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MECHANISMS OF DEVELOPMENT

Interactions of 40LoVe within the ribonucleoprotein complex that forms on the localization element of *Xenopus* Vg1 mRNA

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ABSTRACT

Proline rich RNA-binding protein (Prrp), which associates with mRNAs that employ the late pathway for localization in *Xenopus* oocytes, was used as bait in a yeast two-hybrid screen of an expression library. Several independent clones were recovered that correspond to a paralog of 40LoVe, a factor required for proper localization of Vg1 mRNA to the vegetal cortex. 40LoVe is present in at least three alternatively spliced isoforms; however, only one, corresponding to the variant identified in the two-hybrid screen, can be crosslinked to Vg1 mRNA. *In vitro* binding assays revealed that 40LoVe has high affinity for RNA, but exhibits little binding specificity on its own. Nonetheless, it was only found associated with localized mRNAs in oocytes. 40LoVe also interacts directly with VgRBP71 and VgRBP60/hnRNP I; it is the latter factor that likely determines the binding specificity of 40LoVe. Initially, 40LoVe binds to Vg1 mRNA in the nucleus and remains with the RNA in the cytoplasm. Immunohistochemical staining of oocytes shows that the protein is distributed between the nucleus and cytoplasm, consistent with nucleocytoplasmic shuttling activity. 40LoVe is excluded from the mitochondrial cloud, which is used by RNAs that localize through the early (METRO) pathway in stage I oocytes; nonetheless, it is associated with at least some early pathway RNAs during later stages of oogenesis. A phylogenetic analysis of 2×RBD hnRNP proteins combined with other experimental evidence suggests that 40LoVe is a distant homolog of *Drosophila* Squid.

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1. Introduction

Subcellular gene expression is a means to achieve embryonic and cellular polarity and is frequently accomplished through the controlled translation of localized mRNA (Du et al., 2007; King et al., 2005; Martin and Ephrussi, 2009; St. Johnston, 2005). Localization of specific mRNAs during oogenesis and embryogenesis helps to determine the body plan of the developing embryo. However, localized mRNAs also play essential roles in differentiated cells. For example, in fibroblasts the accumulation of β-actin mRNA at the leading edge underlies directional cell motility (Shestakova et al., 2001), while in neurons the localization of mRNA contributes to synaptic plasticity (Steward and Schuman, 2003).

It has become apparent that, in general, a considerable number of proteins must assemble on the *cis*-acting elements that determine translocation and anchoring of an mRNA. In some instances, localized mRNAs may be associated with ribosomes or other complex structures, thereby forming distinct granules (Antar et al., 2004; Kiebler and Bassell, 2006;

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Kloc et al., 2002; Krichevsky and Kosik, 2001; Smith et al., 2004). Even in cases where the *cis*-acting element is relatively small, such as the 54-nucleotide zipcode sequence in β-actin mRNA, several protein factors appear to participate in the process of localization (Gu et al., 2002). The substantial number of factors associated with any particular localized mRNA certainly reflects the complexity of this process, which usually entails concomitant repression of translation.

Xenopus Vg1 mRNA is transported to the vegetal pole during stage III of oogenesis and then spreads along the cortex of this hemisphere (King et al., 2005). Transport and anchoring require a 340-nucleotide element in the 3′-untranslated region (UTR) referred to as the Vg1 localization element (VLE) (Mowry and Melton, 1992). A 252-nucleotide A:U-rich sequence positioned 118 nucleotides downstream of the VLE mediates translational repression of the mRNA and is termed the Vg1 translational element (VTE) (Otero et al., 2001; Wilhelm et al., 2000). Translation of Vg1 mRNA begins during stage IV of oogenesis and appears to result from a cleavage/polyadenylation reaction that removes the VTE (Kolev and Huber, 2003).

Six different polypeptides can be crosslinked to the VLE upon UV irradiation, indicating that Vg1 mRNA, as expected, requires several protein factors for proper localization (Mowry, 1996). Those *trans*-acting factors that specifically associate with the VLE include Vg1RBP/Vera (Deshler et al., 1998; Havin et al., 1998), VgRBP60/hnRNP I (Cote et al., 1999), Prrp (Zhao et al., 2001), VgRBP71/KSRP (Kroll et al., 2002), 40LoVe (Czaplinski et al., 2005), and a *Xenopus* homolog of Staufen (XStau) (Allison et al., 2004; Yoon and Mowry, 2004). Moreover, the localization RNP complex associates with the plus-end tracking motor proteins kinesin-1 and kinesin-2 (Betley et al., 2004; Messitt et al., 2008; Yoon and Mowry, 2004).

Despite progress in assigning function to some of these *trans*-acting factors, several challenging questions remain, including what determines the directionality of movement, since a pathway to the animal hemisphere operates contemporaneously with the pathway used by Vg1 and other vegetally localized RNAs, and what controls timing of localization, since a group of RNAs move through an earlier (METRO) pathway and seem to use many of the same factors (Chang et al., 2004; Choo et al., 2005; Claussen et al., 2004; Kloc and Etkin, 1998). In addition, the exact composition of the RNP complex, its order of assembly, and its dynamics during transport and anchoring are not fully defined.

To address some of these questions, we have used yeast two-hybrid assays to identify additional constituents of the Vg1 localization RNP complex as well as to delineate interactions within this structure. A yeast two-hybrid screen of a *Xenopus* oocyte library using Prrp as bait retrieved 40LoVe several times. This protein was recently isolated by affinity chromatography on immobilized VLE RNA and shown to be required for efficient localization of Vg1 mRNA (Czaplinski et al., 2005). We present a phylogenic analysis and other evidence that indicate 40LoVe is related to *Drosophila* Squid. We find that 40LoVe associates with Vg1 mRNA in the nucleus; thus, it is one of the first components to join the RNP complex that forms on the VLE. Based on the nonspecific binding activity of 40LoVe *in vitro*, this recruitment likely depends on other *trans*-acting factors that bind to Vg1 mRNA in the nucleus, most likely VgRBP60/hnRNP I (Kress et al., 2004). We demonstrate that 40LoVe, like Vg1RBP/Vera, is excluded from the mitochondrial cloud in stage I oocytes, suggesting factors that bind in the nucleus may determine the temporal pathway utilized in the cytoplasm.

2. Results

2.1. *Prrp interacts with 40LoVe*

Prrp was identified by its ability to bind to and co-localize with *Xenopus* Vg1 mRNA (Zhao et al., 2001). Subsequently, it was used as bait in a yeast two-hybrid screen of a *Xenopus* oocyte cDNA library. Of the 59 colonies that exhibited strong β-galactosidase activity, at least 15 were VgRBP71, which also binds to the VLE (Kroll et al., 2002). The second most frequently recovered protein (at least 5 independent isolates) from the yeast two-hybrid screen is a member of the 2×RBD-Gly family of hnRNP proteins that includes the A/B and D type isoforms (Dreyfuss et al., 1993). None of the clones isolated from the screen contained a full-length open reading frame (ORF); thus, a cRACE strategy (Maruyama et al., 1995) was used to determine the complete 5′ nucleotide sequence that included the remainder of the ORF.

The full-length ORF encodes a 326 amino acid polypeptide (Fig. 1A) having 92% identity with 40LoVe, a protein that was identified by its retention on an RNA affinity column that contained the localization element of Vg1 mRNA (Czaplinski et al., 2005). Antibodies against 40LoVe injected into oocytes decreased localization of Vg1 mRNA, implicating the protein in this process (Czaplinski et al., 2005). It is important to note that we have determined that 40LoVe and the sequence reported here are encoded by distinct genes. Each is well represented in EST databases, indicating that both genes are functional. The tetraploid origin of *Xenopus laevis* means that gene duplication is not uncommon; in most instances, the two copies exhibit 10% or less sequence divergence (Graf and Kobel, 1991).

The interaction between 40LoVe and Prrp was confirmed using a pull down assay (Fig. 1B). Prrp labeled with [^{35}S] was synthesized in rabbit reticulocyte lysate and mixed with 40LoVe that was fused to maltose binding protein (MBP). The two proteins were incubated in the absence (lane 3) or presence (lane 4) of Vg1 RNA, followed by retrieval of the 40LoVe fusion protein using amylose beads. The precipitated samples were analyzed by electrophoresis/autoradiography. Vg1 RNA has no detectable effect on the amount of Prrp associated with 40LoVe, indicating that there is a direct interaction between the two proteins.

The longest version of 40LoVe identified in the two-hybrid screen with Prrp begins at amino acid residue 113 (Fig. 1C, column 4). The shortest clone from the screen begins at valine-193 within the RNP1 sequence of the second RBD. Surprisingly, when a full-length clone of 40LoVe was tested against Prrp, there was no growth on selective medium, suggesting that the N-terminal region of 40LoVe diminishes this interaction (Fig. 1C, column 3). There is an irregular distribution of charged amino acids in 40LoVe. The N-terminus has 13 acidic residues within the first 50 amino acids; whereas, the C-terminal region has a preponderance of basic residues. Thus,

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