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A Consensus RNA Signal That Directs Germ Layer Determinants to the Vegetal Cortex of *Xenopus* Oocytes

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RNA localization is an important mechanism for generating cellular diversity and polarity in the early embryo. In *Xenopus*, the correct localization of the RNA encoding the T-box transcription factor VegT is essential for the correct spatial organization and identity of endoderm and mesoderm. Although localization signals in the 3′ UTR have been identified for many localized RNAs, insight into what constitutes an RNA localization signal remains elusive. To investigate possible common features between signals that direct different RNAs to the same subcellular region, we carried out a detailed analysis of the uncharacterized *VegT* RNA localization signal and compared it with the well-studied *Vg1* localization signal. Both RNAs localize to the vegetal cortex during the same period of oogenesis. Our results suggest a common RNA localization signal at the level of clustered redundant protein-binding motifs and *trans*-acting factors. We propose that what characterizes RNA localization signals in general is not the nucleotide sequence or secondary structure per se, but the critical clustering of specific redundant protein-binding motifs.

Key Words: **RNA localization; 3′ UTR localization signal; *VegT* transcription factor; *Xenopus* oogenesis; maternal determinant; cellular polarity.**

INTRODUCTION

One strategy conserved between *Xenopus* and *Drosophila* for initiating cell diversity and pattern in the embryo is the localization of specific RNAs to opposite poles of the oocyte (reviewed in Jansen, 2001; Kloc *et al.*, 2002; King *et al.*, 1999; Mowry and Cote, 1999). These localized RNAs become inherited by a restricted population of embryonic cells and trigger regional differences in gene expression and fate. In *Xenopus*, the maternal RNAs *Vg1* and *VegT* are localized to the oocyte vegetal cortex. *Vg1* is a member of the TGF-β family of growth factors (Weeks and Melton, 1987), and current evidence supports roles for it in dorsal mesoderm induction, endoderm specification (Joseph and Melton, 1998), and establishing right/left asymmetry (Hyatt and Yost, 1998). *VegT* (also known as Antipodean, Xombi, Brat and, XTbx6r; referenced in Zhang *et al.*, 1998) encodes a T-box transcription factor. Antisense depletion of maternal *VegT* RNA revealed that it is essential for primary germ layer formation as both endoderm and mesoderm are reduced or eliminated in such embryos (Zhang *et al.*, 1998; Kofron *et al.*, 1999). Ectopic expression of either *Vg1* (Dale *et al.*, 1993; Thomsen and Melton, 1993) or *VegT* (Zhang and King, 1996) results in abnormal embryos, underscoring the importance of RNA localization in *Xenopus* development.

During *Xenopus* oogenesis, RNAs are localized to the vegetal cortex by at least two distinct pathways (Forristall *et al.*, 1995; Kloc and Etkin, 1995). During the early pathway, specific RNAs first concentrate into the mitochondrial cloud, which later moves to the vegetal cortex. The mitochondrial cloud is the site of germ plasm formation (Heasman *et al.*, 1984), and one likely purpose of the early pathway is to partition RNAs encoding germ cell determinants into the germ cell lineage (King *et al.*, 1999). The second or late pathway is initiated slightly later in oogen-

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esis and was defined by *in situ* hybridization studies on *Vg1* RNA. Initially, *Vg1* is uniformly distributed in stage I/II oocytes and is localized during midoogenesis to a large domain in the vegetal cortex (Melton, 1987; Forristall *et al.*, 1995; Kloc and Etkin, 1995). *Vg1* localization appears to require intact microtubules for efficient transport to the vegetal cortex (Yisraeli *et al.*, 1990) and may also interact with a vegetal subcompartment of the endoplasmic reticulum (ER) (Deshler *et al.*, 1997). Anchoring to the cortex remains the least understood step in localization, although microfilaments and intermediate filaments have been implicated (Alarcon and Elinson, 2001; Forristall *et al.*, 1995; Yisraeli and Melton, 1990). Recently, additional RNAs, such as *VegT*, *β* TrCP-2/3, and *xBic-C* RNAs (Hudson *et al.*, 1996; Wessely and De Robertis, 2000; Zhang and King, 1996), have been described that also localize to the vegetal cortex during this time period and are thought to follow the same pathway. This common pathway could function to localize RNAs involved in somatic cell differentiation to the vegetal cortex. An important, yet unresolved, issue is what specific features define the RNA localization signal for this critical pathway. One way to address this question is by careful examination of both the *cis*- and *trans*-acting factors required for localizing these RNAs to the vegetal cortex.

Analysis of deletion mutants has revealed *cis*-acting sequences both required and sufficient for RNA localization in a variety of organisms, including *Drosophila* (reviewed in Jansen 2001) and *Xenopus* (Mowry and Melton, 1992; Chan *et al.*, 1999; Kloc *et al.*, 2000; Zhou and King, 1996). Virtually all such localization signals (LS) have been found to reside in the 3′ UTR. Some LS, including those for *bicoid* (Macdonald *et al.*, 1993), *oskar* (Kim-Ha *et al.*, 1993), *orb* (Lantz and Schedl, 1994), and *Vg1* (Gautreau *et al.*, 1997), have proven to be surprisingly large and complex, displaying functional redundancy. Fine deletion mapping revealed that the *bicoid* and *oskar* LS contain subelements that mediate distinct steps in their localization pathways (Macdonald *et al.*, 1993; Macdonald and Kerr, 1997; Kim-Ha *et al.*, 1993). Multiple elements in *Drosophila nanos* RNA (Gavis *et al.*, 1996), mammalian *β-actin* RNA (Kislauskis *et al.*, 1994; Bassell *et al.*, 1998), and *myelin basic protein* RNA (Ainger *et al.*, 1997; Hoek *et al.*, 1998) have been found to play roles in transport and, in some cases, translational regulation as well.

For *Vg1* RNA, deletion analysis identified a 340-nt LS, contained within the 3′ UTR, that was sufficient to direct localization to the vegetal cortex (Mowry and Melton, 1992). Detailed analysis has revealed two redundant subelements located at either end of the signal, and various combinations of these subelements are sufficient for vegetal localization (Gautreau *et al.*, 1997). The subelements found in the *Vg1* 340-nt LS appear to act in concert for localization, rather than mediating distinct steps in the pathway (Gautreau *et al.*, 1997). Small repeated motifs, 5- to 9-nt in length, found within these subelements as well as elsewhere in the LS are important for localization (Deshler *et al.*, 1997; Gautreau *et al.*, 1997). However, simple deletion of any single one of these motifs does not affect localization, reflecting the inherent redundancy of the signal and suggesting a requirement for some critical number or combination of motifs.

Certain small motifs described in the *Vg1* LS have been found to be binding sites for *trans*-acting protein factors. *In vitro* and *in vivo* UV cross-linking analyses revealed six proteins that specifically bound to the *Vg1* LS (Mowry, 1996). One of these proteins, Vg1RBP or Vera, binds the E2 motif (UUCAC and related sequences) required for *Vg1* localization (Schwartz *et al.*, 1992; Elisha *et al.*, 1995; Deshler *et al.*, 1997, 1998; Havin *et al.*, 1998). Vg1RBP/Vera protein is homologous to the zipcode-binding protein (ZBP), which is required for the localization of *β-actin* RNA in chicken fibroblasts (Ross *et al.*, 1997). Another motif, VM1 (UUUCUA and related sequences) was also found to be critical for *Vg1* localization (Gautreau *et al.*, 1997) and is the binding site for hnRNP I (VgRBP60) (Cote *et al.*, 1999). These studies suggest that both somatic cells and germ cells may use the same *trans*-acting protein factors and/or related *cis*-signals. However, importantly, no consensus signal for RNA localization has emerged from these studies either at the nucleotide level or at the level of secondary structures.

To examine the question of a consensus RNA LS more carefully, we have undertaken a comparison between LS with potentially similar functions in different RNAs. This comparison of LS for *VegT* and *Vg1* RNAs should reveal sequences important for vegetal transport in the late pathway. In this work, we have identified an LS within the *VegT* 3′ UTR that is both necessary and sufficient for *VegT* localization. The *VegT* LS has a complex structure, exhibiting a high level of redundancy and consists of two terminal core localization elements with internal regions functionally redundant to the core elements. Each element contains a Vg1RBP/Vera-binding site (E2), along with hnRNP I-binding sites (VM1). Most importantly, we show that, although there is very limited sequence similarity between the two signals, virtually the same proteins, including *Vg1*-RBP/Vera and hnRNP I, specifically bind the *Vg1* and *VegT* LS. Our results suggest a common RNA LS at the level of clustered redundant protein binding motifs and *trans*-acting factors. We propose that what characterizes RNA LS in general is not the nucleotide sequence or secondary structure per se, but the critical clustering of specific redundant protein-binding motifs, which may act to recruit a core set of *trans*-acting protein factors.

MATERIALS AND METHODS

Construction of Mutants Tested for Localization

Initial mapping of the *VegT* LS was done by digesting the *VegT* 3′ UTR with rare cutting restriction enzymes (*Pvu*II, *Sma*I, *Ssp*I, *Xba*I, and *Mun*I). A pSPORT*β*-G construct was made by introducing a 325-nt fragment of the *β-globin* ORF (*Hin*dIII–*Bam*HI frag-

FIG. 1. Schematic representation of RNA transcripts analyzed for localization. Each transcript was tagged with β-globin, as shown for β-globin/3′ UTR construct. (A) Initial mapping of *VegT* 3′ UTR using restriction enzymes. Transcripts are named according to their size and position in the 3′ UTR. (B) Defining the borders of *VegT* LS. Deletions in the 370-nt LS are named from A′ to G, where A′ includes the region 1708–1778; A, 1779–1812; B, 1810–1858; C, 1848–1888; D, 1889–1928; E, 1937–1990; F, 2004–2036; and G, 2043–2078. Numbers refer to the cDNA sequences beginning with the start codon. (C) Mapping the 5′ and 3′ SEs. Abbreviations used are the same as in (B). Heavy black line indicates region deleted. Thin gray lines are included as visual aids. Localization is scored as ++ (completely normal, yellow), +/− (significantly impaired, orange), and − (no detectable localization, red). (*) indicates a severely impaired mutant.

ment of pSP73Xβ G5′; Mowry and Melton, 1992) into the pSPORT1 vector. Each restriction fragment within the *VegT* 3′ UTR was subcloned into the appropriate sites downstream of the pSPORTβ-G vector. The pUM54Ssp construct, which contains a 370-nt fragment sufficient for localization, was subsequently used to make finer 30- to 40-nt deletions as shown in Fig. 1. Deletions were made by PCR amplification of two DNA fragments covering the entire 370-nt fragment, excluding the site of deletion, and the fragments were subcloned back into the vector. Conditions for PCR were essentially as described by MacArthur *et al.* (1999). Briefly, each PCR fragment was synthesized by using the universal primers complimentary to the vector sequences containing a unique restriction site, and internal primers (forward primer specific to 5′ UTR of β-globin: CTCAGAATTCACGCTCAACTTTG at the *Eco*RI site; reverse primer specific to the 1394–1372 region of the *VegT* 3′ UTR: ACCCCAAGCTTTTGGAGTCAATTGT at the *Hin*dIII site) flanking the site of deletion that contained an introduced *Eco*RV site. A pair of fragments corresponding to each one of the internal deletions was subcloned together into the pSPORT1 vector. In some cases, prior to subcloning, PCR fragments were additionally digested with restriction enzymes to produce new and/or larger deletions within the 370-nt 3′ UTR fragment. In this case, the internal ends of the fragments were blunted when necessary, to ligate both fragments together. Each deletion was confirmed by sequence analysis.

RNA Synthesis

For injections, RNAs were synthesized from the linearized plasmids by using T7 RNA polymerase. The pSP73XβG5′ plasmid (Mowry and Melton, 1992) was linearized with *Hin*dIII and transcribed with SP6 RNA polymerase to make the antisense probe. For whole-mount *in situ* hybridizations, DIG-labeled, antisense *β-globin* probe was synthesized according to the procedure of Harland (1991). RNA substrates for RNA-binding assays were transcribed from the *VegT* plasmids 300, 300ΔAB, 300ΔABC, and sE2BG (E2 substitutions),

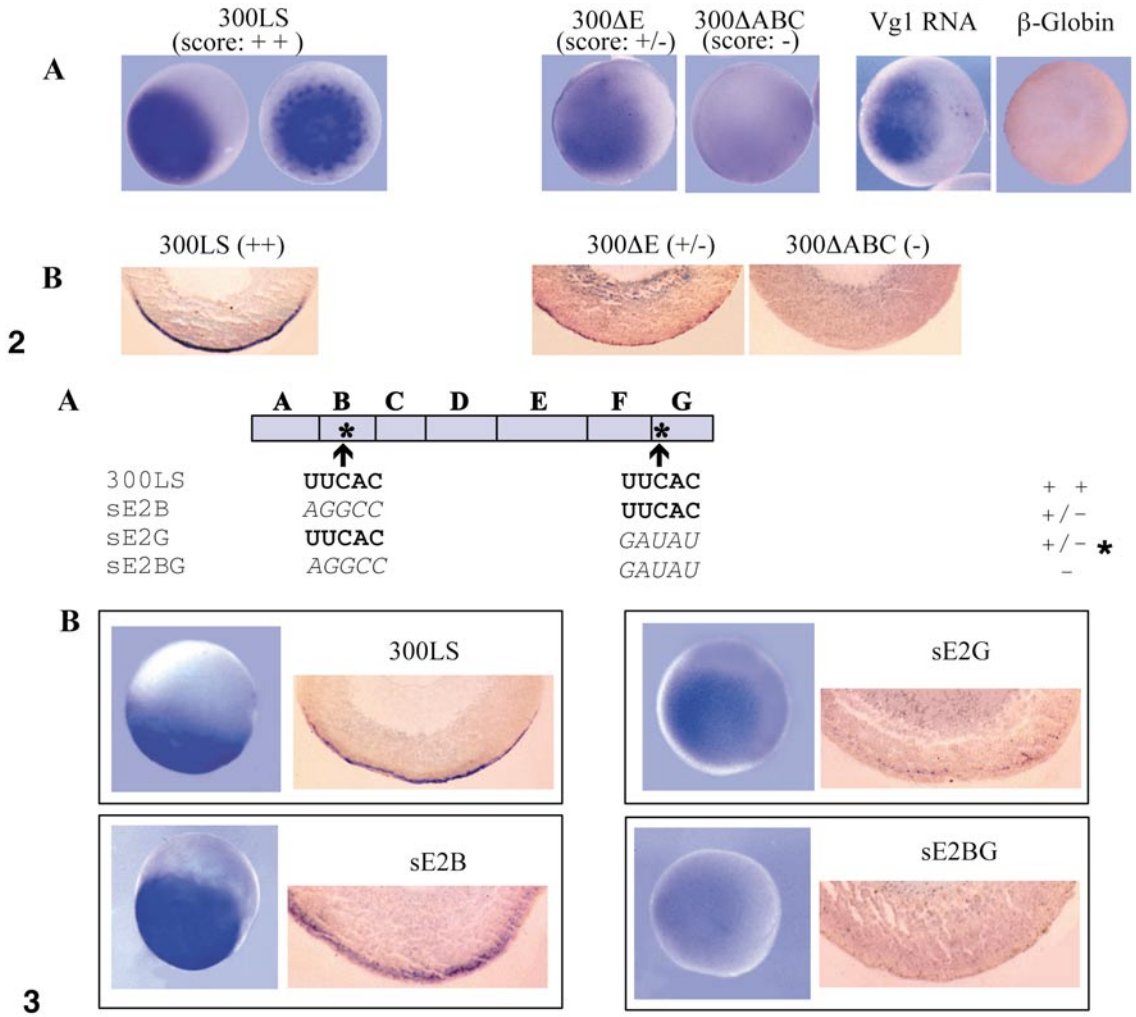

FIG. 2. Localization phenotypes of injected *VegT* mutant transcripts. (A) Vegetal views of injected oocytes assayed by whole-mount *in situ* hybridization showing different degrees of localization. The efficiency of localization is scored as ++ (completely normal), +/− (significantly impaired), and − (no detectable localization). (B) Sections of whole mounts showing internal distribution of injected transcripts for each localization phenotype.

FIG. 3. E2 RNA motif is a component of the *VegT* LS. (A) Diagram of VegT transcripts designed to test the role of UUCAC sequences in *VegT* localization. Original UUCAC sequences are shown in bold, and their positions within the 300-nt LS are indicated by asterisks. Substitutions are italicized. Localization efficiency is scored as described in Fig. 2A. (*) indicates a severely impaired mutant. (B) Localization phenotypes of injected *VegT* mutant transcripts bearing the substitutions diagrammed in (A). Localization was assayed by whole-mount *in situ* hybridization. Corresponding sections of these oocytes are shown on the right.

as well as the *Vg1* plasmid pSP73–340 (Mowry, 1996). Sequence-specific competitor RNA for RNA-binding assays was transcribed from pSP73–340 by using the Megascript *in vitro* transcription kit (Ambion). Nonspecific competitor RNA was either *Xenopus β-globin* or *Escherichia coli* rRNA. Each produced similar results and were used interchangeably.

Microinjection and Culturing of Oocytes

Late stage III–early IV oocytes were manually isolated from ovaries of juvenile *Xenopus laevis* (4–6 cm from *Xenopus* Express), defolliculated, and injected with 6 nl of RNA (100–150 pg) dissolved in water. All oocyte manipulations were done in sterile OR2 supplemented with antibiotics (0.5% streptomycin and penicillin, 10 μg/ml gentamicin). Injected oocytes were cultured in Leibowitz medium as previously described (Wallace *et al.*, 1980; Yisraeli and Melton, 1988).

Whole-Mount in Situ Hybridization and Histology

Whole-mount *in situ* hybridization and histology were performed as previously described (Harland 1991; Houston and King,

TABLE 1
Stability *in Ovo* of RNA Transcripts Showing Negative Phenotype

VegT RNA	RNA stability (% injected RNA recovered after 4 days of culture)
300-LS	98
5′-360	42
3′-500	97
300ΔC	61.4
300ΔABC	55
300ΔCDE	69.5
300ΔEFG	78.3
300ΔAB,FG	59
300ΔAB,G	48
300ΔAB,E	97
300ΔC,FG	40
300ΔD,FG	89
sE2BG	61

2000). Normally, pigmented oocytes were bleached overnight at room temperature in 70% methanol, 10% H_2O_2 under a strong light.

Scoring Localization Phenotypes

After bleaching, the oocytes were scored according to the criteria shown in Fig. 2. In each experiment, a positive (*VegT* 300-nt or *Vg1* 340-nt) and a negative control (*β-globin*) were included and defined these two scores. Three categories were used: ++, clear and sharp localized disc of RNA at the vegetal pole; +/−, impaired localization showing a distinctly weaker and more diffuse signal at the vegetal pole; and −, consistently showing no staining or staining throughout the oocyte. The +/− score includes the range of impaired localizations that we observed. Oocytes were photographed and stored in 100% methanol at –20°C. Histological sections were examined to confirm the scoring. Each transcript was tested for localization by injecting 30–35 oocytes per trial. Experiments were repeated at least 3 times with different donor frogs.

RNA-Binding Assays

UV crosslinking assays were performed as in Mowry (1996), with modifications from Cote *et al.* (1999). RNA-binding reactions contained 1 ng of ^{32}P-labeled substrate RNA, 600 ng of unlabeled competitor RNA, and either S100 extract (10 μg) or heparin-agarose fractionated S100 extract (~4 μg). *Xenopus* oocyte S100 protein extracts were prepared as in Mowry (1996), and heparin–agarose fractionation was performed as in Cote *et al.* (1999). Prior to use in RNA-binding assays, the VgRBP60- and Vg1RBP/Vera-enriched heparin–agarose column fractions were exchanged into 25 mM Hepes (pH 7.9), 40 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.5 mM PMSF.

RNase Protection Assay for RNA Stability

Oocytes scored negative for *VegT* localization were frozen either immediately after injection (zero time point) or after 4 days of culture. The proportion of each injected transcript recovered after 4 days of culture was determined by RNase protection (Ribonuclease Protection Assay, RPAIII kit from Ambion) and quantitated by using a PhosphorImager (Molecular Dynamics). Oocytes injected with the 300-nt LS transcript were used as positive controls for stability and to estimate the efficiency of RNA recovery.

RESULTS

In order to understand what constitutes an RNA LS, our first step was to identify and characterize the LS for *VegT* RNA. For this, a series of mutant derivatives were tagged upstream with a 325-nt coding region of *Xenopus β-globin*, allowing us to monitor localization of each RNA *in vivo* after injection into stage III/IV oocytes. The degree of localization was assayed by whole-mount *in situ* hybridization, followed by analysis in sectioned preparations to characterize the localization phenotype in more detail for each mutant. Localization was scored as normal (++), significantly impaired (+/−), or completely defective (−) (see Materials and Methods; Figs. 1 and 2).

Large Deletion Analysis Identifies 525-nt Localization Domain

The 1280-nt 3′ UTR of *VegT* was found to direct localization to the vegetal cortex in oocytes after injection (Fig. 1A). In order to identify the minimal LS for *VegT*, a series of deletions were created by using well-placed unique restriction sites along the full-length 3′ UTR. While regions at the 5′ and 3′ ends of the 3′ UTR (5′-360 and 3′-500, respectively) did not support localization, an LS is contained within a 525-nt fragment located 360 nt downstream from the stop codon (Fig. 1A). To test whether negative localization phenotypes observed could reflect the instability of the mutant transcripts being tested, we determined the relative amount of the injected RNA over the culture period. Although minor differences in transcript stability could be detected by RNase protection, such differences could not account for the observed localization defects (Table 1). The 525-nt LS was sufficient to localize *VegT* to the vegetal pole (Fig. 1A), and the degree of localization was comparable to or even stronger than the full-length 3′ UTR.

Fine Deletion Analysis Defines the Borders of the LS and Identifies Two Critical Regions

To define critical sequences within the 525-nt LS, successive deletions were tested, both from the 5′ and 3′ ends (Fig. 1B). Deletions from the 3′ end identified a smaller fragment (370 nt) sufficient for localization. To simplify further discussion, the eight segments within the 370-nt fragment were labeled A′ through G. These segments are approximately 40 nt each, except A′ which was 70 nt. Deletion of A′ from the 5′ end had no effect on localization, but removal of additional sequences impaired (300ΔAB) or completely inhibited (300ΔABC) localization. Therefore,

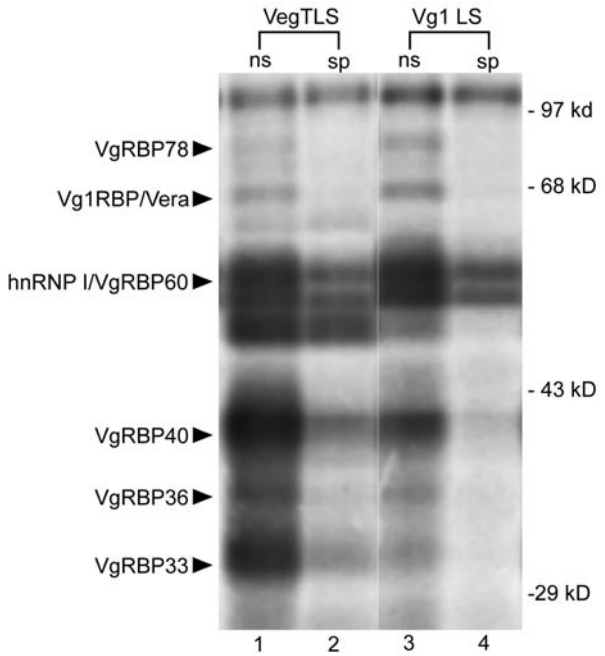

FIG. 4. Specific RBPs recognize the *VegT* LS. RNA binding was assayed *in vitro* by UV cross-linking. RNA-binding reactions contained ^{32}P-labeled *VegT* (lanes 1 and 2) or *Vg1* (lanes 3 and 4) RNA transcripts, S100 extract, and either nonspecific competitor RNA (lanes 1 and 3) or sequence-specific competitor RNA (lanes 2 and 4). Cross-linked proteins were detected by autoradiography after SDS–PAGE. The positions of identified RBPs (left) and molecular weight markers (right) are indicated.

the A′ 70-nt region of the 370-nt fragment was completely dispensable for localization, reducing the size to 300 nt (Fig. 2). Deletion of 77 nt at the 3′ end of the 300-nt *VegT* LS (300ΔFG) was tolerated, while further deletion was not (300ΔEFG), thus defining a possible limit at the 3′ end. However, simultaneous deletion of both ends completely abolished localization (300ΔAB,FG). Thus, FG is required if AB is absent, and deletion of AB abolishes localization in the absence of FG. Therefore, at least one end of the 300-nt LS (AB or FG) is critical for LS function, and these subelements (SEs) appear to function redundantly to one other, at least in the presence of the internal sequences that lie between them.

To assess the importance of the internal sequences (C–E), internal deletions were tested (Fig. 1C). When both SEs are present, the internal sequences can be singly removed without completely eliminating localization, although localization is significantly impaired (Fig. 1C, 300ΔC, 300ΔD, 300ΔE, 300ΔDE). 300ΔE is especially worthy of note as the RNA appears to accumulate at the base of the nucleus in this mutant. However, the internal sequences clearly contribute significantly to LS functioning, as deletion of all three segments abolishes localization (300ΔCDE). Moreover, in the absence of either terminal SE, deletion of each internal sequence (C, D, E) affects localization more strongly (compare, for example, ΔC vs ΔABC and ΔC,FG or ΔE vs ΔAB,E and ΔEFG; Fig. 1C). The results further suggest that, to some degree, the internal sequences are also redundant to the terminal elements. For example, the 300ΔB,FG deletion has only a partial 5′ SE and no 3′ SE, yet it is not completely localization-defective. Taken together, these results indicate considerable functional redundancy across the *VegT* LS. In at least one respect, the overall structure of the *VegT* LS is reminiscent of the *Vg1* LS, as both localization sequences contain critical subelements at their ends and exhibit significant redundancy within the LS. The source of such redundancy within the *Vg1* LS, or any LS is poorly understood, and careful comparison of the *VegT* and *Vg1* LS may provide important insight into this issue.

Comparison of the VegT LS with Other Localized RNAs

We compared the sequences of the *VegT* LS and *Vg1* LS and found no overall homology. However, important features of LS can be present at the level of secondary rather than primary structure (Macdonald, 1990; Seeger and Kaufman, 1990; Serano and Cohen, 1995). Consequently, we analyzed the *VegT* and *Vg1* LS, using the MFOLD program (Jacobson and Zuker, 1993) to look for common secondary structural motifs. The predicted secondary structures are distinctly different from one another (data not shown), revealing no apparent conservation of secondary structure. Another type of sequence motif that has been shown to be important, at least for the *Vg1* LS, is short repeated sequence motifs (Deshler *et al.*, 1997; Gautreau *et al.*, 1997). While the *VegT* and *Vg1* LS show little homology at the level of primary sequence, such motifs could be difficult to identify on the basis of sequence inspection alone without functional analysis.

The UUCAC Motif Is Critical for VegT RNA Localization

Deletion and substitution analysis of the *Vg1* RNA LS has identified several UUCAC or E2 motifs as being Vg1RBP/Vera protein-binding sites and important for the process of localization (Deshler *et al.*, 1997, 1998). This motif is present within both the 5′ and 3′ subelements of the *VegT* LS (Fig. 3A). To directly address the role of these E2 motifs in the localization of *VegT* RNA, each of the sequence motifs (E2B, E2G) or both of them together (E2BG) were replaced with noncognate pentanucleotides (Fig. 3A), and the resulting transcripts were tested for localization *in vivo*. The results from whole-mount *in situ* hybridization indicated that substituting the 3′ UUCAC (sE2G) impaired *VegT* localization while substitution of the 5′ UUCAC (sE2B) was neutral (Fig. 3B). However, histological examination of sections of those same oocytes revealed that both substitutions led to an abnormal phenotype (Fig. 3B). In both cases, the mutant RNAs appeared in a diffuse band

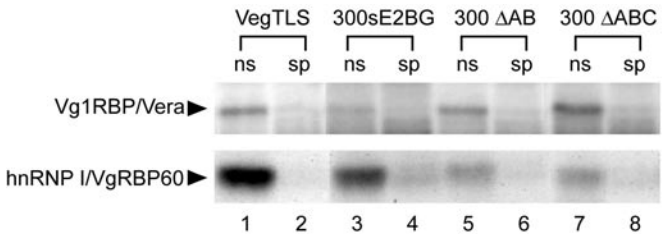

FIG. 5. Binding of Vg1RBP/Vera and hnRNPI/VgRBP60 to *VegT* RNA transcripts. Binding to the wild-type (lanes 1 and 2) and mutated versions (lanes 3–8) of the *VegT* LS was assayed *in vitro* by UV cross-linking. In localization assays (Figs. 1–3), the wild-type LS (lanes 1 and 2) was scored as normal (++), 300sE2BG (lanes 3 and 4) showed no localization (−), 300Δ AB (lanes 5 and 6) showed significant impairment of localization (+/−), and 300Δ ABC exhibited no detectable localization (−). RNA-binding reactions contained ^{32}P-labeled RNA transcripts, partially purified preparations of Vg1RBP/Vera (top) or hnRNPI/VgRBP60 (bottom), and either nonspecific competitor RNA (lanes 1, 3, 5, and 7) or sequence-specific competitor RNA (lanes 2, 4, 6, and 8). Cross-linked proteins were detected by autoradiography after SDS–PAGE.

near the oocyte cortex in contrast to the localization pattern of the control 300-nt LS, which appeared tightly concentrated in a narrow cortical band. Notably, the cortical signal was more markedly reduced in sE2G, the 3′ substitution, in good agreement with the whole-mount analysis. Substitution of both E2 motifs (sE2BG) completely abolished localization (Fig. 3B) with no effect on transcript stability (Table 1). These results support a critical role for E2 motifs in *VegT* RNA localization. Elimination of even a single copy of the E2 motif impairs cortical association without abolishing transport to the vegetal hemisphere, while substitution of two copies of the E2 motif eliminates vegetal localization.

Binding of Specific RNA-Binding Proteins to the VegT LS Is Correlated with Localization

To identify RNA-binding proteins with potential roles in localization of *VegT* RNA, *in vitro* RNA-binding assays were performed. As shown in Fig. 4, the *VegT* LS (lane 1) associates with a set of RNA-binding proteins that are similar to the RNA-binding proteins that have been shown to recognize the *Vg1* LS (lane 3; Schwartz *et al.*, 1992; Mowry, 1996). At least six of these RNA-binding proteins (RBPs), labeled VgRBPs -78, -40, -36, -33, Vg1RBP/Vera, and hnRNPI/VgRBP60, recognize the *VegT* LS in a sequence-specific manner. Binding to these proteins is competed (lane 2) only by the presence of a molar excess of unlabeled sequence-specific competitor RNA (the *Vg1* LS) and is unaffected by sequence-nonspecific competitor RNA (lanes 1 and 3). This is in contrast to the ~56-kDa doublet and the ~97-kDa protein, which are nonspecific, as they are not competed. Moreover, the specific competition by the *Vg1* LS for binding to the *VegT* LS indicates that the *VegT* LS binds *in vitro* to the same set of RBPs that recognize the *Vg1* LS. Yet, the binding profiles are not identical. For example, binding of VgRBP40, VgRBP36, and VgRBP33 appears to be more efficient for the *VegT* LS (lane 1) than for the *Vg1* LS (lane 3). However, while differences are evident with respect to levels of binding for some of the VgRBPs, it is apparent that the *VegT* LS and the *Vg1* LS associate with the same set of RBPs.

Of the RBPs recognizing the *VegT* LS, the 69-kDa Vg1RBP/Vera is of particular interest, as substitution of two of the E2 sequences apparent within the *VegT* LS (Fig. 3) caused profound defects in localization. To address whether Vg1RBP/Vera binding was affected by E2 substitutions, *in vitro* RNA binding was assayed by UV cross-linking, using partially purified preparations of Vg1RBP/Vera. As shown in Fig. 5 (top), Vg1RBP/Vera binds well to the 300-nt *VegT* LS (lane 1), while the same double E2 substitution mutant (sE2BG) that was unable to localize is strongly impaired for Vg1RBP/Vera protein binding (lane 3). The apparent weak binding to Vg1RBP/Vera may be due to an additional predicted E2 site (UUGCAC) (Fig. 6A) or potentially lower affinity sites, as defined by Deshler *et al.* (1998), in the *VegT* LS. Thus, defects in Vg1RBP/Vera binding are correlated with defects in *VegT* RNA localization. Note, however, that Vg1RBP/Vera binding is not sufficient for localization as 300ΔABC transcripts that contain E2 motifs and bind Vg1RBP/Vera (Fig. 5, lane 7) cannot localize (Fig. 1B).

In addition to E2 motifs, the *VegT* LS also contains versions of the VM1 motif identified in the *Vg1* RNA LS as necessary for *Vg1* RNA localization (Gautreau *et al.*, 1997) and critical for binding hnRNPI/VgRBP60 (Cote *et al.*, 1999). Using crude oocyte S100 extracts for RNA-binding analysis, it is difficult to discern hnRNPI/VgRBP60 binding to the *VegT* LS (Fig. 4, lane 1), as it comigrates with two previously characterized sequence nonspecific RNA-masking proteins (Marello *et al.*, 1991). With partially purified preparations of hnRNPI/VgRBP60, however, hnRNP I binding to the *VegT* LS is apparent (Fig. 5, lane 1, bottom). VM1 motifs are present in segments A (UCUCU), B (UUUCU), D (UUUUCC), and E (CUUUU) and are predicted to provide binding sites for hnRNP I (T.L.K., M. L. Rokop, and K.L.M., unpublished results; Anwar *et al.*, 2000). Indeed, deletion of regions A and B (Fig. 5, lanes 5 and 7, bottom) results in a significant reduction, but not elimination, of hnRNP I binding relative to the full-length *VegT* LS (lane 1, bottom). Notably, this same deletion (300ΔAB) reduces but does not abolish *VegT* localization *in vivo* (Fig. 1).

DISCUSSION

While individual RNA LS have been characterized from a variety of cell types and organisms, a consensus signal for any given pathway has not been defined. Consequently, the essential characteristics of an RNA LS remain to be eluci-

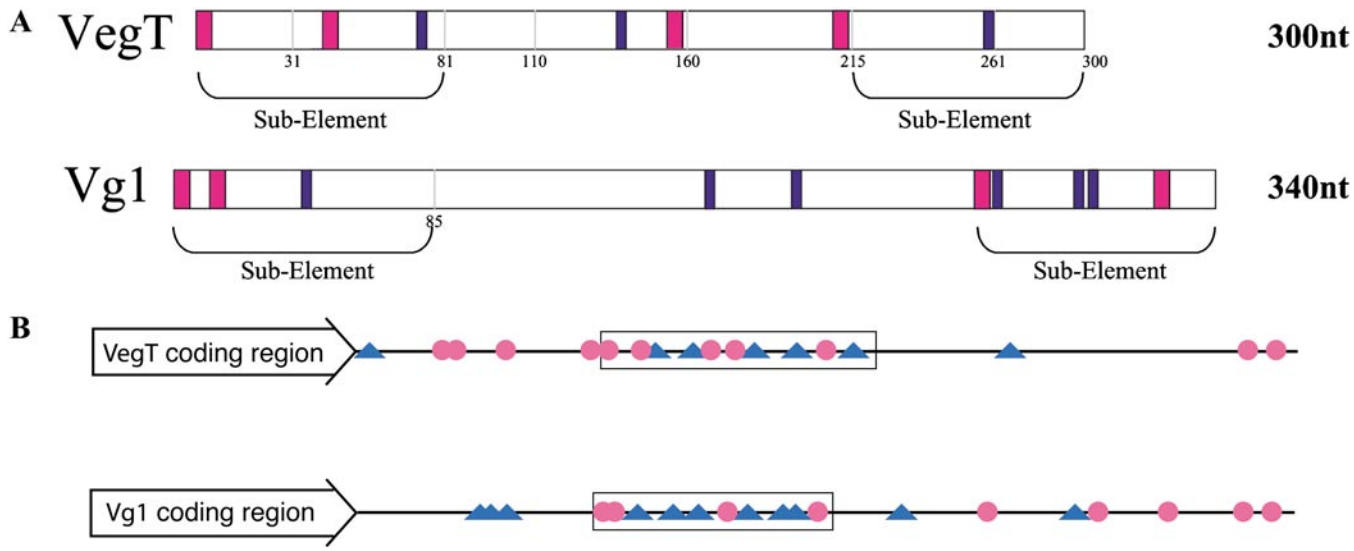

FIG. 6. Features shared between the *Vg1* LS and *VegT* LS. The VM1 sites are UUUCU, CUUCU, UCUCU, UUUUCC, UCCUCC, or CUUUU. The E2 sites are UUCAC, UUGCAC, AUCAC, UUCAU, UCCAC, or UUCAG. (A) Comparison of the *VegT* and *Vg1* LS. E2 sites are shown in blue and VM1 sites are shown in red. The terminal subelements are indicated by brackets. (B) Distribution of E2 and VM1 sites within the 3′ UTRs of *VegT* and *Vg1* RNAs. The E2 sites are depicted as blue triangles, the VM1 sites are shown as red circles, and the *VegT* 525-LS and *Vg1* 340-nt LS are boxed.

dated. In this study, we carried out a detailed analysis of the uncharacterized *VegT* LS and compared it with the well-studied *Vg1* LS to investigate possible common features between their LS. The results of our work indicate a common structural organization for the *VegT* and *Vg1* LS, with a common set of proteins binding these signals. These results point to a consensus signal (diagrammed in Fig. 6) operating during midoogenesis to direct RNAs to the vegetal cortex and suggest that what may characterize RNA LS in general is the critical clustering of specific redundant protein-binding motifs.

Consensus LS Features for Vegetal Localization

We have defined a LS within the 3′ UTR of *VegT* that is sufficient to direct localization to the vegetal cortex of the oocyte. Deletion mutagenesis of the *VegT* LS revealed several levels of functional redundancy within its organization. First, sequences found at either end of the LS could compensate for each other when one was deleted. In the absence of both terminal subelements (5′ SE, 3′ SE), however, the remaining sequences could not support localization, revealing a general bipartite organization to the signal. Secondly, the internal sequences of the LS could compensate for a missing SE. Finally, each deleted region of 30–40 nt could be compensated for by other sequences in the LS. The structural organization of the *VegT* LS is reminiscent of that defined for the *Vg1* LS (Gautreau *et al.*, 1997), as depicted in Fig. 6A. The two signals are similar in size and position relative to the stop codon. The topology of both signals is also similar, with two core elements located at either end, each possessing partial autonomy from the internal sequences.

Sequence comparison of the *VegT* and *Vg1* LS reveals no clear similarity at the level of either overall primary sequence or secondary structure. Yet, analysis of *VegT* and *Vg1* RNA localization has suggested that the LS for these mRNAs may be functionally homologous. Indeed, we have found not only that their overall structural organization is similar, but also that they bind virtually an identical set of proteins. Moreover, there is an apparent conservation of certain short reiterated motifs which represent binding sites for known RBPs. Found in both the *VegT* LS and the *Vg1* LS are two distinct repeated sequences, the E2 and VM1 motifs. It is notable that the distribution of these motifs is similar within both LS. Strikingly, within the 3′ UTRs of *VegT* and *Vg1*, clusters of E2 and VM1 motifs overlap the LS (Fig. 6B). This may provide a clue as to what defines a LS, as clusters of E2 and VM1 are unique to the LS regions.

The reiterated E2 motif has been shown to bind Vg1RBP/Vera (Deshler *et al.*, 1998). Mutational analysis of the E2 motif (UUCAC) showed that Vg1RBP/Vera binding is not affected when the first or second nucleotides are replaced with an A or C, respectively, and that changes at the final position are tolerated as well (Deshler *et al.*, 1998). All consensus E2 sites have been indicated in Fig. 6 and reveal a similar location and spacing for this motif within the two LS. The E2 motif is essential for function of both the *VegT* and *Vg1* LS, as changing this motif results in the loss of localization (Fig. 3, Deshler *et al.*, 1997; Kwon *et al.*, 2002).

For *VegT* localization, we have shown two E2 (UUCAC) sequences, one in each SE, to be critical (Fig. 3). Substitution of either resulted in a diffuse cortical signal (Fig. 3, sE2B and sE2G), suggesting that *VegT* was transported to the vegetal cortex but was not correctly anchored there. It is possible that *trans*-acting factors mediate efficient cortical binding through multiple E2 motifs, with one copy being insufficient for anchoring. Consistent with such an interpretation, substitution of two E2 motifs completely abolished cortical localization. Thus, E2 repeats function as key components in the localization of two late pathway RNAs, *Vg1* and *VegT*.

The other critical RNA motif in the *Vg1* LS, VM1 (UUUCUA), was originally found as three copies within the terminal elements of the *Vg1* LS. The VM1 sequence is the binding site for hnRNP I/PTB (Cote *et al.*, 1999). Mutational and selection analyses have revealed that blocks of pyrimidine residues represent the critical determinants for binding, for example, permutations of YYUCU (T.L.K., M. L. Rokop, and K.L.M., unpublished results; Perez *et al.*, 1997; Anwar *et al.*, 2000). The *VegT* LS is competent to bind hnRNP I (see Fig. 5), and two sites (UUCUCU and UUUCU) are found within the 5′ SE (Fig. 6). Additional VM1 sites are apparent in D and E, and persistent binding of hnRNP I in *VegT* ΔAB mutants is consistent with this assessment. Overall, the E2 and VM1 motifs are moderately conserved in sequence, location, and spacing within the two LS. The conserved overall topology of these two LS explains the high level of functional redundancy also observed for these two signals and the importance of the 5′ subelement of the *Vg1* LS (Gautreau *et al.*, 1997) and *VegT* LS (Fig. 1).

A remarkable feature of the E2 and VM1 motifs is their overall distribution within the *VegT* and *Vg1* 3′ UTRs. While individual copies of the motifs are present throughout the UTRs, the E2 and VM1 sites are found in close proximity within the regions encompassing the LS (Fig. 6B). Could clustering of E2 and VM1 motifs serve to define a LS? It is intriguing that *FatVg* mRNA, which is thought to rely on the same vegetal localization pathway as *Vg1* and *VegT* (Chan *et al.*, 1999), contains versions of both E2 and VM1 sites within the 25-nt LS. While the localization sequences for other RNAs using this pathway have not been defined, inspection of the 3′ UTR sequences of *BicC* and *β TrCP* is provocative; E2 and VM1 sites are present in close proximity (Hudson *et al.*, 1996; Wessely and De Robertis, 2000). Thus, we suggest that clustering of E2 and VM1 sites is a consensus feature of a localization sequence for directing mRNAs to the vegetal cortex during midoogenesis and could potentially be used to predict LS within the 3′ UTRs of RNAs using this pathway.

Vg1 and *VegT* differ as to when they are released from the cortex (Forristall *et al.*, 1995; Stennard *et al.*, 1996) and when they are translated (Tannahill and Melton, 1989; Dale *et al.*, 1989, Stennard *et al.*, 1999; Wilhelm *et al.*, 2000). These important differences could be mediated in part by the structural elements unique to each LS and to the distinct quantitative differences observed in their protein-binding patterns (VgRBP40, VgRBP36, and VgRBP33 in Fig. 4). This is further suggested by localization-defective mutants of *VegT* that are missing a $(GU)_4AUUU(G)_7A(GU)_4$ sequence in the F region; such GU repeats are not found in the *Vg1* LS.

A Core Localization Complex?

One striking conclusion to be made from this study is that, although the *Vg1* LS and the *VegT* LS differ significantly in their overall nucleotide sequence, they bind a virtually identical set of proteins. The following evidence supports this conclusion: The *Vg1* LS effectively competes with the *VegT* LS for the same proteins. Deletion of the UUCAC E2 sites or the regions bearing the VM1 sites eliminated or greatly reduced Vg1RBP/Vera or hnRNP I binding, respectively, to both LS. Lastly, the loss of protein binding was correlated with localization deficiencies for both LS. Our conclusions are in partial agreement with the recent work of Kwon *et al.* (2002). They have suggested that it is E2 motifs, as exclusive binding sites for Vg1RBP/Vera, which characterize the LS for *VegT* and *Vg1*. We find that E2 motifs are indeed important. However, we further find that additional motifs, notably VM1 and perhaps others that are as yet uncharacterized, are critical as well. An important, yet unresolved, issue is whether the localization phenotypes observed by Kwon *et al.* (2002) correlate with changes in binding of Vg1RBP/Vera and hnRNP I. Our results argue that Vg1RBP/Vera and hnRNP I are components of a core localization complex that functions in the late pathway.

A reasonable model is that the structural elements shared in common between the two LS are involved in conferring competence for the late localization pathway. These components could be involved in RNA selection, transport, and anchoring. We hypothesize that RNA-binding sites for the core protein components must be in proximity to one another to promote interactions within the transport particle and that this feature may serve to delimit a functional LS. We are currently testing this hypothesis. It will be of great interest to define precise functions for the common LS-binding proteins identified in this study and to determine how they interact both with one another and with the transport machinery to initiate polarity in the *Xenopus* embryo.

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