interactions with the localization machinery.

Keywords: Vg1; RNP; VgRBP60/hnRNP I; Vg1RBP/vera; RNA-binding protein

1. Introduction

Localization of mRNA molecules to discrete subcellular regions offers a powerful means to generate cell polarity through the asymmetric distributions of specific mRNAs and their protein products (reviewed in Kloc et al., 2002). An increasing number of localized mRNAs have been identified in both somatic cells and oocytes. A well-studied example in somatic cells is the sorting of mRNAs encoding actin isoforms to different subcellular domains, which provides regional functional specification to influence cell motility and morphology (Cheng and Bjerknes, 1989; Hill and Gunning, 1993; Hoock et al., 1991; Kislauskis et al., 1994; Shestakova et al., 2001). The localization of maternal mRNAs in many developing organisms provides the basis for both initial polarities during oogenesis and patterning during embryogenesis. Prominent examples of this phenomenon are found in *Drosophila melanogaster*, where localized mRNAs underlie patterning along both the anterior–posterior and dorsal–ventral axes, and in *Xenopus laevis*, where localized maternal mRNAs generate developmental polarity along the animal–vegetal axis (reviewed in Palacios and St Johnston, 2001). Targeting of mRNA molecules to specific subcellular regions can be regarded as a fundamental mechanism for the spatial regulation of gene expression. Nonetheless, an understanding of the molecular mechanisms governing RNA localization is far from complete.

Studies investigating possible roles for localized mRNA molecules in cell fate determination during *Xenopus* development led to the discovery of Vg1 RNA, which is localized exclusively within the vegetal hemisphere of oocytes and eggs (Rebagliati et al., 1985). Vg1 mRNA is uniformly distributed in early oocytes, becomes localized to the vegetal hemisphere during mid-oogenesis, and is subsequently anchored at the vegetal cortex during late oogenesis where it remains in fully grown oocytes and eggs (Melton, 1987). After fertilization, vegetal blastomeres preferentially inherit Vg1 mRNA and protein (Dale et al.,

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1989; Tannahill and Melton, 1989). Vg1, a member of the transforming growth factor-β (TGF-β) family (Weeks and Melton, 1987), has been implicated in mesoderm induction during embryogenesis (Dale et al., 1993; Joseph and Melton, 1998; Kessler and Melton, 1995; Thomsen and Melton, 1993). Misexpression of the processed Vg1 peptide growth factor in the animal hemisphere leads to the induction of mesodermal cell fates in cells that would normally follow ectodermal lineages (Dale et al., 1993; Thomsen and Melton, 1993), underscoring the importance of regulating transport of this mRNA specifically to the vegetal pole.

Vegetal localization of *X. laevis* Vg1 mRNA is directed by a 340-nucleotide (nt) sequence (VLE) within its 3′ untranslated region (3′ UTR) (Mowry and Melton, 1992). Positioning of *cis*-acting localization elements (LEs) within 3′ UTRs has emerged as a common mechanism for localizing RNAs (reviewed in Jansen, 2001). Detailed analysis of a number of LEs, including those identified for *bicoid* (MacDonald and Kerr, 1997, 1998; Macdonald et al., 1993), *oskar* (Kim-Ha et al., 1993), *orb* (Lantz and Schedl, 1994) and *nanos* (Gavis et al., 1996) mRNAs in *Drosophila*, actin mRNA in fibroblasts (Kislauskis et al., 1994), and Xcat2 (Kloc et al., 2000; Zhou and King, 1996), VegT (Bubunenko et al., 2002; Kwon et al., 2002), and Vg1 (Deshler et al., 1997; Gautreau et al., 1997) mRNAs in *Xenopus*, has revealed multiple, and in some cases redundant, sequence elements that promote localization. In addition to primary sequence, cues for localization may also be provided by secondary structure within the LE. Indeed, conservation of a complex secondary structure has been reported for the *bicoid* LE among several *Drosophila* species (Macdonald, 1990; Seeger and Kaufman, 1990). Additionally, an intermolecular *bicoid* RNA–RNA interaction has been suggested to be critical for localization (Ferrandon et al., 1997). Regardless of whether primary sequence or secondary structure directs localization, information within the LE is recognized by *trans*-acting factors that direct localization.

Several *trans*-acting proteins with potential roles in Vg1 mRNA localization have been identified on the basis of sequence-specific binding to the VLE (Cote et al., 1999; Deshler et al., 1997, 1998; Havin et al., 1998; Kroll et al., 2002; Mowry, 1996; Schwartz et al., 1992; Zhao et al., 2001). Two *trans*-acting factors, Vg1RBP/vera (Deshler et al., 1997, 1998; Havin et al., 1998) and VgRBP60/hnRNP I (Cote et al., 1999), are implicated in Vg1 localization on the basis of their binding to reiterated *cis*-acting sequences in the VLE; base changes within these repeated motifs both abolish in vivo localization and eliminate protein binding in vitro (Cote et al., 1999; Deshler et al., 1997, 1998; Gautreau et al., 1997; Havin et al., 1998). VgRBP60/hnRNP I is a homolog of mammalian hnRNP I and polypyrimidine tract-binding protein (PTB) (Cote et al., 1999), which are spliced isoforms that have been shown to be involved in several aspects of RNA metabolism (reviewed in Valcarcel and Gebauer, 1997). In *Xenopus* oocytes, VgRBP60/hnRNP I colocalizes with Vg1 RNA and binds to VM1 motifs within the VLE (Cote et al., 1999). Vg1RBP/vera binds to the VLE through E2 sites (Deshler et al., 1997, 1998) and is the frog homolog of ZBP1 (Deshler et al., 1998; Havin et al., 1998; Ross et al., 1997), an RNA-binding protein involved in β-actin mRNA localization in fibroblasts. Analysis of *cis*-sequence requirements for the vegetal localization of several mRNAs has implicated either E2 motifs alone (Betley et al., 2002; Kwon et al., 2002) or clusters of VM1 and E2 motifs together (Bubunenko et al., 2002) as signatures of LEs. Thus, controversy exists over the contribution of each of these motifs to a functional RNA LE.

To address this issue, we have compared the *cis*-requirements for vegetal RNA localization of Vg1 RNA from two frog species, *X. laevis* and *Xenopus borealis*. We used mutational analysis to determine a consensus VM1 site for VgRBP60/hnRNP I binding and combined with the previously determined consensus sequence for Vg1RBP/vera binding (Deshler et al., 1998), we were able to analyze more closely the *cis*-sequence requirements for vegetal localization. A *cis*-acting LE within the 3′ UTR of *X. borealis* Vg1 shows a high degree of similarity to that of *X. laevis* Vg1, not only in primary sequence but also in position within the 3′ UTR. Functional conservation of the LEs is indicated as the *X. borealis* Vg1 LE (BVLE) is competent to direct localization in *X. laevis* oocytes and is recognized by the same set of RNA-binding proteins previously implicated in *X. laevis* Vg1 RNA localization. Both VM1 and E2 sites are clustered within the *X. borealis* 3′ UTR but neither VM1 clusters nor E2 clusters alone are sufficient for localization in vivo. Rather, a functional LE is comprised of clusters containing both VM1 and E2 motifs. Supporting the necessity of known *trans*-acting factors for localization, VgRBP60/hnRNP I binds efficiently to VM1 clusters and Vg1RBP/vera binds robustly to E2 clusters. These results suggest that clusters of *cis*-acting sites within the LE, including both VM1 and E2 motifs, are necessary to recruit the *trans*-acting components of the localization machinery and target the RNA for vegetal localization.

2. Results

2.1. The consensus VM1 site for binding of VgRBP60/hnRNP I is YYUCU

Both E2 and VM1 *cis*-acting motifs have been shown to be important for *X. laevis* Vg1 and VegT RNA localization (Bubunenko et al., 2002; Cote et al., 1999; Deshler et al., 1997, 1998; Gautreau et al., 1997; Kwon et al., 2002). It has recently been demonstrated that these sites are not only present but also clustered within the LEs of vegetally localized RNAs (Betley et al., 2002; Bubunenko et al., 2002). Mutations within the E2 motifs have defined the consensus binding requirements for Vg1RBP/vera (Deshler

et al., 1998), allowing identification of putative Vg1RBP/vera binding sites (E2 sites) in LEs through sequence inspection. In contrast to E2 sites, a consensus VM1 motif had not yet been defined. VM1 motifs were originally identified within the VLE as binding sites for VgRBP60/hnRNP I (Cote et al., 1999; Gautreau et al., 1997), but studies in mammalian cells have shown that PTB/hnRNP I binds a variety of pyrimidine-rich sites (Anwar et al., 2000; Perez et al., 1997).

To assess the distribution of VM1 sites within localized RNAs, it was necessary to define the consensus site for *Xenopus* hnRNP I. This was determined by assaying in vitro binding of partially purified VgRBP60/hnRNP I to RNA multimers containing three tandem copies of the VM1 motif (3 × VM1 multimers). The wildtype (WT) VM1 site was originally defined as the hexanucleotide sequence 5′-UUUCUA-3′ (Cote et al., 1999; Gautreau et al., 1997). To evaluate the sequence requirements for VgRBP60/hnRNP I binding to VM1 sites, we created point mutations in each nucleotide position of VM1 and tested in vitro binding by UV crosslinking (Fig. 1). VgRBP60/hnRNP I binds specifically to the WT multimer (Fig. 1A, lanes 1 and 2). Mutation of the U in the second position to a G (U2G) abolishes binding of VgRBP60/hnRNP I (Fig. 1A, lanes 3 and 4). In contrast, mutations of A6U (A in sixth position to a U) and U1C (U in the first position to a C) do not affect VgRBP60/hnRNP I binding (Fig. 1A, lanes 5–8). The results of the mutant analyses are shown in Fig. 1B. At positions 1 and 2, C or U is tolerated, while positions 3, 4, and 5 must be U, C, and U, respectively, and position 6 can be any nucleotide. Thus, the consensus VM1 site is the pentanucleotide 5′-YYUCU-3′.

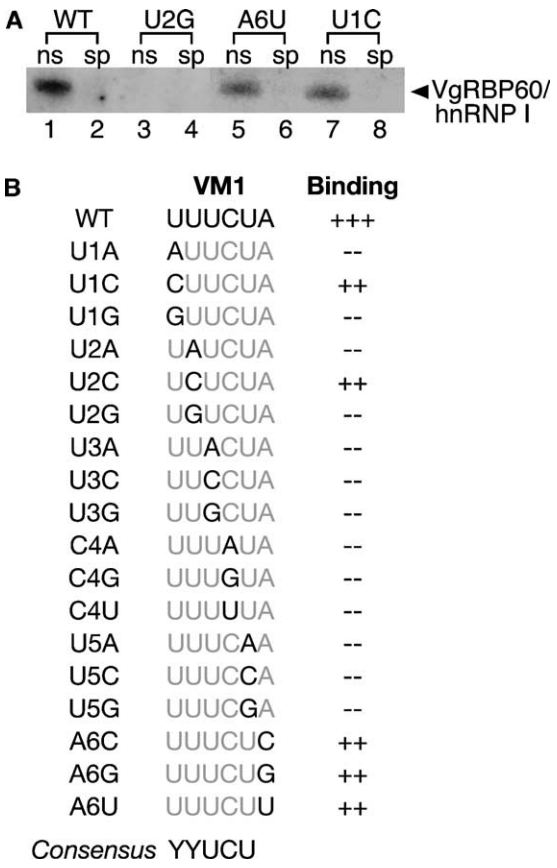

Fig. 1. The consensus VM1 site is 5′-YYUCU-3′. (A) Radiolabeled VM1 RNA multimers were tested by UV crosslinking for the ability to bind in vitro to partially purified VgRBP60/hnRNP I. Specificity of binding was assessed by competition with unlabeled wildtype multimers (sp, even lanes). Shown is in vitro binding of VgRBP60/hnRNP I to wildtype VM1 multimer (WT, lane 1), U2G mutant (lane 3), A6G mutant (lane 5), and U1C mutant (lane 7). ns, unlabeled non-specific competitor. (B) Multimers tested for in vitro binding to VgRBP60/hnRNP I are listed at the left. Each VM1 multimer transcript contained three repeated motifs separated by a two-nucleotide spacer. A single motif for each mutant is shown in gray with the mutated residue in black. The consensus sequence is shown at the bottom (Y = U or C) and the in vitro binding results are summarized at the right. Binding of VgRBP60/hnRNP I to each mutant was scored by comparison to the level of binding to the wildtype multimer (+++, wildtype; ++, significant binding; −−, no binding activity).

2.2. The Vg1 localization element is conserved between X. laevis and X. borealis

To gain insight into how *cis*-acting sequence elements can direct RNA localization, we asked whether localization of Vg1 mRNA might be conserved in other species. Vg1 homologues have been identified in both zebrafish and chicken, but these mRNAs are not localized in a manner similar to that observed in *Xenopus* (Helde and Grunwald, 1993; Selerio et al., 1996; Bally-Cuif et al., 1998). Therefore, we turned to a closer relative of *X. laevis*, the frog *X. borealis*, where we had previously identified a 2.2-kb Vg1 cDNA clone from a *X. borealis* ovarian cDNA library (Accession no. AF041844). The coding region of *X. borealis* Vg1 RNA is 95% identical to that of *X. laevis* and also shares a relatively high degree of conservation with the zebrafish and chick Vg1 RNA homologues, similar to that previously observed for *X. laevis* Vg1 (Helde and Grunwald, 1993; Selerio et al., 1996). However, unlike the relatively short, poorly conserved 3′ UTRs found in both zebrafish and chick Vg1 RNA (Helde and Grunwald, 1993; Selerio et al., 1996), the *X. borealis* Vg1 3′ UTR (Fig. 2A) shares substantial length and sequence identity with the *X. laevis* Vg1 3′ UTR; the 1117-nt *X. borealis* Vg1 3′ UTR is 71% identical to the 1272-nt 3′ UTR of *X. laevis* Vg1 mRNA. These similarities provide a means to assay requirements for VM1 and E2 sites in vegetal localization.

2.3. Clusters of both VM1 and E2 sites are required for localization

Using consensus requirements to identify potential VM1 and E2 motifs, inspection of the 3′ UTR of *X. borealis* Vg1 shows a VM1 cluster, an E2 cluster, and a VM1/E2 cluster (Fig. 2). A single region containing multiple copies of both VM1 and E2 sites is apparent in the 3′ UTR of Vg1 from *X. borealis* (Fig. 2A, box) and corresponds well in position with the *X. laevis* localization element (VLE, bracket).

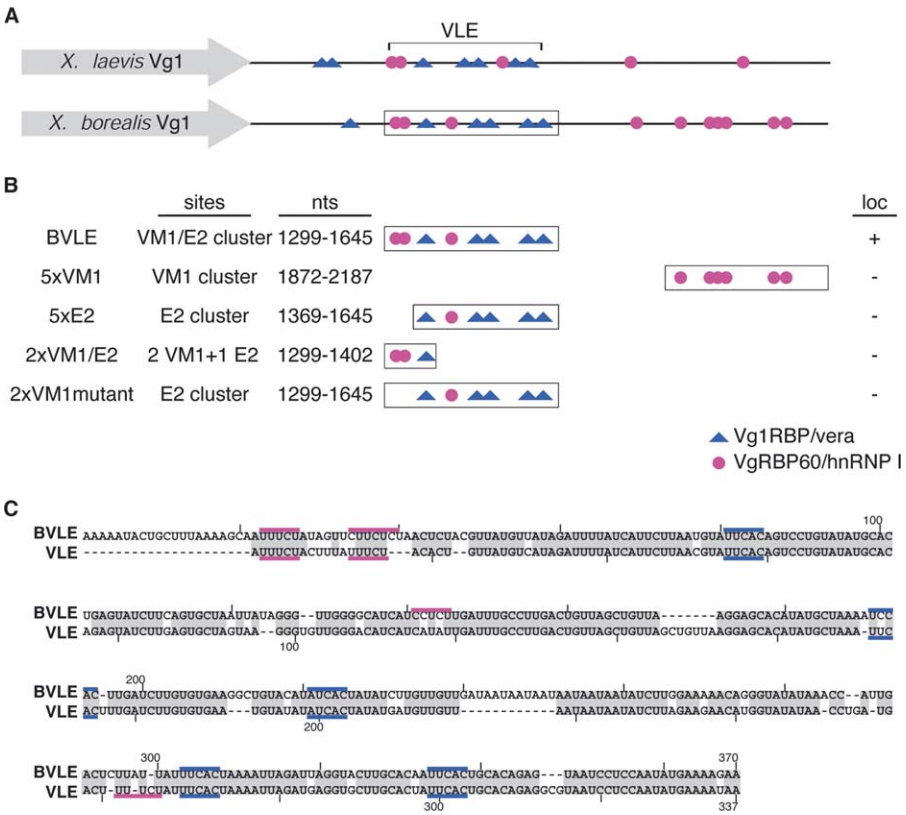

Fig. 2. The Vg1 3′ UTR from *X. borealis* contains clustered *cis*-acting motifs implicated in vegetal RNA localization. (A) A schematic is shown comparing Vg1 RNA from *X. laevis* (top) and *X. borealis* (bottom). Vg1RBP/vera binding sites (E2 motif, WYCAC) are shown as blue triangles and VgRBP60/hnRNP I binding sites (VM1 motif, YYUCU) are depicted as pink circles (Y = U or C, W = U or A). The VLE is indicated by a bracket. Boxed is a cluster VM1 and E2 sites within the 3′ UTR of *X. borealis* Vg1 RNA (BVLE). (B) Schematics of RNA transcripts containing clustered sites are shown: a cluster containing both VM1 and E2 sites (BVLE), 2 VM1 sites plus a single E2 site (2 × VM1/E2), a cluster of 5 E2 sites (5 × E2), a cluster of 5 VM1 motifs (5 × VM1), and the BVLE cluster with two VM1 mutations (2 × VM1mutant). The 3′ UTR nucleotides (nts) contained in each transcript are as indicated. The results of in vivo localization assays (see Fig. 3) are indicated at the right (loc); +, normal localization; −, no localization. (C) Alignment of the Vg1 localization elements from *X. borealis* (BVLE) and *X. laevis* (VLE). Identical residues are shaded in gray; pink and blue lines indicate VM1 and E2 motifs, respectively.

The VM1/E2 cluster in *X. borealis* may represent a functional LE, but additional VM1 and E2 sites are also observed (Fig. 2A). In order to test if RNA localization is mechanistically conserved between the frog species and if clustered motifs can confer localization, we asked whether clusters of VM1 or E2 sites from the *X. borealis* 3′ UTR alone or together were sufficient to direct localization in *X. laevis* oocytes. Synthetic fluorescently labeled RNA transcripts were injected into middle-stage *X. laevis* oocytes and cultured for 2 days, during which time injected Vg1 RNA can be localized to the vegetal hemisphere of the oocyte (Yisraeli and Melton, 1988). Localization of the injected RNA, as summarized in Table 1, was assessed by comparison to an unlocalized negative control RNA (XBM, Fig. 3A), and the *X. laevis* Vg1 LE control (VLE, Fig. 3B). The VM1/E2 cluster (BVLE, Figs. 2B,3C) directed localization in a manner indistinguishable from the *X. laevis* VLE (Fig. 3B). In contrast, neither a VM1 site cluster (5 × VM1, Figs. 2B,3D), nor a cluster of E2 sites (5 × E2, Figs. 2B,3E) are sufficient to direct localization. Although the E2 cluster of the 5 × E2 transcript is not sufficient to localize when injected into oocytes, localization is robust when this cluster is coupled to a pair of VM1 sites (BVLE, Fig. 3B). These VM1 sites are not themselves sufficient for localization, as a transcript containing the pair of VM1 motifs with a single E2 site (2 × VM1/E2) is unable to localize (Figs. 2B,3F). Only the BVLE RNA transcript is sufficient to localize, indicating that the BVLE (*X. borealis* Vg1 localization element), containing clusters of both motifs, represents a functional LE. Alignment of the *X. laevis* Vg1 localization element (VLE) and the BVLE

Table 1
Localization phenotypes of injected transcripts

Transcript	Localization (% oocytes)			*n*
	Normal	Weak	None	
Uninjected	0	0	100	178
XβM	0	0	100	332
VLE	58	0	42	47
BVLE	64	0	36	441
5 × VM1	0	0	100	158
5 × E2	0	9	91	122
2 × VM1/E2	0	0	100	145
2 × VM1mutant	0	10	90	190

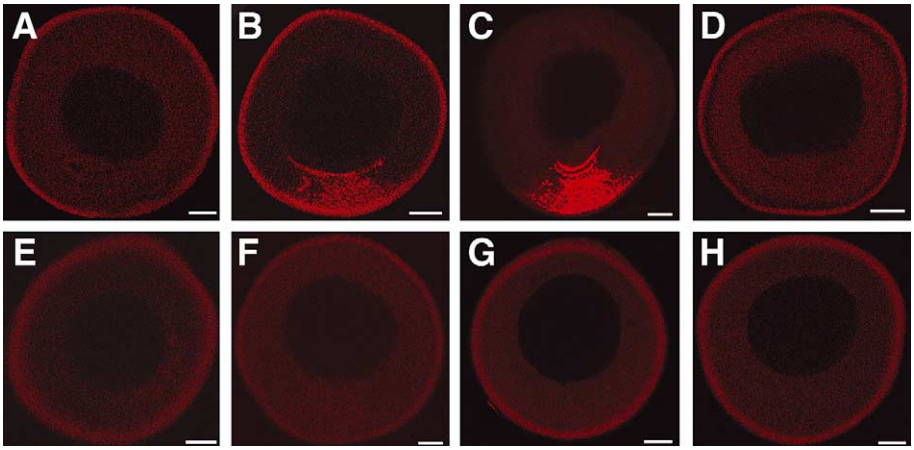

Fig. 3. Clusters of both VM1 and E2 sites are required for localization. *X. laevis* oocytes (stage III/IV) were injected with the following Alexa Fluor-546-labeled RNA transcripts and scored for localization. (A) XβM. (B) VLE. (C) BVLE. (D) 5 × VM1. (E) 5 × E2. (F) 2 × VM1/E2. (G) 2 × VM1mutant. (H) Uninjected. Shown are representative confocal images of injected oocytes with localization of the injected RNA evident as a red wedge in the vegetal cytoplasm. Scale bar = 50 μm.

reveals an overall sequence identity of ~80% (Fig. 2C). Importantly, both the clustering and positioning of the VM1 and E2 sites within the two LEs is highly conserved suggesting a role for the two motifs in vegetal localization. To further test the contribution of the VM1 sites within the BVLE, point mutations were created within the two VM1 motifs at the 5′ end of the BVLE. Strikingly, the 2 × VM1mutant transcript (Fig. 2B) fails to localize when injected into oocytes (Fig. 3G). Thus, point mutations within VM1 motifs abolish wildtype localization, supporting the idea that clusters of both VM1 and E2 sites are necessary for efficient localization.

2.4. Sequence-specific RNA-binding proteins recognize both the X. laevis and X. borealis localization elements

The BVLE can direct vegetal transport in *X. laevis* oocytes suggesting that this sequence is capable of functional interactions with the localization machinery (i.e. *trans*-acting localization factors) of *X. laevis*. To address this issue more directly, we used UV crosslinking analysis to determine if any of the Vg1 RNA-binding proteins (VgRBPs) that bind in a sequence-specific manner to the *X. laevis* Vg1 LE (Cote et al., 1999; Deshler et al., 1997, 1998; Havin et al., 1998; Kroll et al., 2002; Mowry, 1996; Schwartz et al., 1992; Zhao et al., 2001) could also recognize the BVLE RNA sequence. As shown in Fig. 4A, the VgRBPs that crosslink to the BVLE (lane 1) are indistinguishable from those binding to the VLE (lane 3). The VgRBPs, including Vg1RBP/vera and VgRBP60/hnRNP I, bind to both LEs in a sequence-specific manner as they are competed by an excess of unlabeled VLE (Fig. 4A, compare lanes 2 and 4 with lanes 1 and 3). This is in contrast to the ~54–56 kDa doublet, which are non-specific as they are not competable, and the ~31 kDa protein whose binding activity appears to be extract-specific. Moreover, the specific competition by the VLE competitor RNA of the VgRBPs bound to the BVLE RNA (Fig. 4A, lane 2) suggests that the BVLE binds in vitro to the same set of sequence-specific RNA-binding proteins that recognize the *X. laevis* VLE.

Vg1RBP/vera and VgRBP60/hnRNP I have been shown to play roles in Vg1 localization through interactions with E2 sites and VM1 motifs, respectively, within the *X. laevis* VLE (Cote et al., 1999; Deshler et al., 1997, 1998; Gautreau et al., 1997; Kwon et al., 2002). Since clusters of both sites are necessary for localization (Fig. 3) and both proteins bind the LEs (Fig. 4A), we asked whether localization might be correlated with RNA-binding by both Vg1RBP/vera and VgRBP60/hnRNP I. As shown in Fig. 4B, transcripts containing E2 sites (BVLE, 2 × VM1/E2, and 5 × E2) bind to Vg1RBP/vera (odd lanes), this interaction is specific as binding is competed by BVLE RNA (even lanes). As expected, the 5 × VM1 construct, which lacks E2 sites, is not bound by Vg1RBP/vera (data not shown). The binding of Vg1RBP/vera is decreased in 2 × VM1/E2 (Fig. 4B, lane 3), which has only a single E2 site, in comparison with BVLE or 5 × E2, each of which have five E2 sites (Fig. 4B, lanes 1 and 6). Similarly, specific binding of VgRBP60/hnRNP I to the 5 × E2 transcript, which contains a single VM1 site, is weak (data not shown), while robust binding is observed only for transcripts containing multiple VM1 motifs (Fig. 4C; BVLE, 2 × VM1/E2, and 5 × VM1). Notably, only the BVLE shows strong binding to both Vg1RBP/vera and VgRBP60/hnRNP I (Fig. 4B,C), while the other non-localized RNAs exhibit decreased binding of one or both proteins. Thus, in vitro binding of both proteins together, but neither protein alone, correlates with localization. These results suggest that binding of both VgRBP60/hnRNP I and Vg1RBP/vera to clustered VM1 and E2 sites

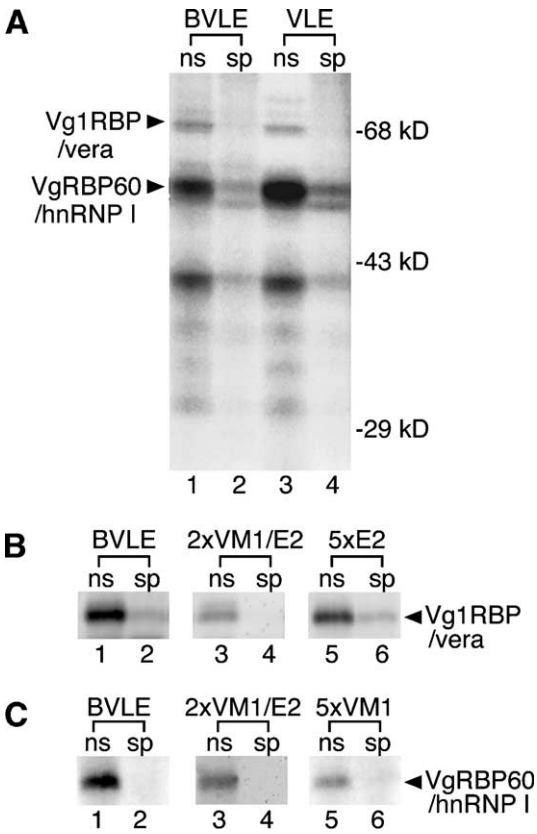

Fig. 4. The BVLE binds to *X. laevis* oocyte factors. (A) Radiolabeled BVLE (lanes 1 and 2) and VLE (lanes 3 and 4) transcripts were tested by UV crosslinking for the ability to be bound in vitro by *X. laevis* oocyte proteins. Specificity of binding was assessed by competition with unlabeled VLE (sp, even lanes) or non-specific competitor RNA (ns, odd lanes). Vg1RBP/vera and VgRBP60/hnRNP I are indicated on the left, and molecular weight markers are shown at the right. (B) Radiolabeled BVLE (lanes 1 and 2), 2 × VM1/E2 (lanes 3 and 4), and 5 × E2 (lanes 5 and 6) transcripts were tested by UV crosslinking for the ability to bind in vitro to partially purified Vg1RBP/vera. Specificity of binding was assessed by competition with unlabeled BVLE (sp, even lanes) or non-specific competitor RNA (ns, odd lanes). (C) Radiolabeled BVLE (lanes 1 and 2), 2 × VM1/E2 (lanes 3 and 4), and 5 × VM1 (lanes 5 and 6) transcripts were tested by UV crosslinking for the ability to bind in vitro to partially purified VgRBP60/hnRNP I. Specificity of binding was assessed by competition with unlabeled BVLE (sp, even lanes) or non-specific competitor RNA (ns, odd lanes).

is required for efficient vegetal localization in *Xenopus* oocytes.

3. Discussion

Transport of specific mRNAs to defined regions within the cell cytoplasm is likely to be initiated by RNA–protein interactions that direct the recognition of the LE and the assembly of a specific RNP transport complex. Previous work using the *X. laevis* Vg1 LE to study the process of cytoplasmic RNA transport in oocytes has defined redundant *cis*-elements required for localization of Vg1 RNA to the vegetal pole (Deshler et al., 1997, 1998; Gautreau et al., 1997; Havin et al., 1998; Kwon et al., 2002; Mowry and Melton, 1992) and has implicated the involvement of certain RNA-binding proteins in this process (Cote et al., 1999; Deshler et al., 1997, 1998; Elisha et al., 1995; Gautreau et al., 1997; Havin et al., 1998; Kroll et al., 2002; Mowry, 1996; Schwartz et al., 1992; Zhao et al., 2001). While this work has provided a foundation for understanding RNA localization, the specific RNP interactions necessary for localization are not yet understood. We have gained insight into two important RNA–protein interactions within the Vg1 RNP complex through comparative functional analysis of Vg1 localization elements from two frog species, *X. laevis* and *X. borealis*. A positionally and functionally conserved *cis*-acting LE within the 3′ UTR of *X. borealis* Vg1 (BVLE) is competent to direct vegetal localization in *X. laevis* oocytes (Fig. 2). The BVLE localizes to the vegetal hemisphere in a manner resembling the *X. laevis* VLE (Fig. 3) and binds both Vg1RBP/vera and VgRBP60/hnRNP I (Fig. 4), supporting potential roles for these proteins in Vg1 localization. But is it surprising that this sequence can functionally interact with the *X. laevis* localization machinery? Vegetal localization of a heterologous RNA in *Xenopus* has been observed with a fragment from the 3′ UTR of tau mRNA which is normally localized to the axonal hillock in neuronal cells (Litman et al., 1996). In addition, the primary sequence of the Vg1 LE is highly conserved between *X. laevis* and *X. borealis*. However, careful comparison of the sequences reveals significant differences within the UTR. In particular, differences are apparent in the distribution of two reiterated sequence motifs, VM1 and E2, implicated in LE function.

In this work, we demonstrate that a functional LE contains both VM1 and E2 sites and that clusters of neither site alone are sufficient for vegetal transport (Figs. 2,3, Table 1). A computational analysis of localized RNAs and ESTs from *Xenopus* revealed an enrichment of CAC-containing motifs within LEs (Betley et al., 2002); notably, the consensus E2 site is a CAC-motif (Deshler et al., 1998). This evidence supports previous findings that binding of Vg1RBP/vera to E2 sites is required for vegetal localization (Bubunenko et al., 2002; Deshler et al., 1997, 1998; Kwon et al., 2002). VgRBP60/hnRNP I binding to VM1 motifs has also been implicated in both Vg1 and VegT localization (Bubunenko et al., 2002; Cote et al., 1999; Gautreau et al., 1997). To determine the distribution of VM1 sites within LEs, we defined the consensus VM1 motif (Fig. 1). The consensus motif for VgRBP60/hnRNP I binding corresponds well with the pyrimidine-rich motifs bound by the mammalian homolog, PTB/hnRNP I (Anwar et al., 2000; Perez et al., 1997). Inspection of the UTRs of vegetally localized RNAs shows an enrichment of VM1 motifs within the LEs, and it was previously been suggested that clusters of VM1 and E2 sites are necessary for localization (Bubunenko et al., 2002). However, clusters of each site lacking the other had not been tested. A recent study (Kwon et al., 2002) suggested that E2 sites alone could be sufficient for localization, as two transcripts lacking consensus VM1

motifs were reported to be capable of localization. The transcripts tested were a mutant Vg1 LE lacking the three consensus VM1 motifs and antisense VegT LE in which the reverse E2 sites were mutated to sense orientation. By contrast, we find that E2 sites are important but not sufficient to direct localization, as the cluster of E2 sites from *X. borealis* Vg1 RNA (5 × E2, Figs. 2,3) fails to localize in most cases (~90%) and localizes weakly in some cases (~9%). The discrepancy between our results and those of Kwon et al. (2002) could represent differences in methods of assaying or scoring localization. Alternatively, pyrimidine-rich sites within the antisense VegT LE could represent binding sites for VgRBP60/hnRNP I, serving as non-consensus VM1 motifs. In support of the latter, we have found that VgRBP60/hnRNP I is capable of binding to both a 3 × VM1 mutant version of the Vg1 LE and the antisense VegT LE (Kress and Mowry, unpublished data). Moreover, the introduced E2 sites within the antisense VegT LE are in close proximity to potential non-consensus VM1 sites, consistent with a model in which clustering of both VM1 and E2 sites is an important feature of LEs directing vegetal localization.

It is possible that VM1 and E2 sites are clustered within the Vg1 LE to promote interactions between VgRBP60/hnRNP I and Vg1RBP/vera that are critical for targeting the RNA for localization. Interestingly, VgRBP60/hnRNP I and Vg1RBP/vera interact not only with Vg1 mRNA but also with one another, in both the nucleus and the cytoplasm (Kress and Mowry, submitted). The in vivo localization data (Fig. 3) correlate well with in vitro binding analyses (Fig. 4B,C) to suggest that interactions between both VgRBP60/hnRNP I and Vg1RBP/vera and their cognate binding sites are required for localization. RNAs containing clustered VM1 motifs bind VgRBP60/hnRNP I but do not bind Vg1RBP/vera, and fail to localize. In contrast, RNAs containing clustered E2 sites bind Vg1RBP/vera but not VgRBP60/hnRNP I, and also fail to localize. While binding by both VgRBP60/hnRNP I and Vg1RBP/vera is necessary for vegetal transport, these factors are not sufficient to direct localization. Both proteins bind the 2 × VM1/E2 transcript (Fig. 4B,C) but this RNA fails to localize. One possible explanation for this result is that the 2 × VM1/E2 transcript may lack *cis*-acting sites for other VgRBPs (Kroll et al., 2002; Mowry, 1996; Zhao et al., 2001). Binding of other *trans*-acting factors may be required for vegetal localization; thus, clustering of VM1 and E2 sites alone may not be sufficient for localization. Alternatively, the single E2 site in 2 × VM1/E2 may be able to bind Vg1RBP/vera in vitro, but may fail to recruit the factor in vivo. This would suggest that clusters of multiple VM1 and E2 sites may be required for RNAs to localize efficiently.

Our evidence supports a view of RNA localization in which conserved and clustered *cis*-sequence motifs within the 3′ UTR of a given RNA may provide the recognition or binding site(s) for critical *trans*-acting factors. The roles of *trans*-acting factors involved in localization may be quite diverse, ranging from recognizing sequences or structures within the LE that demarcate a particular RNA for localization to mediating interactions with cytoskeletal components during later steps of the localization process. The LE from *X. borealis* Vg1 mRNA contains a cluster of both VM1 and E2 sites that are necessary for localization. The clustered *cis*-acting motifs within the BVLE must act in concert with one another as neither VM1 nor E2 clusters alone are sufficient for localization and could provide a spatial means to promote VgRBP60/hnRNP I–Vg1RBP/vera interactions within the LE. We suggest that higher order RNA–protein interactions contribute to the recognition and transport of an RNA to its subcellular destination. The complement of bound factors and their interactions within an RNP complex may confer specificity such that a subset of RNAs destined for localization can be recognized from amongst the many non-localized RNAs for transport to their proper destinations.

4. Experimental procedures

4.1. Cloning, sequencing, and template preparation

A Vg1 cDNA was isolated from a *X. borealis* ovary λZap II cDNA library (a generous gift of D. Brown) by screening with the *X. laevis* Vg1 coding region (Weeks and Melton, 1987). The nucleotide sequence was previously deposited in GenBank (Accession no. AF041844). Sequence alignments were generated using MacVector 6.5.3 (Genetics Computer Group, Madison, WI). A set of 3′ UTR fragments were prepared by PCR amplification as follows: BVLE (nts 1299–1645), 5 × E2 (nts 1369–1645), and 5 × VM1 (nts 1872–2187). The 2 × VM1mutant was created by site-directed PCR mutagenesis, mutating the VM1 motifs at nts 1322–1326 from 5′-UUUCU to 5′-AUACA and nts 1335–1339 from 5′-UCUCU to 5′-ACACA. Each fragment was cloned into pSP73 (Promega), generating pSP73-BVLE, pSP73-5 × E2, and pSP73-5 × VM1, respectively. The template for 2 × VM1/E2 was prepared by digesting pSP73-BVLE with *Dde* I, yielding a transcription product containing nts 1299–1402.

4.2. Definition and analysis of repeated motifs

Point mutations in the 3 × VM1 site multimer (pSP73-3 × VM1WT) (Cote et al., 1999) were created using PCR site-directed mutagenesis such that one nucleotide per VM1 site in all three sites was changed (see Fig. 1B). The PCR products were cloned into pSP73 (Promega). The wildtype and mutant VM1 multimers were assayed for in vitro binding of VgRBP60/hnRNP I by UV crosslinking analysis. The consensus VM1 (YYUCU, Y = U or C) and E2 (WYCAC, W = U or A) (Deshler et al., 1998) sites were used as subsequences in MacVector 6.5.3 (Genetics

Computer Group, Madison, WI) to search the 3′ UTRs of *X. laevis* and *X. borealis*.

4.3. Preparation of RNA transcripts

For microinjection, RNA was transcribed from *X. borealis* 3′ UTR plasmids, pSP73-vg340 (Mowry, 1996), and pSP64-XβM (Krieg and Melton, 1984) in reactions containing 1 × transcription buffer [40 mM Tris–HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermadine, 10 mM DTT, 40 units RNasin RNase inhibitor (Promega)], 0.5 mM each of CTP and ATP, 0.45 mM UTP, 1 mM diguanosine triphosphate, 0.1 mM GTP, 1 μCi of [α-^{32}P]UTP (800 Ci/mmole; DuPont/NEN), and 50 μM Alexa Fluor 546-14-UTP (Molecular Probes). Probes for UV crosslinking were transcribed in reactions containing 1 × transcription buffer, 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/mmole; DuPont/NEN). Sequence-specific competitor RNAs were synthesized from linearized pSP73-vg340 and pSP73-BVLE using the MEGAscript kit (Ambion) as per the manufacturer's protocol. *E. coli* rRNA used as a non-specific competitor was a generous gift from A. Dahlberg.

4.4. Microinjection and in vivo localization assay

Microinjections were performed as described previously (Gautreau et al., 1997). Briefly, stage III–IV oocytes were removed surgically from albino *X. laevis* female frogs (Nasco). The oocytes were microinjected with ~3 nl of 10 ng/μl capped in vitro transcribed RNA and cultured for 2 days (Wallace and Misulovin, 1978), followed by fixation in MEMFA (Harland, 1991) and storage in 100% methanol at −20 °C. For microscopy, oocytes were cleared in 2:1 benzyl benzoate/benzyl alcohol. Confocal images were obtained using a Zeiss LSM 410 Inverted Confocal Microscope.

4.5. In vitro binding assays

Preparation of oocyte S100 extracts was performed as described (Mowry, 1996). Fractionation of oocyte lysate by heparin agarose chromatography was performed as in Cote et al. (1999); VgRBP60/hnRNP I was contained in eluates between ~350 and 380 mM KCl and Vg1RBP/vera was eluted at ~430–450 mM KCl. For in vitro binding, 10 μl reactions containing 5 mg/ml heparin, 1% glycerol, 50 mM KCl, 10 mM DTT, 5.2 mM Hepes (pH 7.9), 1.5 mM ATP, 1–5 mM $MgCl_2$, 0.1 mM EDTA, 40 μg/ml yeast tRNA, and either 10 μg *X. laevis* oocyte S100 lysate or ~4 ng partially purified VgRBP60/hnRNP I or Vg1RBP/vera were pre-incubated for 10 min at 25 °C in the presence of 600 ng of unlabeled competitor RNA followed by the addition of 1 ng ^{32}P-labeled RNA transcript and a 10 min incubation. The binding reactions were crosslinked for 10 min in a Stratalinker (Stratagene) and subsequently treated with RNase A (1 mg/ml, Sigma) for 15 min at 37 °C. The crosslinked proteins were separated by SDS-PAGE and visualized by autoradiography.

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