Orb and a long poly(A) tail are required for efficient oskar translation at the posterior pole of the Drosophila oocyte

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SUMMARY

During Drosophila oogenesis, the posterior determinant, Oskar, is tightly localized at the posterior pole of the oocyte. The exclusive accumulation of Oskar at this site is ensured by localization-dependent translation of oskar mRNA: translation of oskar mRNA is repressed during transport and activated upon localization at the posterior cortex. Previous studies have suggested that oskar translation is poly(A)-independent. We show that a long poly(A) tail is required for efficient oskar translation, both in vivo and in vitro, but is not sufficient to overcome BRE-mediated repression. Moreover, we show that accumulation of Oskar activity requires the Drosophila homolog of Cytoplasmic Polyadenylation Element Binding protein (CPEB), Orb. As posterior localization of oskar mRNA is an essential prerequisite for its translation, it was critical to identify an allele of orb that does localize oskar mRNA to the posterior pole of the oocyte. We show that flies bearing the weak mutation orb mel localize oskar transcripts with a shortened poly(A) that fails to enhance oskar translation, resulting in reduced Oskar levels and posterior patterning defects. We conclude that Orb-mediated cytoplasmic polyadenylation stimulates oskar translation to achieve the high levels of Oskar protein necessary for posterior patterning and germline differentiation.

Key words: Orb, oskar mRNA, Translation, poly(A) tail, Drosophila, Oogenesis

INTRODUCTION

The definition of spatial cues is essential to many biological processes, from cell movement to embryonic development. The ability of cells to sense space and initiate and maintain polarity relies on a set of processes that culminates with the creation of specialized cytoplasmic and cortical compartments, and the establishment of axes of symmetry.

During embryogenesis, the establishment of the embryonic axes is a prerequisite to proper patterning of the embryo. In Drosophila melanogaster, both the anteroposterior (AP) and dorsoventral (DV) axes are established during oogenesis, through the prior asymmetric localization of cytoplasmic determinants in the oocyte. To ensure the tight and exclusive presence of these determinants at the sites where their activity is required, they are often synthesized in loco after prior localization of their transcripts, which are then subject to temporal and spatial translational regulation (St Johnstone, 1995).

oskar (osk) mRNA, which encodes the posterior determinant, is localized at the posterior pole of the oocyte and embryo, where Osk protein is required to assemble the germ plasm (Beams and Kessel, 1974; Ephrussi et al., 1991; Kim-Ha et al., 1991), a cytoplasmic sub-compartment containing the abdominal and germline determinants of the fly (Lehmann and Nüsslein-Volhard, 1986). Females bearing mutations in osk produce embryos lacking both abdomen and germline. Conversely, mislocalization of Osk activity leads to formation of ectopic abdominal structures at the expense of the anterior structures (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992; Smith et al., 1992). Hence, restriction of Osk activity to the posterior pole is also essential for normal development to occur.

Coupling of osk mRNA translational activation to its localization at the posterior pole of the oocyte ensures specific and exclusive accumulation of Osk protein at the posterior pole of the oocyte (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). The oocyte develops in a 16-cell cyst, consisting of 15 transcriptionally active nurse cells and the oocyte itself, all interconnected by cytoplasmic bridges (Spradling, 1993). From the early stages of oogenesis onwards, osk mRNA is transcribed by the nurse cells and accumulates in the oocyte. At mid-oogenesis (stage 8) osk mRNA becomes expressed at high levels at the posterior pole, where it is translated (Ephrussi et al., 1991; Kim-Ha et al., 1991).

Prior to its localization, osk mRNA is translationally repressed through the binding of Bruno (Bru) repressor protein to discrete elements (Bruno Response Elements or BRE) present in the osk 3’ untranslated region (3’UTR) (Kim-Ha et al., 1995; Webster et al., 1997). Upon posterior localization, osk translation is derepressed (Gunkel et al., 1998). The mechanisms that underlie repression and derepression of osk
remain elusive. In many species, however, it appears that the translational status of a regulated transcript is determined by the length of its poly(A) tail, and the switch from a silenced to a translationally active state is controlled by cytoplasmic polyadenylation (reviewed by Richter, 1999). Accordingly, an increase in translation is often associated with poly(A) tail elongation, whereas translational silencing correlates with poly(A) tail shortening (Lieberfarb et al., 1996; Sallès et al., 1994; Sheets et al., 1995). In Xenopus, where this phenomenon has been most extensively studied, the cis-acting elements involved in cytoplasmic polyadenylation have been identified and include the AAUAAA hexanucleotide (also required for nuclear polyadenylation) and the U-rich cytoplasmic polyadenylation element (CPE) (Fox, 1989; McGrew and Richter, 1990). The CPE is specifically bound by the polyadenylation-inducing protein CPEB (Hake and Richter, 1994; Stebbins-Boaz et al., 1996), whose presumed function is to recruit and stabilize the cytoplasmic polyadenylation machinery (Mendez et al., 2000).

In Drosophila, no discrete cis-acting elements involved in cytoplasmic polyadenylation have been identified so far. This has made it difficult to assess directly the involvement of cytoplasmic polyadenylation in translational control of regulated transcripts. A putative CPEB is encoded by the orb binding (orb) locus. Strong orb alleles affect osk mRNA localization (Christerson and McKearin, 1994; Lantz et al., 1994), preventing an analysis of their effect on osk translation. Weaker orb alleles, for which the pattern of osk mRNA localization has not yet been analyzed, are available and might provide a useful tool with which to dissect the role of Orb in modulating osk translation. Among those, the hypomorphic orb allele, orb<sup>mel</sup>, completes oogenesis and has been previously shown to affect the establishment of the AP axis, by interfering with Osk protein accumulation (Christerson and McKearin, 1994). The observation by Chang et al. (Chang et al., 1999) that in this mutant osk poly(A) tail length is shortened supports the idea that cytoplasmic polyadenylation might be involved at least in some aspects of osk translational regulation. However, as posterior localization is required for osk mRNA translation, characterization of the effect of the orb<sup>mel</sup> mutation on osk mRNA localization is an essential prerequisite to any assessment of a possible role of Orb in regulation of osk polyadenylation and Osk protein accumulation.

At least two non-mutually exclusive scenarios may be hypothesized. Polyadenylation of osk transcript might be required to overcome translational repression and to activate translation upon posterior localization, a mechanism widely used to repress/derepress translation of several developmentally regulated transcripts. This would predict that, even upon posterior localization, no Osk activity is synthesized in the absence of polyadenylation. Alternatively, poly(A) tail elongation might not be a prerequisite for translation per se, but rather be required to enhance translation efficiency, allowing for Osk protein accumulation. In fact, it is already established that the poly(A) tail of an mRNA can synergize with its 5’ cap structure to enhance translation initiation. This synergistic effect of the cap and the poly(A) tail arises from the simultaneous binding of the poly(A)-binding protein PABP and the cap-binding protein eIF4E, to the translation initiation factor eIF4G, promoting reinitiation of translation by terminating ribosomes on the same transcript (Gallie, 1991; Tarun and Sachs, 1996; Wells et al., 1998). This second hypothesis predicts that upon posterior localization of osk mRNA, translation would initiate, but with limited efficiency, unless enhanced by polyadenylation. In this light it is interesting that posterior patterning and germline differentiation require different thresholds of Osk activity (Markussen et al., 1995; Rongo et al., 1995). Posterior patterning is robust and requires only low levels of Osk, whereas pole cell formation appears to be very sensitive to any reduction in Osk protein levels. To guarantee production of sufficient amounts of Osk for fulfillment of both functions, translational derepression and enhancement might be involved.

We address the role of cytoplasmic polyadenylation in control of osk translation. We have measured osk poly(A) tail length in vivo and correlated its polyadenylation status to its translation status both in vivo and in vitro. We show that within RNA corresponding to all stages of oogenesis, a discrete population of osk mRNA bears a long poly(A) tail. A poly(A) tail of the maximum length observed for osk mRNA in vivo is necessary and sufficient to enhance translation of a chimeric osk transcript in vitro, in the absence of Bruno repressor protein. However, addition of a poly(A) tail of any length does not suffice to overcome BRE-mediated repression, at least in vitro. We also show that maintenance of a long poly(A) tail on osk transcript requires Orb and is essential for Osk protein accumulation. In orb<sup>mel</sup> mutant egg chambers, the osk poly(A) tail is shorter than in wild type. Shortening of the osk poly(A) tail correlates with a reduction in Osk protein accumulation despite posterior localization of the transcript. This leads to complete sterility and, in extreme cases, to loss of posterior embryonic patterning. However, osk translation in orb<sup>mel</sup> appears to be only attenuated but not abolished. We therefore suggest a role for Orb in posterior patterning by enhancing osk translation through the addition or maintenance of a long poly(A) tail.

**MATERIALS AND METHODS**

**Poly(A) tail measurement**

*Drosophila melanogaster* females were kept at 25°C for 2-3 days on yeast before dissection. Ovaries were recovered in PBT (PBS/0.1% Tween-20) and frozen in liquid N<sub>2</sub> without any buffer. Total RNA was extracted following the RNA clean protocol (Hybaid).

osk poly(A) tail was measured mainly using an RNaseH-based method, as described by Zangar et al. (Zangar et al., 1995), including some modification as described by Lie and Macdonald (Lie and Macdonald, 1999). After denaturation at 85°C for 5 minutes, 20 μg of total ovary RNA were hybridized to 5 μg of a complementary DNA oligo specific for osk mRNA (5’-CGC CAG AAT TCT ACA CTG TG-3’) with and without 5 μg of dT<sub>16</sub> oligo, at 42°C for 10 minutes. The RNA-DNA hybrid was than specifically digested with RNase H (1 U from Gibco) at 30°C for 30 minutes in 20 μl of RNase H buffer (40 mM Tris-HCl pH 8, 4 mM MgCl<sub>2</sub>, 1 mM DTT, 30 ng/ml BSA). Reactions were stopped by phenol-chloroform extraction and ethanol precipitation. The RNA fragments were resuspended in Ambion RNA loading buffer, separated on 5% polyacrylamide denaturing gel and transferred by electro-blotting for 3-4 hours at 20 V to NEN Genescreen hybridization membrane. osk mRNA was detected by northern hybridization. Quantification of the RNA populations bearing different poly(A) tail length was performed on a Macintosh computer using the public domain NIH Image program (developed at...
RNA purified using RNeasy columns from QIAGEN. Prior to template DNA was eliminated by digestion with DNAseI, and the osk-lacZ mRNAs were synthesized using the SP6 Capped chimeric In vitro transcription. C36 luca mRNA was synthesized as described (Gray and Hentze, Transcript (1 than 150A were added by enzymatic polyadenylation, using not be cloned because of instability in bacteria. Poly(A) tails longer original plasmids and differ only in the length of the poly(A) cassette. 32 P]dCTP using the oligodT-anchor and a specific DNA constructs m1m2lacWT was previously described (Gunkel et al., 1998). m1m2lacAABC was derived from m1m2lacWT by deletion of an EcoRI/DraI fragment containing the AB repressor region, and additional deletion of the C region by PCR-directed mutagenesis, using the PCR primers Δbc1Iup (5'-ACT GTC TAG AAC GTT TTT TTT GTG C-3') and T3XL (5'-CGA AAT TAA CCC TCA GTA GGA-3'). This construct lacks nucleotides 3660-3778 and 4416-4487 of the m1m2lacWT chimeric osk-lacZ transcript. Cassettes of 36, 73, 98 and 150 adenosines (As) were cloned downstream of the various 3'UTRs using the unique Nol and KpnI sites in the plasmids. The derived constructs were named after the original plasmids and differ only in the length of the poly(A) cassette. Cassettes encoding a poly(A) tract longer than 150 nucleotides could not be cloned because of instability in bacteria. Poly(A) tails longer than 150A were added by enzymatic polyadenylation, using recombinant yeast Poly(A) Polymerase (γPAP) from Amersham. Transcript (1 μg) was polyadenylated at 30°C for 20 minutes in the presence of 10 mM ATP, 1× PAP buffer and 600 U of γPAP. In vitro transcription Capped chimeric osk-lacZ mRNAs were synthesized using the SP6 mMessage mMachine kit from Ambion. After a 2 hour reaction, the template DNA was eliminated by digestion with DNaseI, and the RNA purified using RNasefree columns from QIAGEN. Prior to transcription, the template was linearized with an appropriate restriction enzyme, cutting at a unique site downstream of the 3'UTR. C36 luca mRNA was synthesized as described (Gray and Hentze, 1994). The RNAs were trace labeled with [γ32P]UTP to facilitate assessment of their concentration and integrity. All RNAs used in the same experiment were synthesized in parallel. In vitro translation assay Translation assays were performed as described previously (Castagnetti et al., 2000). Briefly, 50 ng of template osk mRNA were translated in a 12.5 μl reaction containing 60 μM amino acids, 16.8 mM creatine phosphate, 80 ng/μl creatine kinase, 24 mM HEPES (pH 7.4), 0.6 mM Mg(OAc)2, 60 mM KOAc, 0.1 mM Spermidine, 1.2 mM DTT, 100 ng/μl calf liver tRNA and 40% ovary or embryonic extract. Luciferase mRNA (20 ng) was co-translated as an internal control. The reactions were incubated for 90 minutes at 25°C. The translation efficiency of the osk chimeric mRNAs was quantified using the chemiluminescent β-Gal Reporter Gene Assay (Roche), following the protocol provided by the manufacturer. Luciferase activity was measured according to Brasier et al. (Brasier et al., 1989). In situ hybridization and immunostaining Two- to three-day-old females were dissected in PBS and ovaries were fixed for 20 minutes in 4% paraformaldehyde in PBS. After washing twice in PBT (0.1% Triton X-100), ovaries were extracted for 1.5 hours in PBS 1% Triton X-100, blocked for 1 hour in PBS 0.3% Triton X-100, 0.5% BSA and then incubated overnight with primary antibodies (α-Stau at 1/250, α-Osk at 1/3000). After washing, samples were incubated with FITC/rhodamine-coupled secondary antibodies (1/500 in PBT) for detection. Microscopy was carried out using a Leica TCS SP confocal microscope. For in situ hybridization, ovaries were fixed in 4% paraformaldehyde and processed according to Glotzer and Ephrussi (Glotzer and Ephrussi, 1999) using osk digoxigenin-labeled probes. Immunoprecipitation Total ovarian extract, prepared as for the in vitro translation assay, was incubated with 3 μl of specific antibody in 100 μl of hybridization buffer [20 mM HEPES (pH 7.5), 150 mM NaCl, 2.5 mM MgCl2, 250 mM sucrose, 0.05% NP-40, 0.5% Triton X-100, 1X EDTA-free protease inhibitors cocktail from Roche]. After an overnight incubation, sepharose-protein A beads were added for 50 minutes to allow binding. Beads were washed three times with hybridization buffer without MgCl2. Protein were denatured in Laemmli buffer, run on a 10% SDS polyacrylamide gel and detected using monoclonal Orb antibodies (1.500). The monoclonal antibodies orb6H4 and orb4H8 developed by P. Schell were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. RESULTS An Orb-dependent long poly(A) tail on osk mRNA in vivo Modulation of poly(A) tail length is a powerful and widely employed mechanism in translational control. In several species, translational silencing is correlated with a deadenylated status of the mRNA, and activation of translation parallels poly(A) tail elongation. We decided to address directly the role of the poly(A) tail in osk translational control, measuring the length of the osk poly(A) tail in vivo, and assessing the influence of such a tail on translation both in vivo and in vitro. Previous evaluations of the length of the osk poly(A) tail involved different methods of measurement, yielding differing results (Chang et al., 1999; Lie and Macdonald, 1999; Sallés et al., 1994). We therefore decided to perform the two assays typically used for this purpose on the same samples: the PAT assay, involving PCR amplification; and the more direct, RNaSeH/northern blot-based assay. For the PAT assay, ovaries were divided into early (stage 1-5) and late stages (stage 7-14). During the early stages of oogenesis, osk mRNA is unlocalized and translationally silent. During the late stages, at least a portion of osk mRNA is localized and translated. In both pools, osk poly(A) tails ranging from 100 to 230 A were present (Fig. 1A). Using the RNaSeH-based assay, poly(A) tails 200 in length were also detected in both samples (Fig. 1B and data not shown). Hence, a significant fraction of osk mRNA molecules bears remarkably long poly(A) tails, consistent with some of the previous reports (Chang et al., 1999; Sallés et al., 1994). As a first step towards investigating a potential role of cytoplasmic polyadenylation and the poly(A) tail in osk translational control, we measured the length of the osk poly(A) tail in ovaries of the hypomorphic orb mutant, orbmel, orbmel mutant females complete oogenesis and produce embryos out of which 25% show posterior patterning defects, reflecting the reported reduction in Osk protein accumulation (Christerson and McKearin, 1994; Markussen et al., 1995).
Fig. 1. osk mRNA has a long poly(A) tail in vivo. (A) osk mRNA poly(A) tail measurement using the PAT assay previously described by Sallés et al. (Sallés et al., 1994) and Chang et al. (Chang et al., 1999). The longest detected osk poly(A) tail is about 200 A long, both in early (up to stage 5) and late (5-14) stages of oogenesis. The length of the poly(A) tail is equal to the difference between the top of the smear and the fragment of osk mRNA amplified, both indicated by arrows. (B) Measurement of the osk poly(A) tail length using an RNaseH based assay. Total ovarian RNA was hybridized to a DNA oligonucleotide complementary to the 3'-most region of the osk 3'UTR, in the presence (+) or in the absence (−) of excess oligo dt16. The maximum length present in the osk mRNA population corresponds to the difference between the top of the smear in the ‘−’ lane and the baseline given by the ‘+’ lane. In wild-type ovaries, the osk poly(A) tail reaches a length of 200 A, whereas in orb mel ovaries the osk poly(A) tail is shortened to about 130-150 A. Quantitation using the NIH image program shows that in wild-type ovaries 36% of osk mRNA has a tail length of 150-200 A and that this population is reduced to 4.5% in orb mel homozygous ovaries. The same amount of total RNA was processed in each sample. The result obtained for the wild-type RNA was confirmed using a second osk oligo. (C) Translation efficiency of chimeric osk-lacZ mRNAs bearing poly(A) tails of different lengths in embryo extract. Efficient translation activation was observed when a poly(A) tail longer than 200 A was added to the transcript. Tails of 36, 53, 73 and 150A in length did not activate translation. The difference in translation between A0, A36, A53, A73 and A150 can be explained by a comparable increase in stability of the transcripts. The difference in half-life between the A0 and A36 transcript does not explain the difference in their translation efficiency. Previous reports had suggested that osk translation was poly(A) independent (Castagnetti et al., 2000; Lie and Macdonald, 1999). In both cases the poly(A) tails used in the assay were shorter than 200 A and therefore not competent to activate translation according to our present analysis.

measured by the RNaseH-based assay, the osk poly(A) tail is ~50 A shorter in orb mel mutant females than in the wild type (Fig. 1B), extending to only 150 A. Thus, the presence of a long poly(A) tail on osk mRNA requires orb function. This confirms the observation that osk mRNA is subject to cytoplasmic polyadenylation in vivo (Chang et al., 1999).

The fact that the osk poly(A) tail is somewhat shorter in orb mel than in wild type suggested that this difference might be the basis of the observed defect in Osk protein accumulation in the mutant. However, despite their shorter poly(A) tails in orb mel ovaries, the osk transcripts still bear relatively long tails that should in principle be able to stimulate translation. To determine whether the shorter poly(A) tail of osk mRNA in orb mel could affect Osk accumulation, we tested whether the length of the poly(A) tail is critical for translational stimulation of osk mRNA expression. We compared the in vitro translation efficiency of chimeric osk-lacZ transcripts bearing poly(A) tails of different lengths. As investigation of the role of the poly(A) tail in osk translational stimulation requires an environment in which osk translation is not otherwise silenced, we used the cell-free translation system obtained from Drosophila embryos, which contains no Bru repressor protein. As shown in Fig. 1C, a poly(A) tail as long as 150 A does not stimulate translation above that of transcripts bearing no poly(A) tail. This result is consistent with previous reports from Lie and Macdonald (Lie and Macdonald, 1999) and Castagnetti et al. (Castagnetti et al., 2000) in which poly(A) tails of 36 A and 73 A failed to stimulate translation of osk reporter transcripts in vitro. Remarkably, however, addition of a tail in excess of 200 A enhances translation 20- to 100-fold above that of a transcript bearing no poly(A) tail (Fig. 1C). This increase in translation following polyadenylation is due to a substantial increase in the rate of translation initiation, rather than to stabilization of the transcripts, whose half-lives are comparable (Fig. 1C; data not shown). These results suggest that a tail longer than 150 A may also be necessary for efficient Osk accumulation in vivo, consistent with a role of cytoplasmic polyadenylation and Orb protein in osk mRNA translational regulation.

A poly(A) tail longer than 150A is necessary for Osk accumulation in vivo

Previous reports have shown the involvement of Orb in the establishment of anteroposterior polarity (Chirsterson and McKearin, 1994). To study the potential dependence of osk translation on the poly(A) status of the mRNA in vivo, we first had to determine whether the mRNA is correctly localized in the weak orb mel mutants, as analysis of strong orb alleles has highlighted a role of Orb in osk mRNA posterior localization. In situ hybridization revealed that osk mRNA is correctly
localized in 67% and partially localized in 10% of orb mel oocytes (Fig. 2, upper panel). osk mRNA is not detected at the posterior pole of the remaining 23% of orb mel oocytes. Staufen (Stau) protein, whose distribution has been shown to mirror that of osk mRNA during oogenesis (St Johnston et al., 1991), shows a similar degree of localization in orb mel egg chambers (Fig. 2 lower panel). As shown in Fig. 2, 100% of wild-type and 81% of orb mel oocytes at stage 9/10 accumulate Stau at the posterior pole. In 80% of orb mel oocytes that show posterior Stau accumulation, the protein is fully localized at the posterior pole, and in 20% Stau is also detected in the cytoplasm. Thus, in contrast to the strong orb alleles (Christerson and McKearin, 1994; Lantz et al., 1994), orb mel only mildly affects osk mRNA localization, rendering it suitable for an analysis of the role of cytoplasmic polyadenylation in osk translation.

An indication that Orb is required for osk translation is that, as shown in Fig. 3A and previously reported (Markussen et al., 1995), the amount of both Osk isoforms is dramatically reduced in orb mel when compared with wild type. This reduction could in principle be a consequence of the osk mRNA localization defect. However, the amount of Osk protein in orb mel is at best 25% of the wild type, in spite of the fact that 65% of orb mel oocytes show normal posterior osk mRNA localization. This suggests an involvement of Orb in osk translation. Consistent with this, antibody staining of ovaries reveals that only 67% of orb mel oocytes that localize Stau also accumulate Osk at the posterior (Fig. 3B). In the remaining 33%, no Osk protein is detected. However, trace amounts of Osk are presumably also produced in these oocytes, as maintenance of Stau and osk mRNA at the posterior pole requires Osk protein itself (Rongo et al., 1995; Vanzo and...
chambers, even when levels of Osk sufficient to support abdomen formation are produced, those embryos that develop into adult females are sterile (Table 1).

**Orb interacts with Bic-C and Bru**

Cytoplasmic polyadenylation of mRNA requires CPEB to recruit the enzyme poly(A) polymerase (PAP) on the regulated mRNA. Until recently, only one family of PAP, containing both a catalytic domain and an RRM-like domain, was known. Wang et al. have now identified a novel family of PAP that differs from the canonical PAP for the absence of the RRM-like domain. The prototype of this family is represented by *C. elegans* GLD-2 whose binding to the RNA is mediated by GLD-3, a KH domain containing protein of the BicC family (Wang et al., 2002).

Interestingly, we found that *Drosophila* BicC interacts physically with Orb in co-immunoprecipitation experiments (Fig. 5A). As the phenotype of *BicC* mutants implicates BicC protein as a negative regulator of *osk* translation (Saffman et al., 1998), we tested whether Orb interacts with the translational repressor Bru. Indeed, we could detect a physical interaction between Bru and Orb (Fig. 5B), as revealed by the co-immunoprecipitation of Bru with Orb. By contrast, we detected no direct interaction between Bru and BicC (data not shown) in our assay.

The relevance of these interactions in vivo is further confirmed by the genetic interactions between the *BicC* locus and the *orb* and *aret* loci – the latter encoding Bru protein.

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**Table 1. Phenotypes and fertility of embryos produced by orb*muter* orb*muter* females**

<table>
<thead>
<tr>
<th>Embryonic phenotype</th>
<th>Sterility*</th>
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<tr>
<td>Undeveloped (51%)</td>
<td>–</td>
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<tr>
<td>Posterior patterning defect (27%)</td>
<td>–</td>
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<tr>
<td>Wild type (22%)</td>
<td>100%</td>
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*Sterility was determined by dissection of adult flies and confirmed by Vasa staining of pole cells in embryos.

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**Fig. 4.** A long poly(A) tail is not sufficient to overcome BRE-mediated repression. Translation efficiency of chimeric *osk-lacZ* mRNAs bearing either no poly(A) tail or a tail of >200A in ovary extract. The transcripts used in these experiments bear the 5' region of *osk* mRNA up to the second AUG codon, and either the wild-type *osk* 3'UTR (WT) or an 3'UTR from which the BREs were deleted (ΔABC). The data shown are representative of three independent experiments. The values were normalized for those of a luciferase RNA co-translated as an internal control and the experimental error varies between 3% and 10%.

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**Fig. 5.** BicC, Orb and Bru interact in vitro. (A) Immunoprecipitation experiments were performed on total ovarian extract using α-Orb (lane 1), α-BicC (lane 2) or α-Hrp48 (lane 3) antibodies, or beads alone (lane 4). After precipitation, proteins were detected using α-Orb antibodies. Equal amounts of extract were used in each sample. No Orb protein is retained on the beads alone (lane 4) or with α-Hrp48 (lane 3) antibody. Orb is readily precipitated by α-Orb (lane 1) and α-BicC (lane 2) antibodies. (B) Bru protein is pulled down from total ovarian extract by the α-Orb antibody. The same blot was probed with α-Orb (1) and α-Bru antibodies (2).
Females heterozygous for BicC show a number of AP patterning defects, ranging from head defects to bicaudal embryos (Mahone et al., 1995). The BicC phenotype is suppressed when the mutation is combined with an orb allele or the strong aret allele. aret<sup>Q872</sup> Table 2 shows that 85% of embryos produced by Bic<sup>CYC33/+</sup> females fail to hatch and of those 60% are bicaudal. The null allele orb<sup>F343</sup> efficiently suppresses the Bic-C phenotype and only 13% of the embryos produced by Bic<sup>CYC33/+; orb<sup>F343</sup></sup> females fail to hatch, none of which shows a bicaudal phenotype. As shown in Table 2, the strength of the phenotype and the extent of the suppression depends on the orb allele. Embryonic viability is also improved, up to 72%, in embryos produced by Bic<sup>CYC33/ aretQ872</sup> females.

**DISCUSSION**

Accumulation of Osk activity at the posterior pole of the oocyte is the first step in the assembly of a specialized cytoplasmic sub-compartment, the pole plasm, which contains determinants that direct abdominal patterning and germline differentiation (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992; Kim-Ha et al., 1991; Smith et al., 1992). As mislocalization of Osk activity is detrimental to embryonic development, localization-coupled translational activation ensures specific accumulation of Osk at the posterior pole of the oocyte. Previous studies have shown that Bru is required to silence osk translation during transport, to prevent ectopic Osk accumulation (Kim-Ha et al., 1995). BRE-mediated repression is alleviated upon posterior localization of the transcript, allowing its translational activation (Gunkel et al., 1998). Combining measurement of osk poly(A) tail length in vivo with quantification of the translation activity of the corresponding mRNAs in vivo and in vitro, we have been able to correlate polyadenylation with the translational status of the mRNA. A mutation in Orb, the Drosophila CPEB, leads to shortening of the osk poly(A) tail and to a reduction in Osk accumulation. The fact that, at least in vitro, a long poly(A) tail neither overcomes BRE-mediated repression nor is necessary for repression of osk reporter transcripts, suggests that cytoplasmic polyadenylation is not the decisive event in translational activation of osk at the posterior pole. Rather, it appears that the presence of a 200 A long tail on osk mRNA promotes its efficient translation, allowing accumulation of Osk to levels sufficient for both abdomen and germline formation to proceed.

The prevailing model, which is based on studies of translational control in the Xenopus oocyte, suggests that the polyadenylation status of a transcript correlates with its translational status: a short poly(A) tail corresponding to a silenced mRNA and poly(A) tail elongation triggering translational activation. In Xenopus, upon progesterone treatment a wave of cytoplasmic polyadenylation activates translation of deadenylated and silenced maternally derived mRNAs (Richter, 1999). In Drosophila, translation of bicoid mRNA, which encodes the anterior determinant of the embryo, is repressed until egg activation when poly(A) tail elongation triggers translation initiation (Sallés et al., 1994). Although the correlation between adenylation and translation still holds for several transcripts, a growing body of evidence suggests that the two events may be coincidental but not directly connected. Interestingly, deadenylation and translational repression of Drosophila hunchback (hb) (Chagnovich and Lehmann, 2001) and mouse tPA (Stutz et al., 1998) mRNAs can occur independently of each other. The transcripts are deadenylated concomitant with translational repression, yet repression can occur in the absence of ongoing deadenylation. In arrested primary mouse oocytes, polyadenylation of the tPA mRNA is necessary to counteract the default deadenylation that affects most other oocyte mRNAs, thus preventing its degradation (Stutz et al., 1998).

Our observations suggest that silencing and awakening of osk mRNA translation can occur in the absence of changes in poly(A) tail length and, in fact, osk mRNA bears a long poly(A) tail at all stages of oogenesis, including when it is unlocalized and translationally silent. However, it is still formally possible that at intermediate stages of oogenesis osk mRNA undergoes a deadenylation that goes undetected in our measurements on bulk RNA, and that elongation of the poly(A) tail causes displacement of the repressor complex, leading to translational derepression. This hypothesis is supported by the fact that the repressor protein Bru shares a 50% sequence identity with the Xenopus deadenylation promoting factor EDEN-BP (Kim-Ha et al., 1995). However, we did not detect any obvious pattern of deadenylation in vitro when Bruno was added to the embryonic extract, nor could we observe a shortening of the poly(A) tail of translationally silenced osk transcript recovered form ovarian extract (data not shown). Nevertheless, our results show that BRE-mediated repression is effective independently of the length of the poly(A) tail on osk transcripts, and that a silenced mRNP can be assembled on a naked osk transcript, whether or not it bears a poly(A) tail. These results suggest that polyadenylation is not the sole determining event leading to translational derepression of osk mRNA at the posterior pole, but that the maintenance of a long poly(A) tail, by cytoplasmic polyadenylation, accounts for the enhancement of osk translation and is required for efficient osk translation, to ensure sufficient accumulation of Osk at the posterior pole of the Drosophila oocyte to promote abdominal patterning and germline differentiation.

Furthermore, the physical interaction detected between Orb and Bru, and Orb and BicC suggests the existence of a multi-protein complex containing both positive and negative regulators of osk translation. In this scenario, translational
silencing and polyadenylation are linked through Bru protein, offering a possible explanation as to how CPEB might be recruited to mRNAs in Drosophila, where no canonical CPE has so far been identified. Transcripts properly repressed by Bru, upon localization, could be adenylated by the recruitment of Orb by Bru itself. Loss of Bru repression would, therefore, result in loss of Orb binding with consequent deadenylation and translational silencing. In this model, modulation of the poly(A) tail would be part of the mechanism that regulates translation, ensuring a second level of control over ectopic expression while localizing all the components necessary for efficient translation. Remarkably, mutations in the BRE sites has so far been identified. Transcripts properly repressed by Orb, a maternal gene in Drosophila, Cell 47, 141-152.

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