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

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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-ovitelligenic 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 Johnston 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 osk RNA binding (orb). orb functions at multiple steps during oogenesis (Lantz et al., 1994; Christerson and McKearin, 1994). In the presumed null mutant, orb<sup>343</sup>, oogenesis arrests just before the formation of the 16-cell cyst, and neither oocyte nor nurse cell fates are determined. The orb<sup>303</sup> mutation is slightly less severe than orb<sup>343</sup>, and it arrests oogenesis after the 16-cell cyst is formed. There is also a much weaker hypomorphic allele, orb<sup>mel</sup>, 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<sup>mel</sup>, and homozygous mutant females lay eggs. Many of the eggs produced by orb<sup>mel</sup> 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<sup>mel</sup> mothers often show developmental abnormalities expected from defects not only in DV patterning but also in posterior patterning.

The morphological abnormalities in orb<sup>mel</sup> 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<sup>mel</sup> 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; Christerson and McKearin, 1994). The distribution of 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., 1991; 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<sup>mel</sup> 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<sup>mel</sup> egg chambers is more diffusely distributed.) The fact that the distribution of grk mRNA in orb<sup>mel</sup> is similar to that in K(10) or sqd is surprising as the DV defects in orb<sup>mel</sup> 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<sup>1</sup> is described in Lindsley and Zimm (Lindsley and Zimm, 1992). orb<sup>343</sup> and orb<sup>303</sup> 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<sup>mel</sup> 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'-GCCAGCTCCGGGCCCCGTTTTTTTTTTTTTTT-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'-TAGAAGCCTAGGAGCCAACG-3'), for the grk 3' UTR (5'-TTGTCATTCCATATAGCAGAG-3'), and for the nos 3' UTR (5'-ACTGGTGTAATGCAGGGG-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<sup>mel</sup> egg chambers**

The abnormalities in the distribution of grk mRNA in orb<sup>mel</sup>
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\textsuperscript{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\textsuperscript{mel} egg chambers. To explore this possibility, we examined the expression of Grk and Orb proteins in vitellogenic wild-type and orb\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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).

**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\textsuperscript{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\textsuperscript{mel} ovaries (not shown; Chang et al., 1999). However, defects in early Grk expression are seen in the two stronger mutants orb\textsuperscript{303} and orb\textsuperscript{303}

In the presumed protein null, orb\textsuperscript{42} (Lantz et al., 1994), little or no Grk can be detected (Fig. 2). A different result is obtained for orb\textsuperscript{303}. orb\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{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.
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 gerarium. 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 previtellogenic 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 bicaudal (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 32P-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.

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
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 vitelligenic orb mel chambers, the level of Orb protein in stage 7-10 K(10); orb mel egg 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 vitelligenic 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 oocyte extracts. The results are shown in Fig. 6. Whereas the amount of Orb protein in orb mel oocyte 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 mutation.

### Table 1. Phenotypes of various orb mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild type</th>
<th>DV defects</th>
<th>% DV defect (type)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>w^1</td>
<td>3818</td>
<td>110</td>
<td>2.8 (ventralized)</td>
</tr>
<tr>
<td>K(10)/K(10) orb mel</td>
<td>0</td>
<td>1223</td>
<td>100 (dorsalized)</td>
</tr>
<tr>
<td>orb mel/orb mel</td>
<td>43</td>
<td>643</td>
<td>89.6 (ventralized)</td>
</tr>
<tr>
<td>orb mel</td>
<td>1</td>
<td>643</td>
<td>98.4 (dorsalized)</td>
</tr>
<tr>
<td>(B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>w^1</td>
<td>937</td>
<td>15</td>
<td>1.9 (ventralized)</td>
</tr>
<tr>
<td>Hdl9G, orb mel; Tm3Ser</td>
<td>493</td>
<td>122</td>
<td>19.8 (ventralized)</td>
</tr>
<tr>
<td>K(10)/+; Hdl9G orb^{343}</td>
<td>1374</td>
<td>178</td>
<td>11.5 (ventralized)</td>
</tr>
</tbody>
</table>

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 Hdl9G transgene insert on the third chromosome has the orb 3' UTR fused tolacZ protein coding sequences. Expression of this hybrid mRNA in ovaries is driven by the hsp83 promoter. The hsp83; lacZ orb 3' UTR transgene, Hdl9G, 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 Hdl9G transgene are ventralized. The percentage of ventralized eggs dropped from 20% in the Hdl9G, orb^{343}/+ strain to 12% when the females are also heterozygous for the K(10) mutation.
The orb mRNA expressed by orb<sup>mel</sup> 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:<lacz-<sup>3</sup> 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 orb<sup>mel</sup> transgene fused to the 3′ UTR of masked gurken mRNA is introduced into orb<sup>mel</sup> mutant ovaries. 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<sup>mel</sup> 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 1.0:0.5:0.3=K(10); orb<sup>mel</sup> and 0.05=orb<sup>mel</sup>. The ratios estimated for the double mutant ranged in different experiments from 0.3 to 0.5 and for orb<sup>mel</sup> from 0.05 to 0.1.

**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<sup>342</sup> and orb<sup>303</sup> ovaries. In the presumed Orb protein null, orb<sup>342</sup>, Grk is not detected. In orb<sup>303</sup>, Grk expression parallels the aberrant pattern of Orb<sup>303</sup> protein accumulation. In newly formed 16-cell cysts, all germ cells have high levels of the Orb<sup>303</sup> protein. These germ cells also express much higher than normal levels of Grk protein. In older pseudo-egg chambers, both Orb<sup>303</sup> and Grk disappear. These findings argue that the Orb<sup>303</sup> 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<sup>343</sup>, 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<sup>mel</sup>, 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 orb303 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 checkpoint 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 epistatic 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 dorsIALIZED 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 dorsIALIZED 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 dorsIALIZED (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.

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