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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 pre-localization of their transcripts, which are then subject to temporal and spatial translational regulation (St Johnston, 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; Sallés 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 *oo18 RNA 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^{mel}, 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^{mel} 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 fulfilment 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^{mel} 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^{mel} 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_2 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_{16} 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 $MgCl_2$, 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

the US National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/).

Alternatively, poly(A) tail length was measured using the poly(A) test (PAT). cDNAs were synthesized from 500 ng of total ovary RNA, using the BRL Superscript retrotranscriptase. The protocol used was modified to maintain the poly(A) tract in the cDNA. Prior to first strand synthesis, the poly(A) tail was hybridized to saturation with dT_{16} oligo and the oligo dT_{16} subunits ligated before adding the oligodT-anchor, as described by Sallés et al. (Sallés et al., 1994). Specific *osk* cDNAs were amplified by PCR in the presence of [α^{32}P]dCTP using the oligodT-anchor and a specific *osk* oligo (5′-AAG CGC TTG TTT GTA GCA CA-3′). PCR products were separated on 5% denaturing polyacrylamide gels.

DNA constructs

m1m2lacWT was previously described (Gunkel et al., 1998). m1m2lacΔABC was derived from m1m2lacWT by deletion of an *Eco*RI/*Dra*I fragment containing the AB repressor region, and additional deletion of the C region by PCR-directed mutagenesis, using the PCR primers ΔCbclIIup (5′-ACT GTC TAG AAC GTT TTT TTT GTC C-3′) and T3XL (5′-CGA AAT TAA CCC TCA CTA AAG 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 *Not*I and *Kpn*I 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 (yPAP) 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 yPAP.

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 RNeasy 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. C36luca mRNA was synthesized as described (Gray and Hentze, 1994). The RNAs were trace labeled with [α^{32}P]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 embryo 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/rhodhamine-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 $MgCl_2$, 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 $MgCl_2$. 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. Schedl 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, orb^{mel}. orb^{mel} 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). As

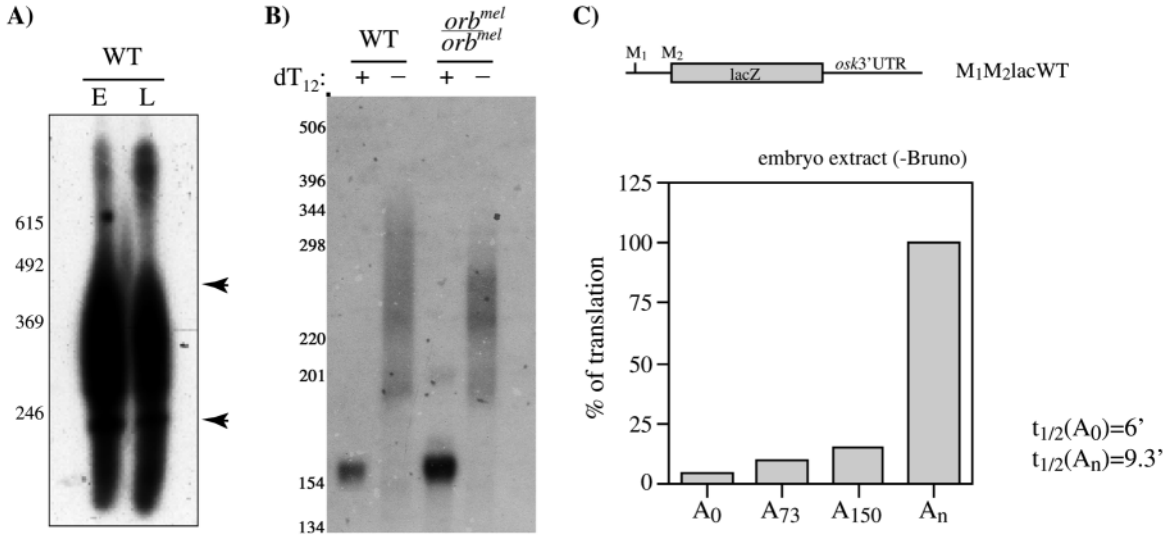

Fig. 1. *osk* mRNA has a long poly(A) tail in vivo. (A) *osk* mRNA poly(A) tail measurement using the PAT assay previously described by Sallés et al. (Sallés et al., 1994) and Chang et al. (Chang et al., 1999). The longest detected *osk* poly(A) tail is about 200 A long, both in early (up to stage 5) 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 dT_{16}. 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 A_0, A_{36}, A_{53}, A_{73} and A_{150} can be explained by a comparable increase in stability of the transcripts. The difference in half-life between the A_0 and A_n 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 (Christerson 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

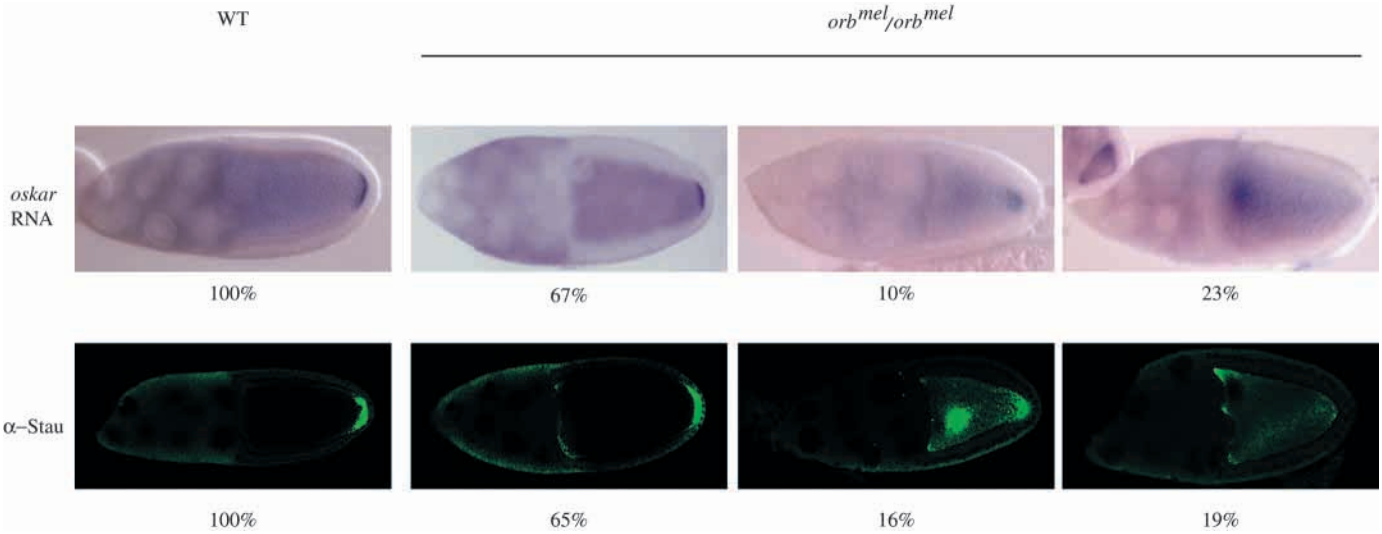

Fig. 2. *osk* mRNA localization is only mildly affected in *orb*^{mel} mutants. Whole-mount in situ hybridization (upper panel) was performed on ovaries from wild-type and *orb*^{mel} homozygous flies, using a DIG-labeled *osk* probe. The result obtained by RNA in situ hybridization was confirmed by immunostaining with α-Stau antibody (lower panel).

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

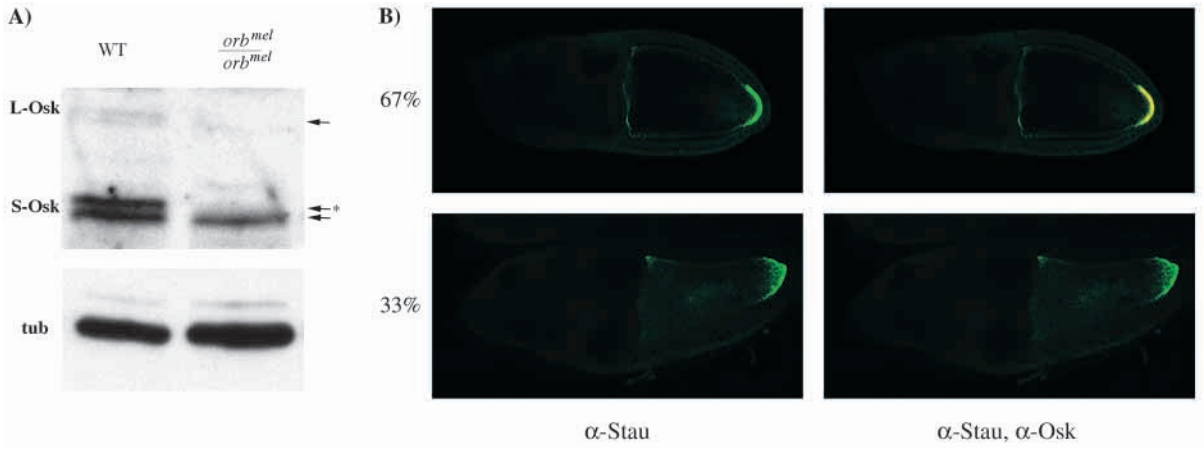

Fig. 3. Osk accumulation in *orb*^{mel} homozygous egg chambers. (A) Western blot analysis was performed on total ovarian protein extracts of similarly aged wild-type and *orb*^{mel} flies. The reduction in Osk phosphorylation is presumably a consequence of the reduction in Osk accumulation, as Osk protein has been shown to be required for its own phosphorylation and stabilization (Markussen et al., 1997; Riechmann et al., 2002). (B) Whole-mount antibody staining of *orb*^{mel} egg chambers with Stau, and Stau and Osk antibodies.

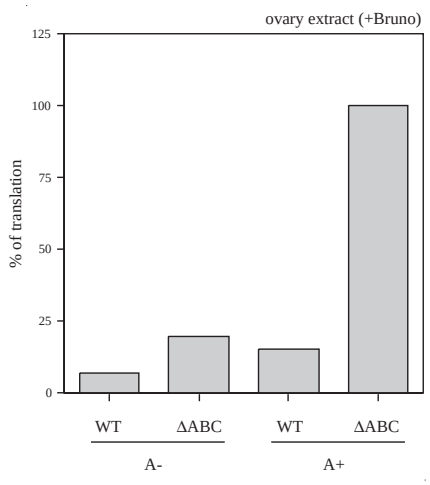

Fig. 4. A long poly(A) tail is not sufficient to overcome BRE-mediated repression. Translation efficiency of chimeric *osk*-lacZ mRNAs bearing either no poly(A) tail or a tail of >200A in ovary extract. The transcripts used in these experiments bear the 5′ region of *osk* mRNA up to the second AUG codon, and either the wild-type *osk* 3′UTR (WT) or an *osk* 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%.

Ephrussi, 2002). It therefore appears that, in orb^{mel} ovaries, *osk* translation is impaired but not abolished. In these egg chambers, *osk* mRNA bears a poly(A) tail that is insufficient to support efficient translation in vitro. Taken together these observations suggest that Orb-mediated cytoplasmic polyadenylation is required to enhance translation, but not for translational derepression of *osk* mRNA.

To confirm this hypothesis, we checked if the addition of a long poly(A) tail could overcome BRE-mediated repression in vitro. For this purpose, we used the ovarian extract, which contains Bru and recapitulates BRE-mediated repression. As shown in Fig. 4, addition of a >250 A tail does not overcome Bru-mediated repression, as BRE-containing transcripts are less efficiently translated than their BRE-deleted counterparts, whether or not they bear a poly(A) tail. As in the embryo extract, the polyadenylated BRE+ and BRE– transcripts are translated more efficiently than the corresponding transcripts lacking a poly(A) tail. Taken together, these results suggest that addition of a long poly(A) tail is not sufficient to overcome repression, but has a stimulatory effect on translation of both BRE+ and BRE– transcripts. The physiological relevance of cytoplasmic polyadenylation in *osk* regulation is strengthen by the observation that, in orb^{mel} egg-chambers, even when levels of Osk sufficient to support abdomen formation are produced, those embryos that develop into adult females are sterile (Table 1).

Table 1. Phenotypes and fertility of embryos produced by orb^{mel}/orb^{mel} females

Embryonic phenotype	Sterility*
Undeveloped (51%)	–
Posterior patterning defect (27%)	–
Wild type (22%)	100%

*Sterility was determined by dissection of adult flies and confirmed by Vasa staining of pole cells in embryos.

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.

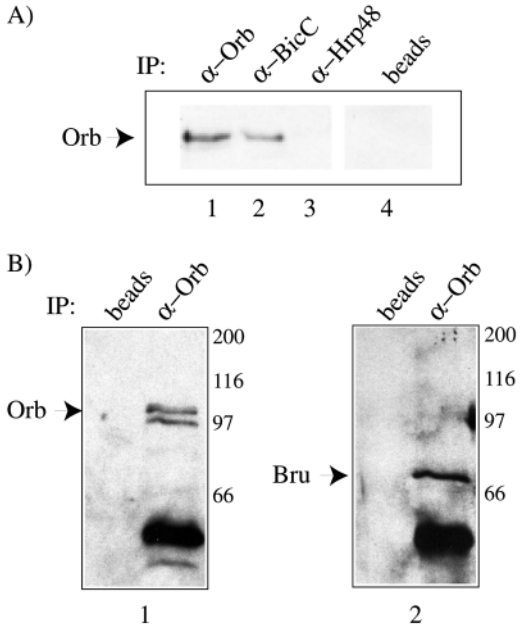

Fig. 5. BicC, Orb and Bru interact in vitro. (A) Immunoprecipitation experiments were performed on total ovarian extract using α-Orb (lane 1), α-BicC (lane 2) or α-Hrp48 (lane 3) antibodies, or beads alone (lane 4). After precipitation, proteins were detected using α-Orb antibodies. Equal amounts of 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).

Table 2. Hatching rate and phenotype of embryos

			Phenotype of non-hatchers	
	Number*	Hatching rate (%)	Bicaudal (%)	Head defects (%)
$BicC^{YC33}$/cyO	624	15	60†	14†
$aret^{QB72}$/cyO	267	74	0	0
orb^{mel}/TM3	236	65	0	0
orb^{F303}/TM3	276	75	0	0
orb^{F343}/TM3	536	85	0	0
$BicC^{YC33}/aret^{QB72}$;+/+	604	72		
$BicC^{YC33}$/cyO; orb^{mel}/TM3	766	65	39†	52†
$BicC^{YC33}$/cyO; orb^{F303}/TM3	746	30	62†	30†
$BicC^{YC33}$/cyO; orb^{F343}/TM3	662	87	0	0

*Number of eggs scored.
†Embryos to 100% either undeveloped or unfertilized.

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, $aret^{QB72}$. Table 2 shows that 85% of embryos produced by *Bic-*C^{YC33}/+ females fail to hatch and of those 60% are bicaudal. The null allele orb^{F343} efficiently suppresses the *Bic-C* phenotype and only 13% of the embryos produced by $BicC^{YC33}$/+; orb^{F343}/+ 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 $BicC^{YC33}/aret^{QB72}$ 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 do not result in ectopic *osk* translation (Kim-Ha et al., 1995), suggesting the existence of a second layer of translational control. Moreover, during embryonic development when *osk* translation is no longer required, both Orb and Bru proteins are depleted in the embryo and *osk* mRNA undergoes complete deadenylation (Sallés et al., 1994).

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