Development 128, 3169-3177 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV4552

Functioning of the *Drosophila orb* gene in *gurken* mRNA localization and translation

Jacqueline S. Chang*,‡, Lihua Tan*, Melisande R. Wolf* and Paul Schedl

Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA
*These authors contributed equally to this work
‡Present address: Department of Biology, DCMB Program, Duke University, Durham, NC 27708, USA

Accepted 25 May 2001

SUMMARY

The *orb* gene encodes an RNA recognition motif (RRM)-type RNA-binding protein that is a member of the cytoplasmic polyadenylation element binding protein (CPEB) family of translational regulators. Early in oogenesis, *orb* is required for the formation and initial differentiation of the egg chamber, while later in oogenesis it functions in the determination of the dorsoventral (DV) and anteroposterior axes of egg and embryo. In the studies reported here, we have examined the role of the *orb* gene in the *gurken* (*grk*)-*Drosophila epidermal growth factor receptor* (*DER*) signaling pathway. During the pre-vitellogenic stages of oogenesis, the *grk-DER* signaling pathway defines the posterior pole of the oocyte by specifying posterior follicle cell identity. This is accomplished through the localized expression of Grk at the very posterior of the oocyte. Later in oogenesis, the *grk-DER* pathway is used to establish the DV axis. Grk protein synthesized at the dorsal anterior corner of the oocyte signals dorsal fate to the overlying follicle cell epithelium. We show that *orb* functions in both the early and late *grk-DER* signaling pathways, and in each case is required for the localized expression of Grk protein. We have found that *orb* is also required to promote the synthesis of a key component of the DV polarity pathway, K(10). Finally, we present evidence that Orb protein expression during the mid- to late stages of oogenesis is, in turn, negatively regulated by *K(10)*.

Key words: Orb, *Drosophila*, DER signaling pathway, *gurken*, mRNA localization

INTRODUCTION

mRNA localization pathways play a central role in axis determination in *Drosophila* (St Johnson and Nusslein-Volhard, 1992). The posterior axis of the embryo is specified by a Nanos (Nos) protein gradient, which is generated from the translation of *nos* mRNA anchored at the posterior pole (Lasko, 1999). *nos* mRNA is localized at the pole during oogenesis by a mechanism that depends upon the *oskar* (*osk*) gene (Ephrussi and Lehmann, 1992; Smith et al., 1992). In stage 8-10 egg chambers, *osk* mRNA is transported to the posterior pole of the oocyte by Staufen (Stau) (St Johnston et al., 1991; St Johnston, 1995; Theurkauf, 1994) where it is activated for translation (Rongo et al., 1995). Newly synthesized Osk protein then nucleates the assembly of structures required to localize *nos* mRNA and to form the pole plasm.

The establishment of the dorsoventral (DV) axis of the *Drosophila* egg and embryo also depends upon mRNA localization (see Nilson and Schupbach, 1999; Shulman and St Johnston, 1999 for reviews). Around stage 7 of oogenesis, the microtubule network is reorganized, and the oocyte nucleus moves from the posterior of the oocyte to the dorsal-anterior corner (Theurkauf, 1994). Shortly thereafter, *K(10)* and *squid* (*sqd*) begin concentrating *gurken* (*grk*) mRNA, which encodes a transforming growth factor α (TGFα) homolog, in a cap just above the oocyte nucleus (Neuman-Silberberg and Schupbach, 1993; Gonzales-Reyes et al., 1995; Serano et al., 1995; Saunders and Cohen, 1999). Translation of *grk* mRNA in stage 9 egg chambers results in the localized production of Grk protein. Grk is secreted through the oocyte plasma membrane and it signals dorsal fate to the overlying follicle cell epithelium by interacting with the *Drosophila* epidermal growth factor receptor (DER; Egfr – FlyBase) (Price et al., 1989; Schejter and Shilo, 1989). Mutations in either *grk* or *DER* disrupt the DV signaling pathway, leading to the production of eggs with a ventralized chorion that either lack or have fused dorsal appendages. The mis-specification of follicle cell identity during oogenesis also disrupts embryonic development (Roth and Schupbach, 1994). Although the loss of *grk* activity in the developing oocyte or *DER* in the follicle cells results in the ventralization of the egg shell and embryo, mutations in *K(10)* and *sqd* have the opposite effect on DV polarity, giving a gain-of-function dorsalization of the egg chamber (Kelly, 1993; Neuman-Silberberg and Schupbach, 1993; Serano et al., 1995). The dorsalized phenotype arises because *grk* mRNA is distributed all along the anterior margin of the oocyte. Translation of the mislocalized mRNA results in the secretion of Grk protein around the entire circumference of the oocyte, and it signals dorsal fate to follicle cells on both the dorsal and ventral sides.

Another gene required for the establishment of polarity in the egg and embryo is *oo18 RNA binding* (*orb*). *orb* functions at multiple steps during oogenesis (Lantz et al., 1994; Christerson and McKearin, 1994). In the presumed null mutant, orb^{343}, oogenesis arrests just before the formation of the 16-cell cyst, and neither oocyte nor nurse cell fates are determined. The orb^{303} mutation is slightly less severe than orb^{343}, and it arrests oogenesis after the 16-cell cyst is formed. There is also a much weaker hypomorphic allele, orb^{mel}, which was generated by the imprecise excision of a P-element inserted into 5′ UTR sequences in the second female-specific exon (Christerson and McKearin, 1994). Unlike the stronger alleles, oogenesis is not irreversibly blocked in orb^{mel}, and homozygous mutant females lay eggs. Many of the eggs produced by orb^{mel} females have ventralized or lateralized chorions that are indicative of a failure in the *grk-DER* signaling pathway (Christerson and McKearin, 1994; Roth and Schupbach, 1994). When fertilized, the embryos from orb^{mel} mothers often show developmental abnormalities expected from defects not only in DV patterning but also in posterior patterning.

The morphological abnormalities in orb^{mel} egg shells and embryos can be attributed to defects in the execution of the mRNA localization pathways responsible for axes determination. In the case of the posterior pathway, the target for Orb function appears to be *osk* mRNA. Instead of being localized in a cap at the posterior pole, *osk* mRNA is distributed throughout much of the oocyte in stages 8-10 orb^{mel} chambers (Christerson and McKearin, 1994). As Orb protein is predicted to have RNA-binding activity (the C-terminal half of the ~100 kDa female Orb protein has two 90 amino acid RNA recognition motif (RRM) domains and a short 60 amino acid cysteine- and histidine-rich region that resembles a zinc finger; Lantz et al., 1992) it might be expected to play a direct role in the *osk* mRNA localization pathway. Indeed, *osk* mRNA is found in an immunoprecipitable complex with Orb protein in vivo (Chang et al., 1999). Although one function may be to help anchor localized mRNAs such as *osk*, Orb also appears to play a central role in controlling the translation of localized mRNAs. Orb is a member of the cytoplasmic polyadenylation element binding protein (CPEB) family of translation regulators (Hake and Richter, 1994). During oogenesis, CPEB proteins bind to the 3′ UTRs of masked maternal mRNAs and control their translation. Initially this interaction is thought to help repress the translation of the masked mRNAs; however, at egg maturation, the CPEB proteins activate translation by promoting polyadenylation (Fox et al., 1989; Paris and Richter, 1990; Paris et al., 199l; Hake and Richter, 1994; Sheets et al., 1994; Barkoff et al., 1998; Minshall et al., 1999). Although a role in repressing *osk* translation has not yet been demonstrated, Orb is required for the translation of *osk* mRNA that has been transported to the posterior pole (Markussen et al., 1995; Chang et al., 1999). In addition to interacting with and promoting the translation of *osk* mRNA, Orb autoregulates its own expression by localizing *orb* mRNA in the oocyte and then activating the translation of the localized message. Both localization and translational regulation seem to be mediated through sequences in the *orb* mRNA 3′ UTR (Tan et al., 2001).

orb is also required for mRNA localization in the DV polarity pathway. Previous studies have shown that the localization of mRNA for two genes in this pathway, *K(10)* and *grk*, is affected in *orb* mutant ovaries (Christerson and McKearin, 1994; Lantz et al., 1994; Roth and Schupbach, 1994). The distribution of *grk* mRNA in vitellogenic orb^{mel} chambers shows similarities to that seen in *K(10)* and *sqd* mutant ovaries: instead of being concentrated in a crescent at the dorsal anterior corner of the oocyte, *grk* mRNA is distributed around the anterior margin of the oocyte. (While the mislocalized *grk* mRNA in *K(10)* and *sqd* mutants remains tightly associated with the oocyte cortex, giving a ring in cross sections, *grk* mRNA in orb^{mel} egg chambers is more diffusely distributed.) The fact that the distribution of *grk* mRNA in orb^{mel} is similar to that in *K(10)* or *sqd* is surprising as the DV defects in orb^{mel} more closely resemble those in *grk* or *DER* mutants. In the studies reported here, we have examined the role of the *orb* gene in this *grk-DER* signaling pathway, as well as earlier in oogenesis, when the *grk-DER* pathway functions in anteroposterior (AP) polarity.

MATERIALS AND METHODS

Fly stocks

The w^1 is described in Lindsley and Zimm (Lindsley and Zimm, 1992). orb^{343} and orb^{303} are from a collection of female steriles on the third chromosome (a gift from C. Nusslein-Volhard) and have been described previously (Lantz et al., 1994). orb^{mel} has been described previously (Christerson and McKearin, 1994). The *hsp83 lacZ* transgenes containing DNA sequences from the *orb* 3′ UTR have been described in detail elsewhere (Lantz and Schedl, 1994; Tan et al., 2001).

Immunoprecipitations and PCR assays

Immunoprecipitation procedures for detecting mRNAs in Orb complexes from ovaries were as described previously (Tan et al., 2001). Total ovary RNA and the mRNAs from immunoprecipitations (IPs) with anti-Orb or anti-Dorsal antibodies were reverse transcribed using an anchored-oligo(dT) primer (5′-GCGAGCTCCGCGG-CCGCGTTTTTTTTTTTT-3′; adapted from Salles et al., 1994). PCR was performed using the anchor primer paired with a gene-specific primer for the *K(10)* 3′ UTR (5′-TAGAGCCTAGGGGACCCAACG-3′), for the *grk* 3′ UTR (5′-TTGGTTTCACCTATATAAGCCTCC-3′) and for the *nos* 3′ UTR (5′-ACTTGTTCAATCGTCGTGGCCG-3′). The amplification was as follows: one cycle of 4 minute at 94°C, 25 cycles of 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C, followed by 1 cycle of 15 minutes at 72°C. The PCR products were analyzed on several different gel systems and transferred to nitrocellulose or Zeta Probe membrane (BioRad) using standard techniques. The blots were hybridized with random primed probes made from *K(10)*, *grk* and *nos* DNA.

Immunocytochemistry and western analysis

Ovaries were dissected in phosphate-buffered saline (PBS), fixed and stained for Orb, Grk and K(10) proteins (essentially as described by Lantz et al., 1994). The Orb antibody is a mouse monoclonal, while the Grk and K(10) antibodies are from rats. Imaging was by laser scanning confocal microscopy (Krypton-Argon Laser, BioRad MRC 600 or a Zeiss Confocal). Western analysis was as described previously (Lantz et al., 1994).

RESULTS

Grk is not properly expressed in orb^{mel} egg chambers

The abnormalities in the distribution of *grk* mRNA in orb^{mel}

would be expected to result in the dorsalization of the egg chamber like that in *K(10)* and *sqd* mutants. However, the DV phenotypes of *orb*^{mel} more closely resemble those expected for loss-of-function mutations in *grk* or *DER*. Given the similarity of Orb to CPEB proteins in other species, a plausible explanation for this discrepancy is that mislocalized *grk* mRNA is not properly translated in *orb*^{mel} egg chambers. To explore this possibility, we examined the expression of Grk and Orb proteins in vitellogenic wild-type and *orb*^{mel} ovaries. In wild-type vitellogenic chambers, high levels of Orb protein accumulate in the cortical cytoplasm around the entire circumference of the oocyte. In *orb*^{mel} ovaries, the pattern of Orb protein accumulation varies between vitellogenic chambers. In many mutant chambers, the level of Orb protein is substantially reduced and little protein is observed around the oocyte cortex (see Fig. 1). In other chambers, the distribution of protein is quite irregular, with nearly normal levels of Orb in some parts of the oocyte and greatly reduced levels in the remainder of the oocyte. Finally, as might be expected from the fact that up to 20% of the eggs laid by *orb*^{mel} mothers can develop into adults, a few of the chambers have nearly normal Orb protein expression (not shown).

While high levels of Grk are found concentrated in a cap above the oocyte nucleus in wild-type chambers, there are severe abnormalities in Grk expression in most *orb*^{mel} chambers (see Fig. 1). In many of the mutant chambers, there is no detectable Grk protein. In other chambers, a small amount of Grk can be seen in the region just above the oocyte nucleus; however, the level of Grk protein is greatly reduced compared with that seen in wild-type chambers. These findings indicate that *orb* is required in the DV signaling pathway for both the localization of *grk* mRNA and the proper expression of Grk. Similar results have been reported elsewhere (Neuman-Silberberg and Schupbach, 1996).

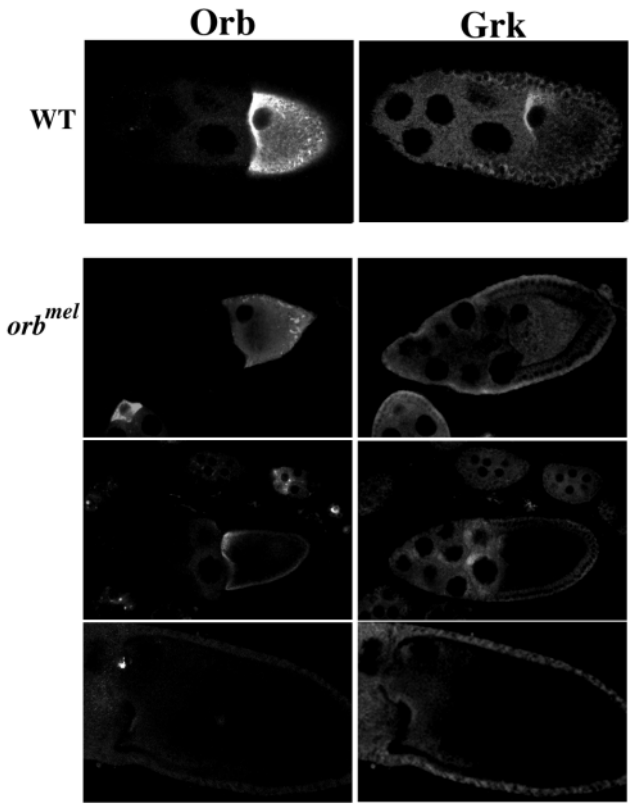

Fig. 1. Defects in Orb and Grk expression in late stage *orb*^{mel} egg chambers. Ovaries from wild-type and *orb*^{mel} females were stained with antibodies against Orb protein (panels on left) and Grk protein (panels on right). The genotypes of the ovaries are as indicated. In wild-type stage 9 egg chambers (top panels), Orb protein accumulates at high levels in the cortical cytoplasm around the entire circumference of the oocyte. Grk protein is localized in a cap above the oocyte nucleus in the anterior corner of the oocyte. Note that the Grk antibody gives 'nonspecific' background staining in nurse cells and follicle cells. This background staining is observed *grk* null chambers (not shown). Representative *orb*^{mel} chambers are shown in the three lower panels. In the first of the group of *orb*^{mel} chambers, the level of Orb protein appears reduced in most of the oocyte except at sites near the posterior pole. Little or no Grk protein is detected. In the panels in the middle, only low levels of Orb protein are observed, while there appears to be a small amount of Grk protein along anterior margin of the oocyte near the oocyte nucleus. The lower panel shows a stage 10 egg chamber with little or no Orb or Grk protein.

orb is required for *grk* expression at the posterior of the oocyte in pre-vitellogenic stages

In addition to its role in DV polarity, the *grk-DER* signaling pathway also functions earliemr in oogenesis in the establishment of A-P polarity (Gonzales-Reyes et al., 1995; Roth et al., 1995). This process begins in region 3 of the germarium, which contains incompletely budded stage 1 chambers, and continues during the pre-vitellogenic stages. Grk protein translated from *grk* mRNA localized at the posterior of the oocyte signals the posterior-most follicle cells, specifying posterior identity (see Fig. 2). As high levels of Orb are concentrated in the posterior cortical cytoplasm of the oocyte in stage 1 and older pre-vitellogenic chambers, it seemed possible that *orb* might also function in the AP *grk-DER* signaling pathway.

The pre-vitellogenic stages of oogenesis in the *orb*^{mel} mutant appear to be completely normal, and the pattern of Orb protein accumulation before stages 7-8 is indistinguishable from wild type. For this reason, we did not expect, nor did we observe, any abnormalities in Grk expression during the early stages of oogenesis in *orb*^{mel} ovaries (not shown; Chang et al., 1999). However, defects in early Grk expression are seen in the two stronger mutants *orb*^{303} and *orb*^{303}.

In the presumed protein null, *orb*^{343}(Lantz et al., 1994), little or no Grk can be detected (Fig. 2). A different result is obtained for *orb*^{303}. *orb*^{303} expresses only one of the two female Orb isoforms and this protein exhibits an abnormal pattern of accumulation (Lantz et al., 1994). In wild-type ovaries, Orb protein begins concentrating in the presumptive oocyte in region 2 of the germarium, which contains newly formed 16-cell cysts. Unlike wild type, the mutant Orb^{303} protein does not properly localize to the presumptive oocyte and instead is distributed at abnormally high levels in all 16 germ cells (Fig. 2). The level of Orb^{303} protein remains elevated when the aberrant cysts exit the germarium; however, as these pseudo-egg chambers 'mature', the mutant protein begins to disappear. As can be seen in Fig. 2, Grk expression is prematurely induced in *orb*^{303} ovaries. Newly formed 16-cell cysts in region 2 of the germarium not only have elevated amounts of the mutant Orb protein but also have abnormally high levels of Grk.

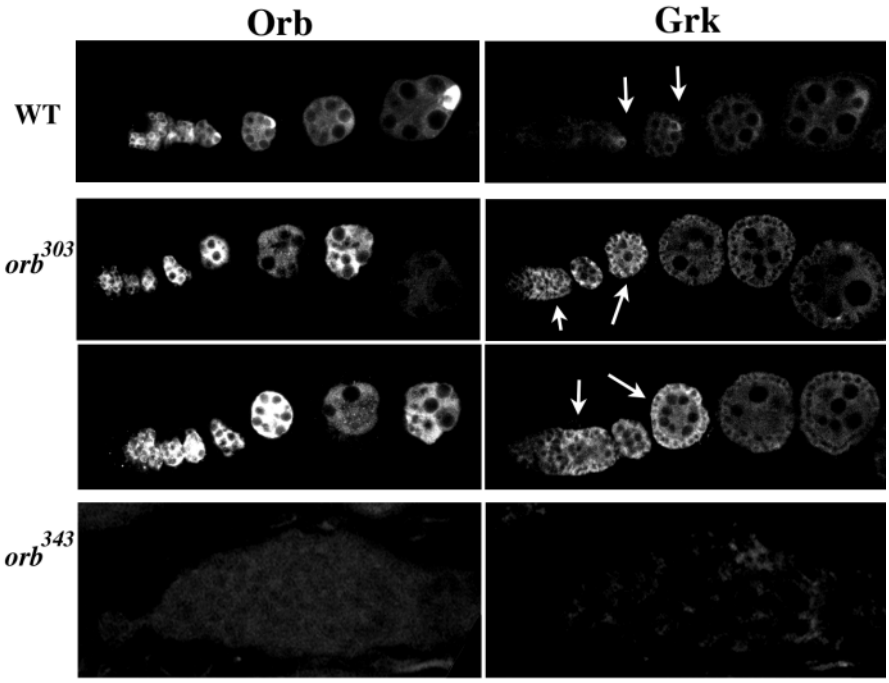

Fig. 2. Orb and Grk are improperly localized in early stage egg chambers in *orb*^{303} and completely absent in *orb*^{343}. Ovaries from wild-type and *orb* mutant females were stained with antibodies to Orb and Grk protein. Genotypes are as indicated. The two panels at the top show the pattern of Orb and Grk protein accumulation in the germarium, and early stages of a wild-type ovariole. Note that Orb protein can be detected in region 2 of the germarium, where it begins concentrating in the presumptive oocyte. In the stage 1 chamber in region 3 and older chambers, much of the Orb protein is in the oocyte at the posterior end. Grk protein can be detected in the stage 1 chamber that has not yet completely budded off from the germarium (left arrow) and in slightly older stages (right arrow) at the posterior of the oocyte. The two sets of panels in the middle show the Orb and Grk protein staining pattern in *orb*^{303} ovarioles. The Orb protein distribution in *orb*^{303} cysts and chambers is abnormal. Unusually high levels of Orb protein are observed in the cysts in region 2 and 3 of the germarium, and this protein appears to be distributed in all germ cells. High levels of uniformly distributed protein can be seen in pseudo-egg chambers just budded off from the germarium, but then the level of Orb protein begins to decline as the aberrant chambers age. Grk also shows an anomalous pattern of accumulation (panels on right). As indicated by the arrows on the left, Grk is expressed prematurely and abnormally high levels of Grk protein are observed in cysts in region 2 of the germarium. High levels of Grk protein are also visible in stage 1 pseudo-egg chambers that have just budded off from the germarium (arrows on right). When the level of Orb protein begins to decline, Grk protein is also reduced. The two panels at the bottom show that there is little or no Orb or Grk protein in *orb*^{343} ovarioles.

Moreover, instead of being localized in only a single germ cell (the presumptive oocyte), as in wild type, Grk is distributed throughout the cyst and can also be detected in the surrounding somatic tissue. This can be seen most readily in pseudo-egg chambers that have just budded off from the germarium. As Grk is thought to help polarize the early egg chamber, the uniform distribution of Grk protein in these early mutant cysts could help explain why the germ cells in mutant cysts do not undergo the rearrangements that position the oocyte at the posterior. In older pseudo-egg chambers that have little Orb protein, Grk also disappears. These findings indicate that wild-type *orb* function is required for the localized expression of Grk protein during early oogenesis.

Orb is required for K(10) protein expression

In wild-type stage 8-10 egg chambers, *K(10)* mRNA is concentrated along the anterior margin of the oocyte. While *K(10)* mRNA also accumulates along the anterior margin of *orb*^{mel} vitellogenic chambers, it is not as tightly concentrated as in wild type and spreads towards the center of the oocyte. To test whether *orb* is also required for the expression of K(10) protein, we stained wild-type and *orb*^{mel} ovaries with antibodies against K(10) and Orb proteins (Fig. 3). In wild-type ovaries, K(10) protein can first be detected in the oocyte in previtellogenic stages. By the time Grk is first expressed at the dorsal anterior corner of the oocyte in stage 8-9 chambers, high levels of K(10) are found concentrated in the oocyte nucleus. The pattern of K(10) protein expression in pre-vitellogenic *orb*^{mel} chambers appears to be normal. In contrast, in most stage 8-10 *orb*^{mel} chambers, little or only low levels of K(10) are observed in the oocyte nucleus (Fig. 3). We also examined K(10) expression in *orb*^{303} ovaries. Low levels of K(10) could be detected in cysts that had high levels of Orb^{303} protein (not shown). These findings indicate that *orb* is required for the proper expression of K(10) protein.

K(10) but not *grk* mRNA is associated with Orb protein in vivo

Orb is predicted to be an RNA-binding protein and consequently might be expected to interact with its regulatory targets. Consistent with this suggestion, two known orb regulatory targets, *osk* and *orb* mRNA, are found in immunoprecipitable complexes with Orb protein in ovary extracts (Chang et al., 1999; Tan et al., 2001). The complexes seen in ovary extracts seem to be specific, as two other localized mRNAs, *nanos* (*nos*) (see Fig. 4) and *bicoid* (*bcd*) (not shown) cannot be detected in Orb immunoprecipitable complexes. To test whether *grk* and *K(10)* mRNAs are associated with Orb protein, we immunoprecipitated Orb protein complexes from wild-type ovaries with Orb antibody. RNA isolated from the immunoprecipitated complexes was reverse transcribed with an ‘anchored’ oligo dT primer and the reverse transcription products were then PCR amplified using an upstream primer specific for the 3′ UTRs of *grk*, *K(10)* or, as a control, nos and a downstream primer corresponding to just the ‘anchor’ sequence. The PCR products were then detected with ^{32}P-labeled grk, K(10) and nos probes. Because the anchored oligo dT primer can bind to different sites in the poly A tail, a smear of PCR products should be observed that extends upwards from the fragments generated by the

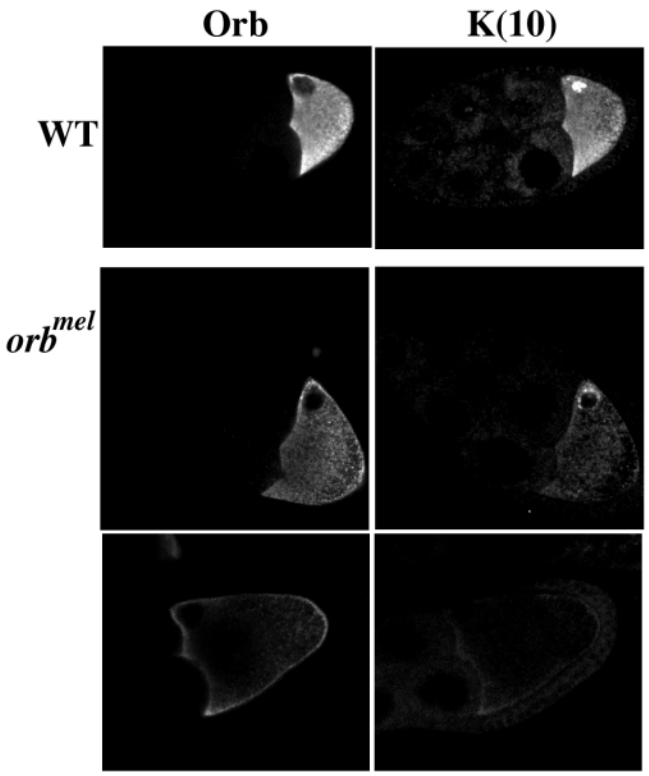

Fig. 3. Defects in K(10) protein expression in *orb*^{mel} ovaries. Wild-type and *orb*^{mel} mutant ovaries were stained with Orb and K(10) antibodies. The two panels at the top show a stage 9 wild-type egg chamber stained with Orb (left) or K(10) antibody. Orb protein can be detected in the oocyte cortex, while K(10) protein is concentrated primarily (though not exclusively) in the oocyte nucleus. The four lower panels show Orb and K(10) proteins in stage 9 and early stage 10 *orb*^{mel} egg chambers. Though the level of Orb protein in these two egg chambers is reduced compared with wild type, the extent of reduction is not great as seen in other mutant chambers (see, for example, Figs 1, 5). In the top *orb*^{mel} chamber, some residual K(10) protein can be seen around the oocyte nucleus. In the bottom *orb*^{mel} chamber there is little K(10) protein.

hybridization of the anchor close to the polyA addition site in each RNA. To control for the nonspecific association of mRNAs with antibody-bound beads, we reverse transcribed and PCR amplified (using the same primers) RNA immunoprecipitated from ovary extracts using Dorsal antibody.

As shown in Fig. 4, *K(10)* mRNA seems to be associated with Orb protein in ovary extracts. The expected smear of K(10) 3′ UTR-specific amplification products is observed in total RNA and in the Orb immunoprecipitate, while these amplification products are absent in the Dorsal immunoprecipitate. Unexpectedly, the results for *grk* were the same as for *nos*; we were unable to detect 3′ UTR sequences from *grk* mRNA in the Orb immunoprecipitates (see Fig. 3). One possible explanation for this result could be that the bound mRNAs are partially hydrolyzed during the immunoprecipitation procedure. For example, in the case of ~5 kb *orb* mRNA, we found sequences from the ~1.0 kb 3′ UTR in Orb immunoprecipitates, while sequences upstream of the 3′ UTR in the protein-coding region were not detected (Tan et al., 2001). Though *grk* mRNA is only about ~1.5 kb in length, it is possible that we failed to detect grk in the immunoprecipitates because Orb complexes are associated with sequences close to the 5′ end of *grk* mRNA rather than with the 3′ UTR. To test this possibility, we reverse transcribed and PCR amplified with primers complementary to sequences in the *grk* 5′ UTR. While appropriate amplification products were observed in the total RNA control, we did not detect amplification products in the Orb immunoprecipitates (data not shown).

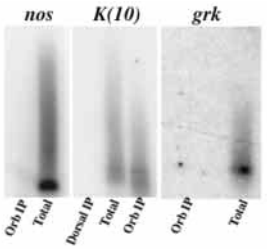

Fig. 4. *K(10)* but not *grk* mRNA is in an immunoprecipitable complex with Orb protein. Anti-Orb or anti-Dorsal antibody was used for immunoprecipitation of wild-type ovary extracts. RNA isolated from the immunoprecipitates (IP) and from the total extracts was reverse transcribed with an anchored oligo(dT) primer (see Materials and Methods) and the resulting cDNAs were subjected to PCR amplification using the anchored primer and gene-specific primers for either *K(10), grk* or *nos*. The amplification products were displayed by electrophoresis and blotting to nitrocellulose and the filters were then hybridized with gene specific probes. *K(10)* sequences can be RT-PCR amplified from the Orb IP sample, as well as from the total RNA pool, but not from the Dorsal IP sample (middle panel). In both the Orb IP and the total samples, the *K(10)* probe hybridizes to a prominent band and an upward smear. The prominent band corresponds in size to a PCR amplification product extending from the K(10) primer in the 3′ UTR to the beginning of the poly(A) tail. The smear arises from hybridization of the anchored oligo dT primer at different sites in the poly(A) tail. Neither *nos* PCR products (left panel) nor *grk* PCR products (right panel) could be detected in the Orb IP lanes, but could be amplified from the total RNA pool. As expected, no amplification products were observed when the RT step was omitted.

Is *orb*^{mel} epistatic to K(10)?

If the reduction in K(10) protein expression were the only effect of the *orb*^{mel} mutation on the *grk-DER* DV signaling pathway, it would be expected to result in dorsalized egg chambers. However, the fact that *orb*^{mel} produces ventralized rather than dorsalized eggs can be explained by the finding that Grk protein expression is also substantially reduced in *orb*^{mel} vitellogenic chambers. As *grk* mRNA is mislocalized in a *K(10)*-like pattern in *orb*^{mel} egg chambers, it would be reasonable to suppose that the *orb*-dependent activation of Grk protein expression is downstream of the *K(10)*-dependent step in the *grk-DER* pathway. In this case, *orb*^{mel} should be epistatic to *K(10)* – that is, the double mutant should have essentially the same defects in DV polarity as observed in *orb*^{mel} alone. To test this prediction, we generated females homozygous for both the *orb*^{mel} and *K(10)* mutations, and examined their eggs. The results of this analysis are presented in Table 1. Contrary to our expectations, the vast majority of the eggs produced by the double mutant females have the dorsalized phenotype of *K(10)*, rather than the ventralized phenotype of *orb*^{mel}.

Grk protein is upregulated in the *K(10); orb*^{mel} double mutant

If *K(10)* is epistatic to *orb*^{mel}, then the pattern of Grk protein expression in the double mutant ovaries should resemble that

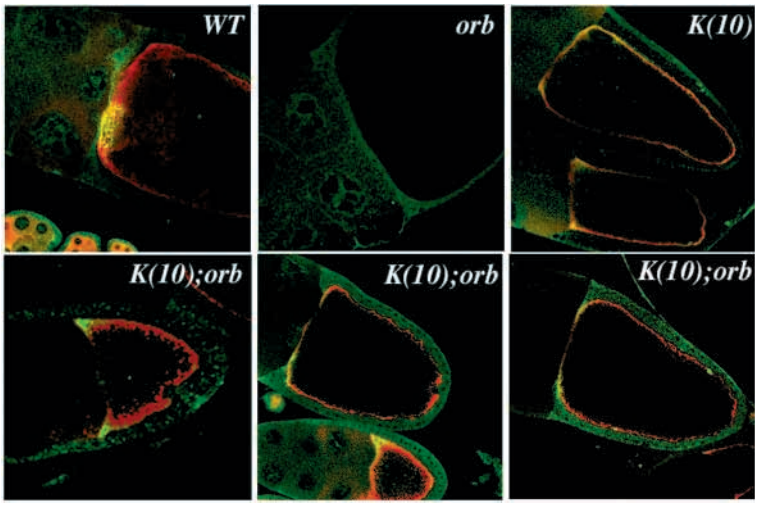

Fig. 5. Orb and Gurken expression in *K(10); orb^{mel}* double mutant ovaries. Ovaries from wild-type (*w^{1}*), *orb^{mel}*; *K(10)* and *K(10); orb^{mel}* females were simultaneously stained with antibodies against Orb (red) and Grk (green). To make protein levels comparable between the different ovaries, the ovaries were stained in parallel and analyzed by confocal microscopy using identical conditions. Background staining by the Grk antibody is nonspecific and present in the same pattern in *grk*-null egg chambers (data not shown). The genotypes are as indicated in each panel. In the wild-type stage 10 egg chamber (top left panel) Grk protein is localized to the anterior corner of the oocyte above the nucleus, while Orb protein can be seen as a ring around the edges of the oocyte. In the *orb^{mel}* stage 10 egg chamber (top middle), neither Grk nor Orb is observed. In the *K(10)* stage 8-9 egg chambers (top right), the level and localization of Orb resembles that seen in wild type, while Grk protein can be seen along the anterior margin of the oocyte (as yellow staining). In the *K(10); orb^{mel}* egg chambers shown in the panels at the bottom, the level of Orb protein is greatly increased compared with that seen in the *orb^{mel}* chamber, and the localization pattern appears to be quite similar to that seen in wild-type egg chambers. Note that red staining can be seen around the circumference of each of the double mutant oocytes. As in *K(10)* mutant chambers, Grk protein can be seen along the entire anterior margin of the double mutant oocytes.

observed in *K(10)* not *orb^{mel}* mutant ovaries. In the experiment shown in Fig. 5, we stained *K(10)*, *orb^{mel}* and *K(10); orb^{mel}* ovaries with antibodies against Grk protein. We also monitored Orb protein expression in these different genetic backgrounds. The *K(10)* mutant differs from wild type in that Grk protein is expressed all along the anterior margin of the oocyte in stage 8-10 chambers, instead of being restricted to the dorsal anterior corner. As expected from the egg shell phenotype of the double mutant, we found that Grk protein expression in *K(10);orb^{mel}* egg chambers resembles that seen in *K(10)* not that of *orb^{mel}*: high levels of protein are observed all along the anterior margin of the oocyte (see Fig. 5).

Orb protein expression in *orb^{mel}* is upregulated by the *K(10)* mutation

These findings indicate that the *K(10)* mutation suppresses the *orb^{mel}* defect in the translation of *grk* mRNA, and consequently Grk protein expression. A possible mechanism is suggested by a comparison of the Orb expression pattern in *K(10); orb^{mel}* and *orb^{mel}* mutant ovaries. While little Orb is detected in most vitellogenic *orb^{mel}* chambers, the level of Orb protein in stage 7-10 *K(10);orb^{mel}* chambers is close to that seen in wild type (compare Orb antibody staining in the different chambers shown in Fig. 5). As the defect in *grk* mRNA translation in *orb^{mel}* is thought to be a consequence of the greatly reduced levels of Orb protein in vitellogenic mutant chambers, it is reasonable to think that the increase seen in the double mutant would be sufficient to restore Grk expression.

To provide further evidence that the level of Orb protein accumulation is close to that of wild type in the double mutant, we probed western blots of ovary extracts. The results are shown in Fig. 6. Whereas the amount of Orb protein in *orb^{mel}* ovary extracts is greatly diminished (estimated to be about 1/20th that in wild type), the level of protein in the double mutant is much closer to wild type (estimated to be about 1/3rd the level in wild type). The increased accumulation of Orb protein seen in both whole mounts and western blots is consistent with the idea that *K(10)* negatively regulates Orb expression.

We also examined Orb protein expression in *K(10)* mutant ovaries that are wild type for the *orb* gene. However, we were unable to detect any obvious difference in the amount of protein. As Orb is already present in substantial quantities in wild-type chambers, there could be other, *K(10)*-independent mechanisms that limit accumulation above a certain level. Alternatively, it is possible that the effects of the *K(10)* mutation on Orb protein expression are specific for the *orb^{mel}*

Table 1. Phenotypes of various *orb* mutants

	Chorion phenotype		
Genotype	Wild type	DV defects	% DV defect (type)
(A)			
w^{1}	3818	110	2.8 (ventralized)
K(10)/K(10	0	1223	100 (dorsalized)
orb^{mel}/orb^{mel}	5	43	89.6 (ventralized)
K(10)/K(10); orb^{mel}/orb^{mel}	1	643	98.4 (dorsalized)
(B)			
w^{1}	937	15	1.9 (ventralized)
Hd19G,orb^{ere}; Tm3Ser	493	122	19.8 (ventralized)
K(10)/+; Hd19G orb^{343}	1374	178	11.5 (ventralized)

Six females flies of the indicated genotype were placed on apple juice-agar plates with yeast for 24 hours at 22-23°C.

(A) *K(10)* suppresses the dorsoventral defects associated with *orb* mutants. Eggs laid by *w^{1}*, *orb^{mel}*, *K(10)* and the *K(10); orb^{mel}* double mutant were examined and scored for chorion defects. The *w^{1}* strain present in the laboratory shows a background level of 1-3% ventralized eggs.

(B) The *K(10)* mutation weakly suppresses the chorion defects induced by reduced Orb protein expression. The *Hd19G* transgene insert on the third chromosome has the *orb* 3′ UTR fused to *lacZ* protein coding sequences. Expression of this hybrid mRNA in ovaries is driven by the *hsp83* promoter. The *hsp83:lacZ orb* 3′ UTR transgene, *Hd19G*, is a dominant negative and interferes with *orb* autoregulation (Tan et al., 2001). Approximately 20% of the eggs produced by *orb^{343}*/+ females carrying a single copy of the *Hd19G* transgene are ventralized. The percentage of ventralized eggs dropped from 20% in the *Hd19G, orb^{343}*/+ strain to 12% when the females are also heterozygous for the *K(10)* mutation.

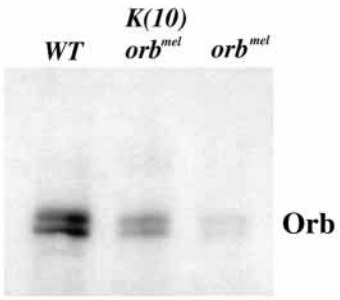

Fig. 6. Orb protein expression is increased in *K(10); orb^{mel}* double mutants. Ovary extracts were prepared from wild-type *w^{1}*, *orb^{mel}* and *K(10); orb^{mel}* females. The Orb protein level was then analyzed by western blotting. A representative blot is shown in this figure. The two bands seen in this blot correspond to the two Orb protein isoforms typically seen in wild-type and *orb^{mel}* mutant ovaries. To control for the total protein in the extracts, the blots were reprobed with antibodies against the snRNP protein Snf or Armadillo (data not shown). Using NIH Image, the ratio of Orb protein to the loading control, Snf or Armadillo was calculated for each extract, and then normalized to that in wild-type ovaries. In the experiment shown here, the ratio is 1=*w^{1}*; 0.3=*K(10); orb^{mel}* and 0.05=*orb^{mel}*. The ratios estimated for the double mutant ranged in different experiments from 0.3 to 0.5 and for *orb^{mel}* from 0.05 to 0.1.

allele. The *orb* mRNA expressed by *orb^{mel}* lacks sequences from the 5′ UTR and this might make this message especially sensitive to the repressive activity of the K(10) protein.

To further test the effects of *K(10)* on *orb*, we took advantage of the dominant negative activity of a transgene, *hsp83:Lac-Z orb* 3′ UTR, which constitutively expresses a transcript that contains *lacZ*-coding sequences fused to the *orb* 3′ UTR. Previous studies (Tan et al., 2001) have shown that this transgene RNA interferes with *orb* autoregulation by competing with the endogenous mRNA for *orb* function. Because Orb activity is required for its own synthesis, this competition downregulates Orb expression. The downregulation of Orb protein disrupts the *grk-DER* signaling pathway, giving ventralized eggs. A single copy of the transgene has only very modest phenotypic effects, inducing DV polarity defects in a few percent of the eggs. However, when the *hsp83:lacZ orb* 3′ UTR transgene is introduced into females heterozygous for *orb^{343}*, about 20% of the eggs have DV defects. If *K(10)* functions as a negative regulator of the wild-type *orb* gene, then a *K(10)* mutation might act as a suppressor, reducing the frequency of DV defects in eggs from *hsp83:lacZ orb* 3′ UTR, *orb^{343}*/+ females. As can be seen in the second part of Table 1, *K(10)* is a weak suppressor. When *hsp83:lacZ orb* 3′ UTR, *orb^{343}*/+ females have only a single wild type *K(10)* gene, the frequency of DV defects is reduced.

DISCUSSION

In the studies reported here, we have investigated the role of the *orb* gene in *grk-DER* signaling. We find that *orb* functions at several levels in this signaling pathway through effects on both K(10) and Grk translation and Grk localization. Early in oogenesis, the *grk-DER* pathway is used to specify posterior identity to the follicle cells at the posterior end of the oocyte. It appears that *orb* activity is required for this early signaling pathway, as abnormalities in Grk expression are observed in *orb^{343}* and *orb^{303}* ovaries. In the presumed Orb protein null, *orb^{343}*, Grk is not detected. In *orb^{303}*, Grk expression parallels the aberrant pattern of Orb^{303} protein accumulation. In newly formed 16-cell cysts, all germ cells have high levels of the Orb^{303} protein. These germ cells also express much higher than normal levels of Grk protein. In older pseudo-egg chambers, both Orb^{303} and Grk disappear. These findings argue that the Orb^{303} protein inappropriately activates translation of *grk* mRNA, and that the mutant Orb protein must be present to sustain Grk expression. Later in oogenesis, after the oocyte nucleus moves from the posterior of the oocyte to the dorsal anterior corner, the *grk-DER* pathway is used to signal dorsal identity to the follicle cells above the oocyte. At this stage *orb* is required for the proper expression not only of Grk but also of K(10).

How does *orb* function in regulating translation and localization? Orb homologs in other organisms, the CPEB proteins, interact with elements in the 3′ UTRs of masked mRNAs, and activate their translation by a mechanism that is thought to involve polyA addition (see Richter, 1999 for a review). As the translational function of the CPEB proteins is conserved in animals as diverse as clams and mice, it would be reasonable to suppose that the role of the *orb* gene in the *Drosophila grk-DER* signaling pathway also involves translational activation. Accordingly, the defects in the expression of both Grk and K(10) proteins would arise because wild type *orb* activity is required to properly regulate the translation of *grk* and *K(10)* mRNAs. In the case of *K(10)*, it seems possible that Orb protein might act directly on the mRNA. First, *K(10)* mRNA is associated with Orb protein in an immunoprecipitable complex (see above) and second, *K(10)* mRNA is mislocalized in *orb* mutant ovaries (Christerson and McKearin, 1994; Lantz et al., 1994). For *grk*, the situation appears to be more complicated and will be considered further.

As translational activation by CPEB proteins in other systems has been tied to polyadenylation, an obvious question is whether the polyA tails of *K(10)* mRNA are affected in *orb* mutants. Unfortunately, experiments aimed at testing this point have been inconclusive. Using the anchored-dT RT-PCR procedure of Salles et al. (Salles et al., 1994), we found that *K(10)* mRNA isolated from the strong loss-of-function *orb* mutant, *orb^{343}*, had shorter poly(A) tails than wild type (J. S. C., unpublished). However, we cannot excluded the possibility that the short poly (A) tails in this mutant arise because *K(10)* mRNA is targeted for deadenylation in the absence of translation. For *orb^{mel}*, the average poly(A) length appeared, at most, to be only marginally shorter than wild type. Of course, as K(10) protein is expressed normally in pre-vitellogenic stages in this mutant, the presence of mRNAs with extended poly(A) tails is not altogether surprising. Further studies will be required to determine whether the mechanism used to promote the translation of *K(10)* mRNA depends upon polyA addition as is thought to be the case in other organisms.

In contrast to *K(10)*, *grk* mRNA was not found in Orb immunoprecipitates. Although there are many reasons why an Orb protein:*grk* mRNA complex might not be detected, this result forces us to consider the possibility that *orb* acts on *grk* only indirectly. In this case, we would have to propose other mechanisms to account for the defects in both the localization and translation of *grk* mRNA that are observed in *orb* mutants.

It seems possible that the mislocalization of *grk* mRNA in *orb*^{mel} could arise, at least in part, because the expression of K(10) protein is greatly reduced in stage 8-10 *orb*^{mel} chambers. However, as the localization defects in *orb*^{mel} are more severe than those seen in *K(10)*, *orb* may regulate some other factor in addition to K(10) that helps direct the proper localization of *grk* mRNA. An obvious candidate is *sqd*. Although we did not detect any alterations in Sqd protein expression in *orb*^{mel} chambers, it should be noted that only one of the three Sqd isoforms seems to be involved in *grk* mRNA localization (Norvell et al., 1999). Consequently, any effects on the expression of this specific isoform could be obscured by the other isoforms.

We must also explain why *grk* mRNA is not properly translated in *orb* mutant ovaries. Orb protein could be required for the expression of factors that activate translation of *grk* mRNA. In *orb*^{303} this factor(s) could be prematurely produced throughout the cyst, leading to the very high levels of unlocalized Grk seen in this mutant. As *K(10)* and *sqd* do not seem to function in the localization or translation of *grk* mRNA at the posterior of the oocyte in pre-vitellogenic stages, the *orb* regulatory target(s) early in oogenesis could be different from that used later in DV signaling. Another possibility is that *orb* regulates the expression of a signal(s) that coordinates the activation of *grk* mRNA translation with other events in oogenesis. This function is suggested by the fact that CPEB activity in other organisms helps govern progression through oogenesis (Sheets et al., 1995; De Moor and Richter 1999; Barkoff et al., 2000; Groisman et al., 2000) and by the finding that *grk* expression in the DV pathway is sensitive to check points that monitor progression through meiosis (Gonzalez-Reyes et al., 1997; Ghabrial and Schupbach, 1999). In this case, signals crucial for translation of *grk* mRNA might not be produced in the absence of *orb* activity.

Role of K(10) in Orb protein expression

The epsitatic relationship between *orb*^{mel} and *K(10)* is rather surprising. As *orb* is required for the localization and translation of *grk* mRNA, we expected that *orb*^{mel} would be epistatic to *K(10)*. However, contrary to this expectation, eggs produced by *K(10);orb*^{mel} double mutant females have the dorsalized egg shell phenotype that is characteristic of *K(10)* mutations, rather than the ventralized phenotype of *orb*^{mel}. This result implies that the loss of *K(10)* function rescues the *orb*^{mel} defect in *grk* mRNA translation (but not the localization defect). Interestingly, a similar epistatic relationship is found for *K(10)* and mutations in the *spindle* (*spn*) genes (Gonzalez-Reyes et al., 1997). Mutants in the *spn* genes resemble *orb* in that *grk* mRNA is mislocalized in a *K(10)*-like pattern but is not properly translated, giving ventralized eggs. Moreover, the defects in *grk* mRNA translation in *spn* mutants can also be rescued by mutations in *K(10)* and double mutant females produce dorsalized eggs. To explain these findings, Gonzalez et al. have postulated that the function of the *spn* genes is to alleviate *K(10)*-dependent repression of *grk* mRNA translation (Gonzalez-Reyes et al., 1997).

Although *orb* could have a similar role in alleviating *K(10)*-dependent repression of *grk*, an alternative (or additional) explanation for the epistatic relationship between *orb*^{mel} and *K(10)* is that *K(10)* negatively regulates Orb protein expression. This possibility is suggested by the finding that the amount of Orb protein in vitellogenic chambers from the double mutant is close to that seen at equivalent stages in wild-type ovaries. The restoration of near wild-type levels of Orb protein in these *orb*^{mel}*;K(10)* chambers would in turn be expected to produce a concomitant increase in Grk expression, giving the observed gain-of-function phenotype.

Complicating our conclusion that *K(10)* negatively regulates Orb expression is the finding that K(10) protein does not properly accumulate in the oocyte nucleus of vitellogenic *orb*^{mel} chambers. One might have expected that this reduction in the level of K(10) protein would alleviate the *K(10)*-dependent repression of Orb protein expression, leading to an increased accumulation of Orb protein in the *orb*^{mel} mutant and a dorsalized (not ventralized) DV phenotype. However, it does not. One explanation for this paradox is that *orb*^{mel} is wild type for *K(10)*, whereas this is not the case in the double mutant. In addition, there are no apparent defects in K(10) expression in pre-vitellogenic *orb*^{mel} chambers. It is possible that there is sufficient residual K(10) protein remaining at later stages to effectively repress *orb* (see examples in Fig. 3), or that *K(10)* repression of *orb* is linked to a process that occurs before the time when the accumulation of K(10) protein drops below some critical threshold value in the *orb*^{mel} chambers. In this context, it is interesting to note that the most severe defects in both *orb* mRNA localization and Orb protein expression in *orb*^{mel} occur after the reorganization of the cytoskeleton and the concomitant movement of the oocyte nucleus from the posterior to the anterior of the oocyte. This marks a shift in the localization of *orb* mRNA and the site of Orb protein synthesis from the posterior of the oocyte to the anterior. As the expression of K(10) protein before this time is normal in *orb*^{mel} ovaries, its possible that K(10) repression may be somehow linked to this spatial transition in *orb* regulation.

Although the *K(10)* mutation had quite dramatic effects on Orb expression in *orb*^{mel} ovaries, there were no obvious changes in Orb expression in *K(10)* mutant ovaries that are wild type for *orb*. It seems possible that there may be some special features of the *orb*^{mel} mutation that make it especially sensitive to K(10) repression. However, our genetic interaction experiments suggest that *K(10)* also negatively regulates expression of the wild-type *orb* gene. An important unanswered question is the mechanism of regulation. Here, there is a problem of compartmentalization. For example, as *orb* mRNA is thought to be synthesized in nurse cells, K(10) protein is unlikely to influence transcription. Even effects on the localization/translation of *orb* mRNA must be indirect. Further studies will clearly be required to understand how *K(10*) regulates *orb* expression.

We thank Drs R. Cohen, L. Gavis, E. Mohier and T. Schupbach for antibodies, genomic fragments, cDNAs and fly stocks. We also thank members of the Schedl Laboratory for helpful discussions and suggestions, Mr J. Goodhouse for technical assistance with the confocal microscopy, and Mr G. Grey for preparing fly food. L. T. was supported by a NIH postdoctoral Fellowship. This research was supported by a grant from NIH.

REFERENCES

Barkoff A., Ballantyne S. and Wickens, M. (1998). Meiotic maturation in Xenopus requires polyadenylation of multiple mRNAs. *EMBO J.* **17**, 3168-3175.

Barkoff, A. F., Dickson K. S., Gray, N. K. and Wickens, M. (2000). Translational control of cyclin B1 mRNA during meiotic maturation: coordinated repression and cytoplasmic polyadenylation. *Dev. Biol*. **220**, 97-109.

Chang, J. S., Tan, L. and Schedl, P. (1999). The *Drosophila* CPEB homolog, Orb, is required for Oskar protein expression in oocytes. *Dev. Biol*. **215**, 91-106.

Christerson, L. B. and McKearin, D. M. (1994). orb is required for anteroposterior and dorsoventral patterning during Drosophila oogenesis. *Genes Dev.* **8**, 614-628.

De Moor, C. H. and Richter, J. D. (1999). Cytoplasmic polyadenylation elements mediate masking and unmasking of cyclin B1 mRNA. *EMBO J.* **18**, 2294-2303.

Ephrussi, A. and Lehmann, R. (1992). Induction of germ cell formation by oskar. *Nature* **358**, 387-392.

Fox, C. A., Sheets, M. D. and Wickens, M. P. (1989). Poly(A) addition during maturation of frog oocytes: distinct nuclear and cytoplasmic activities and regulation by the sequence UUUUUAU. *Genes Dev.* **3**, 2151-2162.

Ghabrial, A. and Schupbach T. (1999). Activation of a meiotic checkpoint regulates translation of Gurken during Drosophila oogenesis. *Nat. Cell Biol.* **6**, 354-357.

Gonzalez-Reyes, A., Elliott, H. and St Johnston, D. (1995). Polarization of both major body axes in Drosophila by gurken-torpedo signalling. *Nature* **375**, 654-658.

Gonzalez-Reyes, A., Elliott, H. and St Johnston, D. (1997). Oocyte determination and the origin of polarity in Drosophila: the role of the spindle genes. *Development* **124**, 4927-4937.

Groisman I., Huang, Y. S., Mendez, R., Cao, Q., Theurkauf, W. and Richter JD. (2000). CPEB, maskin, and cyclin B1 mRNA at the mitotic apparatus: implications for local translational control of cell division. *Cell* **103**, 435-447.

Hake, L. E. and Richter, J. D. (1994). CPEB is a specificity factor that mediates cytoplasmic polyadenylation during *Xenopus* oocyte maturation. *Cell* **79**, 617-627.

Kelley R. L. (1993). Initial organization of the *Drosophila* dorsoventral axis depends on an RNA-binding protein encoded by the *squid* gene. *Genes Dev.* **7**, 948-960.

Lantz, V. and Schedl, P. (1994). Multiple *cis*-acting targeting sequences are required for orb mRNA localization during Drosophila oogenesis. *Mol. Cell. Biol*. **14**, 2235-2242.

Lantz, V., Ambrosio, L. and Schedl, P. (1992). The *Drosophila orb* gene is predicted to encode sex-specific germline RNA-binding proteins and has localized transcripts in ovaries and early embryos. *Development* **115**, 75-88.

Lantz, V., Chang, J. S., Horabin, J. I., Bopp, D. and Schedl, P. (1994). The *Drosophila orb* RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. *Genes Dev.* **8**, 598-613.

Lasko, P. (1999). RNA sorting in *Drosophila* oocytes and embryos. *FASEB J.* **13**, 421-433.

Lindsley. D. L. and Zimm, G. G. (1992). *The Genome of* Drosophila melanogaster. New York: Academic Press.

Markussen, F-H., Michon, A-M., Breitwieser, W. and Ephrussi, A. (1995). Translational control of *oskar* generates short OSK, the isoform that induces pole plasm assembly. *Development* **121**, 3723-3732.

Minshall, N., Walker, J., Dale, M. and Standart, N. (1999). Dual roles of p82, the clam CPEB homolog, in cytoplasmic polyadenylation and translational masking. *RNA* **5**, 27-38.

Neuman-Silberberg, F. S. and Schupbach T. (1993). The *Drosophila* dorsoventral patterning gene *gurken* produces a dorsally localized RNA and encodes a TGF alpha-like protein. *Cell* **75**, 165-174.

Neuman-Silberberg F. S. and Schupbach T. (1996). The *Drosophila* TGF-alpha-like protein Gurken: expression and cellular localization during Drosophila oogenesis. *Mech. Dev.* **59**, 105-113.

Nilson L. A. and Schupbach T. (1999). EGF receptor signaling in Drosophila oogenesis. *Curr. Top. Dev. Biol.* **44**, 203-243.

Norvell A., Kelley R. L., Wehr K., Schupbach T. (1999). Specific isoforms of squid, a Drosophila hnRNP, perform distinct roles in Gurken localization during oogenesis. *Genes Dev.* **13**, 864-876.

Paris, J. and Richter, J. D. (1990). Maturation-specific polyadenylation and translational control: diversity of cytoplasmic polyadenylation elements, influence of poly A tail size and formation of stable polyadenylation complexes. *Mol. Cell. Biol*. **10**, 5634-5645.

Paris, J., Swenson, K., Piwnica-Worms, H. and Richter, J. D. (1991). Maturation-specific polyadenylation: *in vitro* activation by p34cdc2 and phosphorylation of a 58-kD CPE-binding protein. *Genes Dev.* **5**, 1697-1708.

Price J. V., Clifford R. J. and Schupbach T. (1989). The maternal ventralizing locus *torpedo* is allelic to *faint little ball*, an embryonic lethal, and encodes the *Drosophila* EGF receptor homolog. *Cell* **56**, 1085-1092.

Richter, J. (1999). Cytoplasmic polyadenylation in development and beyond. *Microbiol. Mol. Biol. Rev.* **63**, 446-456.

Rongo, C., Gavis, E. R. and Lehmann, R. (1995). Localization of *oskar* RNA regulates *oskar* translation and requires Oskar protein. *Development* **121**, 2737-2746.

Roth S. and Schupbach T. (1994). The relationship between ovarian and embryonic dorsoventral patterning in Drosophila. *Development* **120**, 2245-2257.

Roth S., Neuman-Silberberg F. S., Barcelo G. and Schupbach T. (1995). cornichon and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in Drosophila. *Cell* **81**, 967-978.

St Johnston, D. (1995). The intracellular localization of messenger RNAs. *Cel1* **81** 161-170.

St Johnston, D. and Nusslein-Volhard, C. (1992). The origin of pattern and polarity in the Drosophila embryo. *Cell* **68**, 20

St Johnston, D., Beuchle, D. and Nusslein-Volhard, C. (1991). *Staufen*, a gene required to localize maternal RNAs in the Drosophila egg. *Cell* **66**, 51-63.

Salles, F. J., Lieberfarb, M. E., Wreden, C., Gergen, J. P. and Strickland, S. (1994). Coordinate initiation of *Drosophila* development by regulated polyadenylation of maternal messenger RNAs. *Science* **266**, 1996-1999.

Saunders C. and Cohen R. S. (1999). The role of oocyte transcription, the 5′UTR, and translation repression and derepression in Drosophila *gurken* mRNA and protein localization. *Mol. Cell.* **3**, 43-54.

Schejter E. D. and Shilo B. Z. (1989). The *Drosophila* EGF receptor homolog (DER) gene is allelic to *faint little ball*, a locus essential for embryonic development. *Cell* **56**, 1093-1104.

Serano T. L., Karlin-McGinness M. and Cohen R. S. (1995). The role of *fs(1)K10* in the localization of the mRNA of the TGF alpha homolog gurken within the Drosophila oocyte. *Mech. Dev*. **51**, 183-192.

Sheets M. D., Wu M. and Wickens M. (1995). Polyadenylation of c-mos mRNA as a control point in Xenopus meiotic maturation. *Nature* **374**, 511-516.

Sheets M. D., Fox C. A., Hunt T., Vande Woude G. and Wickens M. (1994). The 3′-untranslated regions of c-mos and cyclin mRNAs stimulate translation by regulating cytoplasmic polyadenylation. *Genes Dev.* **8**, 926-938.

Shulman J. M. and St Johnston D. (1999). Pattern formation in single cells. (1999 *Trends Cell Biol*. **9**, 60-64.

Smith, J. L., Wilson, J. E. and Macdonald, P. M. (1992). Overexpression of *oskar* directs ectopic activation of nanos and presumptive pole cell formation in Drosophila embryos. *Cell* **70**, 849-859.

Tan, L., Chang, J., Costa, A. and Schedl, P. (2001) An autoregulatory feedback loop directs the localized expression of the *Drosophila* CPEB protein Orb in the developing oocyte *Development* **128**, 1159-1169.

Theurkauf, W. E. (1994). Microtubules and cytoplasm organization during Drosophila oogenesis. *Dev. Biol*. **165**, 352-360.