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An autoregulatory feedback loop directs the localized expression of the *Drosophila* CPEB protein Orb in the developing oocyte

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SUMMARY

The RRM-type RNA binding protein Orb plays a central role in the establishment of polarity in the *Drosophila* egg and embryo. In addition to its role in the formation and initial differentiation of the egg chamber, *orb* is required later in oogenesis for the determination of the dorsoventral (DV) and anteroposterior (AP) axes. In DV axis formation, Orb protein is required to localize and translate *gurken* mRNA at the dorsoanterior part of the oocyte. In AP axis formation, Orb is required for the translation of *oskar* mRNA. In each case, Orb protein is already localized at the appropriate sites within the oocyte before the arrival of the mRNAs encoding axis determinants. We present evidence that an autoregulatory mechanism is responsible for directing the on site accumulation of Orb protein in the *Drosophila* oocyte. This *orb* autoregulatory activity ensures the accumulation of high levels of Orb protein at sites in the oocyte that contain localized *orb* message.

Key words: Orb, Oogenesis, Autoregulation, Translation, *Drosophila*

INTRODUCTION

Axis determination in the *Drosophila* embryo depends upon mRNA localization pathways that operate during oogenesis (reviewed by St. Johnson and Nusslein-Volhard, 1992). The posterior axis is established by the posterior organizing factor Oskar (Osk; Ephrussi et al., 1992; Smith et al., 1992). At the onset of vitellogenesis (stage 8), masked *osk* mRNA is transported from the nurse cells to the posterior pole of the oocyte by the double strand RNA binding protein Staufen (Stau) (St. Johnston et al., 1991; St. Johnston et al., 1995; Theurkauf, 1994). After ariving at the pole, *osk* message is activated for translation (Rongo et al., 1995). Newly synthesized Osk anchors its own message and nucleates the assembly of structures required to localize mRNA encoding the posterior determinant Nanos (Nos). The dorsoventral (DV) axis also depends upon mRNA localization. *gurken* (*grk*) mRNA, which encodes a TGFα homolog, is concentrated in a cap above the oocyte nucleus at the dorsoanterior part of the oocyte by the K(10) and Squid proteins (Squ; Neuman-Silberberg and Schupbach, 1993). Grk protein translated from the localized *grk* message signals dorsal fate to the overlying follicle cell epithilium by interacting with the receptor *torpedo* (*top*) (Roth et al., 1995; Sapir et al., 1998).

One gene that functions in both the *osk-nos* and *grk* mRNA localization pathways is *oo18 RNA-binding protein* (*orb*). *orb* encodes sex- and germline-specific mRNAs and proteins (Lantz et al., 1992). The ovarian Orb protein is approx. 100 kDa and in the C-terminal half has two 90 amino acid (aa) RRM-type RNA binding domains and a short 60 aa cysteine-histidine rich region that resembles a zinc finger. Strong loss-of-function mutations block oogenesis at an early stage (Lantz et al., 1994). In the presumed null, orb^{343}, oogenesis arrests just before the formation of the 16-cell cyst and neither oocyte nor nurse cell fates are determined. orb^{303} is slightly less severe than orb^{343}. It arrests oogenesis after the 16-cell cyst is formed; however, the presumptive oocyte remains in the center of the cyst instead of migrating to the posterior pole. Consequently the mutant cysts never establish one of the earliest morphological asymmetries. In both mutants, there are defects in the localization and translation of mRNAs that normally accumulate in the early oocyte.

There is also a much weaker allele, orb^{mel}, which was generated by imprecise excision of a P element inserted into the 2nd (female-specific) exon (Christerson and McKearin, 1994). The excision event eliminates this exon, which encodes much of the 5′ UTR and an in frame AUG located close to the 5′ splice junction. However, the protein species produced by orb^{mel} are identical in size to wild type, suggesting that a downstream AUG in exon 3 is normally used for translation initiation. Unlike the stronger alleles, oogenesis is not irreversibly blocked in orb^{mel} and mutant females lay eggs. While Orb appears to be synthesized at near normal levels in early stages, severe reductions in expression become evident around stage 7. The proper accumulation of Orb protein after this stage is important for the establishment of polarity. Many of the eggs produced by orb^{mel} females have ventralized chorions like those in *grk* or *top* mutants. When fertilized, the orb^{mel} embryos often show developmental abnormalities expected from a failure in posterior patterning. However,

orb^{mel} is not fully expressed and almost 20% of the embryos hatch as larvae. The morphological abnormalities in *orb*^{mel} egg shells and embryos can be directly attributed to an improper execution of the mRNA localization pathways responsible for axes determination during oogenesis.

How *orb* functions in mRNA localization is best understood in the posterior pathway. Christerson and McKearin (Christerson and McKearin, 1994) found that *osk* mRNA is transported to the posterior end of the oocyte in *orb*^{mel} chambers, but does not seem to be properly anchored to the pole. This RNA localization defect is likely due to a failure in the translation of *osk* message when it arrives at the pole. This possibility was first suggested by the discovery that Orb is homologous to the Cytoplasmic Polyadenylation Element Binding protein (CPEB) found in maturing *Xenopus leavis* eggs (Hake and Richter, 1994; Stebbins-Boaz et al., 1996). CPEB is required to activate translation of masked maternal mRNAs. *orb* seems to have an analogous function in the fly posterior pathway, activating the translation of *osk* mRNA when it arrives at the pole (Chang et al., 1999). When Orb is absent, Osk protein is not synthesized, and *osk* message diffuses away. *orb* appears to play a similar role in DV polarity, in this case controlling the translation of both *K(10)* and *grk* mRNA (Neuman-Silberberg and Schupbach, 1996; unpublished data).

In each of these polarity pathways, Orb protein is already on site before the mRNAs encoding the axes determinants arrive. In fact, Orb is one of the first proteins localized to the oocyte (Lantz et al., 1994). In the germarium, it can be detected in the oocyte as soon as the 16-cell cyst is formed. From this stage through the remaining previtellogenic stages (stages 1-7) Orb accumulates at the posterior of the oocyte forming a graded cap that extends anteriorly along the cortex. By the time *osk* mRNA is transported to the posterior pole at stages 8-9, high levels of Orb are already present. Similarly, when the oocyte nucleus moves to the dorsoanterior part of the oocyte, there is a rapid accumulation of Orb protein along the entire oocyte cortex. As a result Orb is already present at the oocyte-nurse cell margin when K(10) and Squid localize *grk* mRNA around the oocyte nucleus.

If Orb protein localization precedes and is independent of the two polarity pathways, what directs its distribution? The most likely mechanism is an mRNA localization pathway that targets *orb* mRNA to specific regions of the oocyte and then activates translation. *orb* mRNA exhibits a dynamic pattern of accumulation during oogenesis that closely parallels if not anticipates the protein distribution (Lantz et al., 1992, Lantz et al., 1994). In pre-vitellogenic stages, *orb* mRNA accumulates in a cap at the posterior pole while at the onset of vitellogenesis, this cap disappears and the message shifts to the anterior margin. While an mRNA localization pathway is likely to direct Orb accumulation in the oocyte, very little is known about this pathway. The long 3′ UTR of the female *orb* mRNA has been shown to contain *cis*-acting localization elements (Lantz and Schedl, 1994), however, the *trans*-acting factors that bind to the message, target it to different regions of the oocyte and control translation remain to be elucidated. We have investigated the role of Orb protein in the *orb* mRNA localization pathway. We show that Orb protein autoregulates its own expression through sequences in the 3′ UTR of the *orb* mRNA. This autoregulatory activity provides a mechanism for ensuring the on site accumulation of high levels of Orb protein.

MATERIALS AND METHODS

Fly stocks

The *w*^{1} is described by Lindsley and Zimm (Lindsley and Zimm, 1992). *orb*^{343} is from a collection of female steriles on the third chromosome (a gift from C. Nusslein-Volhard) and is described by Lantz et al. (Lantz et al., 1994). *orb*^{mel} is described by Christerson and McKearin (Christerson and McKearin, 1994). The *hsp83 lacZ* transgenes containing DNA sequences from the *orb* 3′ UTR have been described in detail by Lantz and Schedl (Lantz and Schedl, 1994). The *hsp83:lacZ* reporter transgene with the *male specific lethal 2 (msl2)* 3′ UTR instead of the *orb* 3′ UTR was a gift from J. Yanowitz.

Immunoprecipitation

Ovaries of well-fed 2- to 3-day old wild-type females (30) were dissected in 1× PBS and frozen immediately on dry ice. Ovary extract was prepared by adding 100 μl of ice-cold IP buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 2.5 mM $MgCl_2$, 250 mM sucrose, 0.05% NP40, 0.5% Triton X-100, 1 mM PMSF, 1 μg/ml pepstatin A, 10 μg/ml aprotinin, 1 μg/ml leupeptin) and homogenizing with a plastic pestle in a 1.5 ml microfuge tube with 3-4 freeze/thaw cycles. The homogenate was filtered through glass wool in the end of a 200 μl pipette tip and centrifuged at 3000 rpm for 5 minutes. The supernatant was removed and mixed with 40 units of RNasin, 30 μl of antibody-coupled protein-A beads (Bio-Rad) for 2 hours to overnight at 4°C on a rotator. The IP mix was centrifuged at low speed in the cold room to remove the supernatant. The protein-A beads were then washed 4-5 times with 20 volumes of IP buffer without $MgCl_2$. 5-10 μl of the protein-A beads were analyzed on a western blot for the presence of Orb protein by detection with enhanced chemilluminesence system from Amersham. The remainder of the beads were phenol/chloroform extracted and precipitated with glycogen to isolate mRNAs. The RT-PCR assay was performed as described below.

PCR Poly(A) assay

Total ovary RNA and the mRNAs from IP with anti-Orb and anti-Dorsal antibodies were reverse transcribed using an anchored oligo(dT) primer (as in Salles et al., 1994). PCR was performed using the anchor primer paired with a gene-specific primer for the *orb* 3′ UTR: 5′-CATAGCCAAGCCCCCGACTCG-3′, starting at base no. 4128 of the published sequence in Lantz et al. (Lantz et al., 1992), for *nos* 3′ UTR: 5′-ACTTGTTCAATCGTCGTGGCCG-3′ starting approximately at base no. 2387 of the sequence published by Wang and Lehmann (Wang and Lehmann, 1991) and for *bcd* 3′ UTR: 5′-CTAGTCAGCA-GGCCGCACGG-3′, starting at base no. 4172 of the sequence published by Berleth et al. (Berleth et al., 1988) as follows: 1 cycle of 4 minutes 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 for 15 minutes at 72°C. The PCR products were analyzed on several different gel systems (non-denaturing agarose gels with TBE buffer; alkaline agarose gels with 10 mM EDTA, 50 mM NaOH; 1.2% vertical formaldehyde agarose gels in MOPs buffer) and transferred to nitrocellulose or Zeta Probe membrane (from Bio-Rad) by standard techniques. The blots were hybridized with random primed probes made from *orb*, *nos* and *bcd* 3′ UTR sequences cloned in BS plasmid vectors.

UV cross-linking

UV cross-linking experiments were preformed as described by Chang et al. (Chang et al., 1999) with only minor modifications; the reaction buffer contained two divalent cations. In addition to 1.5 mM $MgCl_2$, 0.2 mM $ZnCl_2$ was included.

Immunocytochemistry and western analysis

Ovaries were dissected in PBS, fixed and stained for Orb protein as described previously (Lantz et al., 1994). Alexa-546 (Molecular Probes, Inc.) -conjugated anti-mouse antibody was used with mouse anti-Orb and mouse anti-β-galactosidase antibodies. Yo-Pro was used

for staining the nuclei. Imaging was done by laser scanning confocal microscopy (Krypton-Argon Laser, Bio-Rad MRC 600). Western analysis was done as described previously (Lantz et al., 1994).

RNA in situ hybridization on whole-mount ovaries

Ovaries were dissected in cold 1× PBS, teased apart, fixed and hybridized as described by Tautz and Pfeifle (Tautz and Pfeifle, 1989) and modified by Suter and Steward (Suter and Steward, 1991). Digoxigenin-labeled antisense RNA probes were synthesized by use of the RNA genius kit (Boehringer Mannheim). Ovaries were mounted in Aqua-polymount (Polysciences, Inc.).

RESULTS

Antisense RNA complementary to the *orb* 3′ UTR interferes with *orb* gene function

Previous studies have shown that *orb* mRNA localization depends upon sequences in the approx. 1 kb 3′ UTR of the female transcript (Lantz and Schedl, 1994). When a tagged *orb* female cDNA is ectopically expressed in the germline, the tagged transcript displays a localization pattern like that of the endogenous *orb* message. Removal of the 3′ UTR sequences from the tagged *orb* cDNA prevents the localization. Conversely, the localization pattern of the endogenous message can be recapitulated by fusing the UTR to a heterologous transcript encoding *E. coli* β-galactosidase. Deletion mapping pinpoints the localization elements to an approx. 300 nucleotide sequence within the UTR.

While these findings indicate that the 3′ UTR is required to localize *orb* mRNA, they do not reveal whether localization is important for *orb* activity. Since attempts to generate *orb* cDNA rescue constructs have been unsuccessful, we decided to address this question by testing whether antisense RNAs complementary to sequences in the female *orb* 3′UTR interfere with *orb* gene function. We reasoned that the antisense RNAs would hybridize to the 3′ UTR of the endogenous *orb* message, preventing components of the localization pathway from recognizing their target sites.

To assess the feasibility of this strategy, we asked whether an *hsp83* construct, *NH*, which expresses β-*gal* coding sequences fused to a near full length (815 bp) antisense *orb* 3′ UTR (plus a sense SV40 polyadenylation signal; Fig. 1), has any phenotypic consequences. As shown for two different *NH* transgenic lines in Table 1A, between 3 and 6% of the eggs produced by transgenic females had ventralized chorions (Fig. 1), while the frequency of similar defects in eggs from the wild-type control was less than 0.5% (Table 1A).

The ventralized chorions are indicative of a failure in the *grk-top* signaling pathway. Since precisely the same phenotype is found in eggs from females homozygous for the weak orb^{mel} allele (Christerson and McKearin, 1994), a plausible hypothesis is that the antisense RNA interferes with the functioning of the endogenous *orb* gene. In this case the frequency of chorion defects should be increased by reducing the *orb* gene dose. *orb* is weakly haplo-insufficient for its function in the *grk-top* pathway and females heterozygous for the null allele orb^{343} produce a significant number of eggs which have ventralized chorions. The frequency of chorion defects in eggs from orb^{343}/+ mothers is 10-15%. As shown in Table 1A, a single copy of the *NH* transgene in orb^{343}/+ females increases the frequency of chorion defects to over 20% for the *NH4B1* line and to 45% for the *NH24C* line. These findings indicate that the antisense *NH* transgene acts as a dominant negative.

Table 1. Egg shell phenotypes induced by different transgenes

Genotype	Total	DV defects*	% DV defects*
A. 3′ UTR antisense transgene *NH*			
w/w	480	2	0.4
orb^{343}/TM3	560	60	10.7
NH4B1/NH4B1	261	17	6.5
NH4B1 orb^{343}/TM3	1,112	257	23.0
NH24C1/NH24C1	472	17	3.6
NH24C1 orb^{343}/TM3	1,428	642	45.0
B. 3′ UTR sense transgene *HD*			
w/w	360	1	0.3
HD19G/TM3	815	37	4.5
HD19G/HD19G	250	29	11.6
orb^{343}/TM3	1,383	162	11.7
HD19G orb^{343}/ TM3	2,133	578	27.1
Hd19G orb^{343}/*HD19G*	1,325	989	74.6
C. 3′ UTR sub-fragments			
w/w	350	2	0.6
orb^{343}/TM3	655	60	9.2
AN61D/AN61D	1,103	99	9.0
An61D/+: orb^{343}/TM3	908	387	42.6
AE48A orb^{343}/TM3	1,331	293	22.0

*Frequency of ventralized egg shells.

w/w is the parental stock for isolating transgenic lines for the different *lacZ* reporter constructs. These constructs have a *mini-white* marker.

NH4B1 and *NH24C1* are two independent transgenic lines for the *NH* construct (see Fig. 1A). Both are inserted on the 3rd chromosome.

NH4B1/NH4B1 are females that are homozygous for the *NH4B1* insert; *NH4B1* orb^{343} is a recombinant between the transgene and the orb^{343} allele. In this experiment the recombinant chromosome is over the TM3 balancer.

HD19G is a transgenic line for the *HD* construct (see Fig. 1A) which is inserted on the third chromosome. Other genotypes are as for *NH4B1*. Two transgenes, *AN* and *AE*, carrying sub-fragments from the *orb* 3′ UTR (see Fig. 1A) were also tested either as homozygotes or when combined with orb^{343}. Note that *AN61D* is inserted on the 2nd chromosome.

Total: number of eggs scored; DV defects: the number of eggs exhibiting a ventralized egg shell phenotype; %DV defects: the percentage of eggs exhibiting ventralized egg shells.

Transgenes expressing the *orb* 3′ UTR in the sense orientation also interfere with *orb* gene function

As a control for the antisense *NH* transgene, we examined eggs produced by females carrying a second transgene, *HD*, which ectopically expresses *lacZ* coding sequences fused to the female *orb* 3′ UTR in a sense, rather than an antisense orientation (Fig. 1; Lantz and Schedl, 1994). The UTR sequences in *HD* are sufficient to recapitulate the complex localization pattern of the endogenous *orb* mRNA. (*HD* is an *orb* genomic fragment and also contains sequences downstream of the female *orb* polyadenylation site.)

Since transcripts from the sense transgene should not hybridize to *orb* mRNA, we expected that they would have no effect on *orb* gene function. However, contrary to this expectation, the sense 3′ UTR transgene induced chorion defects at a frequency similar to that seen for the antisense transgene. Nearly 5% of the eggs have ventralized chorions when the *HD* transgene is present in a single copy, while about 11% have a ventralized chorion when there are two copies (Table 1B).

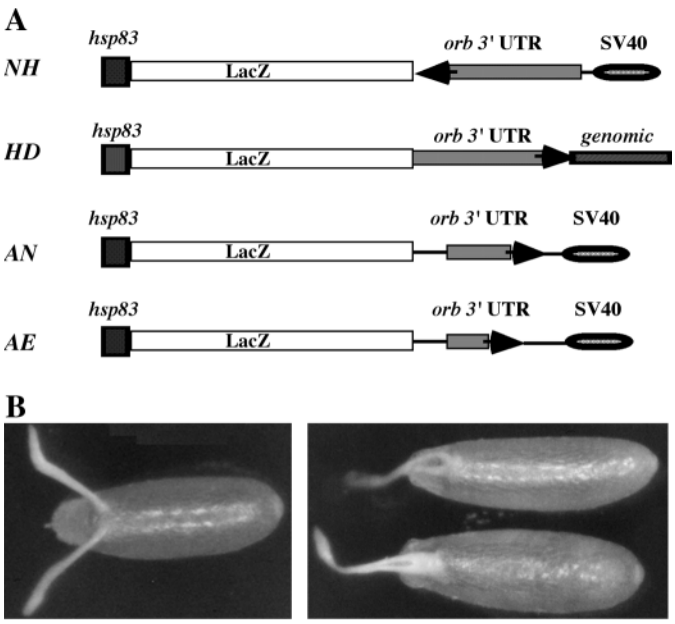

Fig. 1. *orb* 3′ UTR transgenes induce dorsoventral polarity defects. (A) Sequence organization of the *hsp83 lacZ orb* 3′ UTR transgenes. All transgenes have *mini-white* as a marker and an *hsp83* promoter to drive expression of *lacZ* mRNA expression. *NH*: The *NH* transgene has an *Hin*dIII-*Nde*I fragment from the *orb* 3′ UTR inserted in an antisense orientation (*Nde*I-*Hin*dIII) in between the *lacZ* mRNA coding sequences and an SV40 poly(A) addition signal. *HD:* The *HD* transgene has a genomic 2.2 kb *Hin*dIII fragment inserted downstream of the *lacZ* mRNA coding sequences. The *Hin*dIII fragment contains most of the *orb* 3′ UTR plus downstream genomic sequences. *AN:* The *AN* transgene has a 570 bp *Asn*I-*Nde*I restriction fragment from the *orb* 3′ UTR (which contains the localization region) inserted between the *lacZ* mRNA coding sequences and the SV40 poly(A) signal. *AE*: The *AE* transgene has a 365 bp restriction fragment beginning at the *Asn*I restriction site and extending to site generated by exonuclease III digestion. It contains the entire *orb* mRNA localization region. (B) Photomicrographs of a wild-type egg (left) and two ventralized eggs (right). The ventralized eggs were induced by the *HD lacZ orb* 3′ UTR transgene. Similar ventralized eggs were observed with the antisense transgene *NH*, and the two smaller 3′ UTR transgenes, *AN* and *AE*.

As suggested for the antisense transgene, the upsets in DV polarity could be explained if the sense transgene interferes with *orb* function. If this hypothesis is correct, the phenotypic effects of the *HD* transgene should be exacerbated by reducing the dose of the *orb* gene relative to the transgene. This is the case. When a single copy of the sense UTR transgene is introduced into heterozygous orb^{343}/+ females, the frequency of chorion defects doubles from 12% to 25%, while it increases to nearly 75% of the eggs when there are two copies of the sense transgene (Table 1B).

In both the antisense and sense transgenes, *orb* sequences are fused to a *lacZ* coding sequence. However, the *lacZ* sequence is not responsible for the dominant negative activity of these transgenes since an *hsp83: lacZ* transgene which carries the SV40 3′ UTR instead of the *orb* 3′ UTR does not induce the production of ventralized eggs nor does it show any synergistic interactions with *orb* mutations.

Sequences conferring dominant negative activity overlap with the *orb* mRNA localization signal

Since the sense *HD* transgene contains the female *orb* 3′UTR plus downstream genomic sequences, the formal possibility exists that the non-coding genomic sequence and not the *orb* 3′ UTR is responsible for the dominant negative activity. To exclude this possibility, we tested transgenes in which fragments from the *orb* 3′ UTR are inserted between the *lacZ* protein coding sequences and an SV40 polyadenylation sequence. As previous studies showed that sequences near the middle of the UTR are sufficient to localize *lacZ* RNA in a pattern like the endogenous message (Lantz and Schedl, 1994), we examined two transgenes, *AN* and *AE*, which have UTR sequences spanning this critical region (Fig. 1). *AN* has a 570 nt UTR while the *AE* transgene has a 364 nt UTR.

The phenotypic effects of the *AN* transgene are comparable to the original *hsp83:lacZ orb 3′ UTR* transgene (Table 1C). When present in two copies in a wild-type background, about 10% of the eggs produced by *AN61D* mothers have ventralized chorions. When a single copy of the *AN* transgene is introduced into females heterozygous for orb^{343}, about 50% of the eggs are defective. While the phenotypic effects of the *AN61D* line are stronger than the *HD* transgene, position effects might account for this difference as a second *AN* line is weaker (not shown). The smaller *AE* transgene also disrupts DV polarity (see Table 1C). These findings demonstrate that sequences from the *orb* 3′ UTR are responsible for the dominant negative activity of the *orb* sense transgenes and suggest that elements conferring this activity overlap with those involved in localizing *orb* mRNA during oogenesis.

β-galactosidase expression from the *orb* 3′ UTR transgene depends upon *orb* gene function

We suppose that both the sense and antisense transgenes act as dominant negatives because they interfere with the proper expression of Orb protein; however, since RNA from the sense transgenes cannot hybridize to *orb* message, the mechanism must be different from that of the antisense transgene. One plausible hypothesis is that the sense *lacZ orb* 3′UTR transcript competes with the endogenous *orb* mRNA for some limiting factor that is essential for Orb protein expression. While a number of factors could be limiting, the fact that *orb* is haploinsufficient makes Orb itself a good candidate. This would imply that Orb is required to promote the translation of *orb* mRNA. In this case the dominant negative activity of the *lacZ orb* 3′UTR mRNA would arise from a competition with endogenous *orb* mRNA for Orb function. When Orb acts on the *lacZ orb* 3′UTR mRNA instead of *orb* mRNA, it would promote β-galactosidase not Orb expression. As a consequence, Orb protein production would be downregulated by the transgene mRNA.

If this autoregulatory hypothesis is correct, then the expression of β-galactosidase from the *lacZ orb* 3′UTR transgenes should depend on *orb* function. To determine if this is the case we compared the level of β-galactosidase expressed from the *HD* transgene in orb^{343}/+ females with orb^{343}/orb^{mel} females. As in orb^{mel} homozygotes, *orb* activity in the *343/mel* mutant combination appears comparatively normal in previtellogenic stages; however, by the onset of vitellogenesis defects in *orb* dependent mRNA localization pathways become evident (Christerson and McKearin, 1994; Chang et al., 1999).

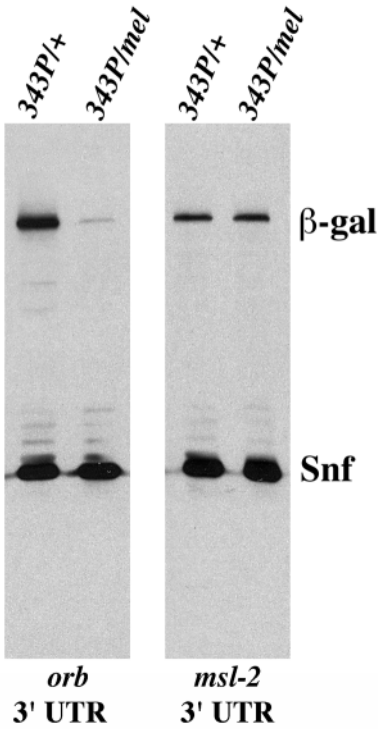

Fig. 2. β-galactosidase expression from the *lacZ orb* 3′ UTR transgene depends upon *orb* gene function. The *hsp83 lacZ orb* 3′ UTR transgene and the control *hsp83 lacZ msl-2* 3′ UTR transgene were introduced into either $orb^{343}/+$ or orb^{343}/orb^{mel} females. β-galactosidase expression was assayed by western blotting ovary extracts. To control for total protein in the extracts the blot was probed with antibodies against the snRNP protein, Snf. Left panel: the *lacZ orb* 3′ UTR transgene. Expression of β-galactosidase requires *orb* gene function and is substantially reduced in orb^{343}/orb^{mel} ovaries. Right panel: the *lacZ msl-2* 3′ UTR transgene. Expression of β-galactosidase does not require *orb* gene function and is the same in $orb^{343}/+$ and orb^{343}/orb^{mel}.

As a control for a general reduction in protein synthesis or a change in β-galactosidase stability in the absence of wild-type *orb* function, we introduced a *lacZ* transgene which has a 3′ UTR from the *msl-2* mRNA into the same genetic backgrounds.

Western blots of ovaries from $orb^{343}/+$ and orb^{343}/orb^{mel} females carrying either the *orb* or *msl-2* transgenes were probed with antibody directed against β-galactosidase and, as a loading control, the snRNP protein Snf (Fig. 2). Loss of *orb* function has no effect on β-galactosidase expression from the *msl-2* 3′ UTR transgene; the level of β-galactosidase is the same in ovaries lacking a wild-type *orb* gene as in ovaries that have a wild-type copy. (Similar results were obtained for another *lacZ* control, the *hsp83:lacZ SV40* 3′ UTR transgene; not shown.) In contrast, β-galactosidase expression from the *HD* transgene depends upon *orb* and the level of β-galactosidase is substantially reduced in ovaries from females lacking a wild-type gene (compare *343P/+* with *343P/mel*).

To confirm and extend these findings we examined β-galactosidase expression from the *HD lacZ orb* 3′ UTR transgene in wild-type and *orb* mutant backgrounds using confocal microscopy. In wild-type ovaries, β-galactosidase from the *lacZ orb* 3′ UTR transgene can be detected throughout much of oogenesis (Fig. 3). Though cytoplasmic protein is observed in wild-type chambers, the highest levels of β-galactosidase are seen in nuclei. There appears to be a very slight but consistent decrease in the level of β-galactosidase in vitellogenic $orb^{343}/+$ egg chambers, while the level of protein in earlier chambers is generally close to that seen in a wild-type background. The most striking reduction in β-galactosidase expression is seen in orb^{343}/orb^{mel} ovaries. As expected from the defects in *orb* function evident in vitellogenic orb^{343}/orb^{mel} chambers, very little β-galactosidase is observed after stage 7. Moreover, even in earlier stages the level of β-galactosidase is less than in wild type. These findings indicate that the expression of β-galactosidase from the *lacZ orb* 3′ UTR transgene, particularly in vitellogenic stages, requires wild type *orb* gene function.

Orb protein expression is inhibited by the *orb* 3′ UTR transgene

A second prediction of the autoregulatory model is that mRNA produced by the *lacZ orb* 3′ UTR transgenes should down-regulate translation of the endogenous *orb* mRNA by competing for *orb* activity. To test this prediction, we sought to maximize the dominant negative effects of the *HD* transgene. In wild-type females the frequency of DV defects induced by even two copies of the transgene is only a few percent (see Table 1). Consequently, we did not expect, nor did we observe, transgene-induced changes in Orb expression in a wild-type background (data not shown). However, if there is only a single functional *orb* gene, the frequency of D-V defects in the presence of two copies of the *HD* transgene can be more than 50%. Under these conditions clear cut changes in Orb expression could be observed.

The level and distribution of Orb protein in previtellogenic

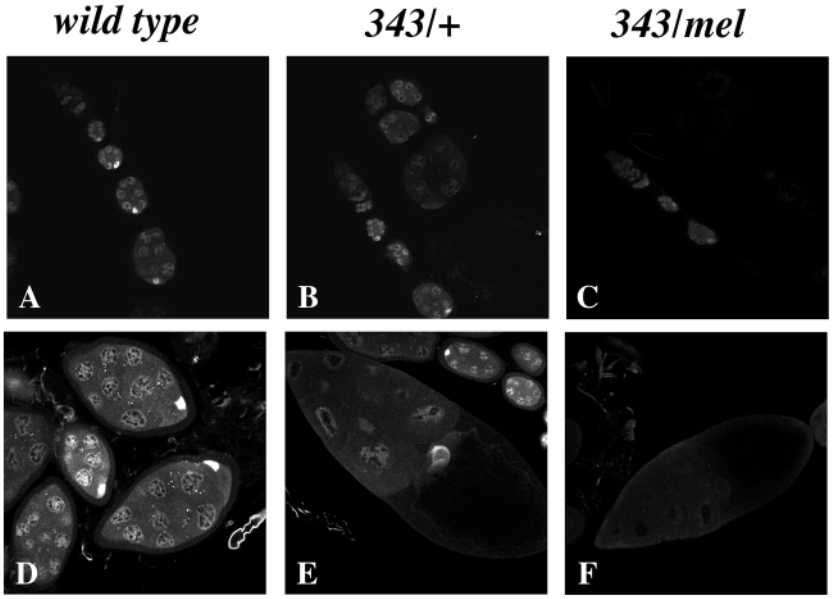

Fig. 3. β-galactosidase expression from the *lacZ orb* 3′ UTR transgene in *orb* mutant ovaries. Ovaries from *lacZ orb* 3′ UTR transgenic wild-type, $orb^{343}/+$ and orb^{343}/orb^{mel} females were stained with antibodies against β-galactosidase. To compare expression levels, the samples were stained in parallel and analyzed by confocal microscopy under identical conditions. β-galactosidase expression is reduced in ovaries from $orb^{343}/+$ and orb^{343}/orb^{mel} females compared to the wild-type control. (A-C) previtellogenic chambers; (D-F) vitellogenic chambers.

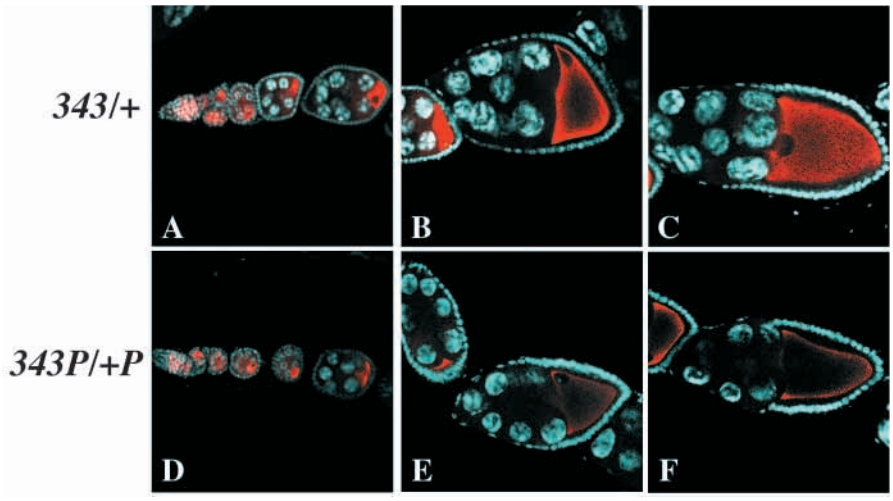

Fig. 4. The *hsp83 LacZ-orb*3′UTR transgene interferes with *orb* protein expression. Expression of Orb protein in ovaries from orb^{343}/+ and orb^{343} *P/P* females; (A,D) previtellogenic chambers; (B,C,E,F) vitellogenic chambers. Staining in red is Orb protein while nuclei are stained in blue. In orb^{343}/+ ovaries, the pattern of accumulation is similar to that observed in wild-type ovaries (data not shown; see Lantz et al., 1994). When two copies of the *lacZ orb* 3′ UTR transgene are present, the expression of Orb protein in orb^{343}/+ ovaries is reduced. The effects of the transgene on Orb protein accumulation are most evident after stages 6-7.

stages of orb^{343}/+ ovaries appears to be similar either with or without the transgene (Fig. 4A,D). However, after the onset of vitellogenesis, the accumulation of Orb protein in chambers from transgene ovaries is reduced compared to chambers from ovaries lacking the transgene (compare the $orb^{343}P/+P$ with orb^{343}/+ chambers in the middle and right panels of Fig. 4). We also examined ovaries from orb^{343}/+ females that had a single copy of the *lacZ orb* 3′ UTR transgene. Although vitellogenic chambers with abnormally low levels of Orb protein were observed, the Orb staining pattern in most vitellogenic chambers was similar to that of the orb^{343}/+ control without transgene. Presumably this reflects the fact that the frequency of eggs with DV defects is lower when orb^{343}/+ females have only a single copy of the transgene then when they have two copies. Based on these findings and the results described in the previous sections, we conclude that Orb protein positively autoregulates its own expression, most probably by activating the translation of *orb* mRNA through sequences in the 3′ UTR.

orb function is required for *orb* mRNA localization

Since Orb protein accumulates at sites containing *orb* mRNA, we reasoned that *orb* function might be required not only for translation but also for *orb* mRNA localization. In wild type ovaries, *orb* mRNA can first be detected in the germarium where it preferentially accumulates in the presumptive oocyte of newly formed 16 cell cysts (Lantz et al., 1992 and $orb^{343}P/+$ ovaries in Fig. 5A). Between stages 1 and 7, the highest concentrations of mRNA are found in a cap at the posterior of the oocyte. This posterior cap disappears at the onset of vitellogenesis, and most *orb* mRNA is found in a ring along the oocyte-nurse cell border (Lantz et al., 1992 and Fig. 5A).

To determine if Orb plays a role in localizing its own mRNA, we examined the distribution of *orb* message in orb^{343}/orb^{mel} ovaries either with (Fig. 5A) or without (not shown) the *HD* transgene. Orb protein expression in the germarium and in stages 1-6 egg chambers of mutant ovaries

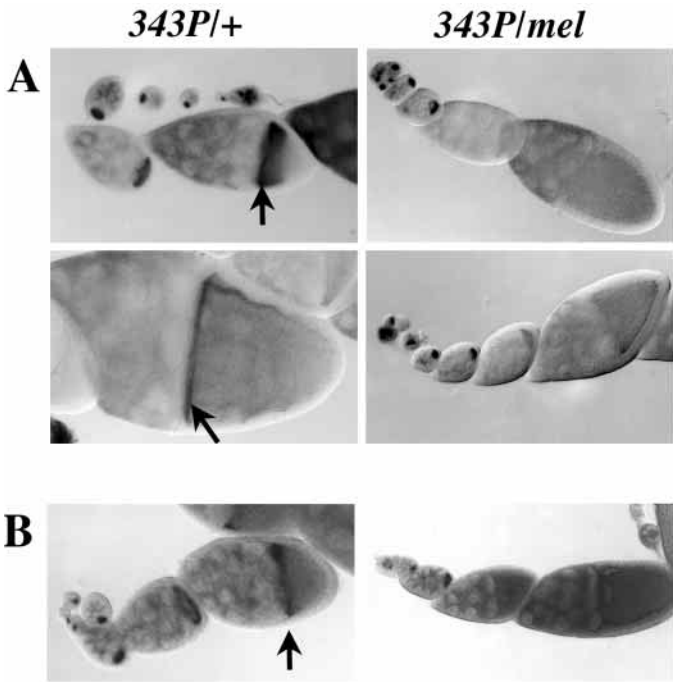

Fig. 5. *orb* mRNA localization requires *orb* gene function. (A) Four examples of *orb* mRNA localization in $orb^{343}P/+$ and $orb^{343}P/orb^{mel}$ ovaries. Ovaries were hybridized with an antisense RNA probe synthesized from an *orb* cDNA. The localization of *orb* mRNA in the orb^{343} *P/+* ovaries is similar to that previously described for wild-type ovaries (Lantz et al., 1992; data not shown). In the *orb* mutant combination, $orb^{343}P/orb^{mel}$, *orb* mRNA localization in early stages is not discernibly altered; however, from stage 5-6 onward, defects in the pattern of the *orb* mRNA localization are evident. Not shown here is the localization pattern of *orb* mRNA in ovaries from orb^{343}/+ and orb^{343}/orb^{mel} females that do not carry the *lacZ orb* 3′ UTR transgene. As indicated in the text, similar results were obtained. (B) *lacZ orb* 3′ UTR mRNA localization in $orb^{343}P/+$ and $orb^{343}P/orb^{mel}$ ovaries. In this experiment whole mounts of $orb^{343}P/+$ and $orb^{343}P/orb^{mel}$ ovaries were hybridized with an antisense RNA probe synthesized from a *lacZ* clone. The localization of *lacZ orb* 3′ UTR mRNA in the orb^{343} *P/+* ovaries is the same as that previously described in wild-type transgenic ovaries (Lantz and Schedl, 1994; data not shown). In the *orb* mutant combination, $orb^{343}P/orb^{mel}$, the localization pattern of *lacZ orb* 3′ UTR mRNA in early stages is the same as that in orb^{343} *P/+* ovaries; however, from stage 5-6 onward, abnormalities are evident. Arrows indicate hybridization (to *orb* or *lacZ* mRNA) along the anterior margin of the oocyte.

resembles wild type; however, defects in Orb protein accumulation can be detected around stage 7 (data not shown; Christerson and McKearin, 1994; Chang et al., 1999). Many vitellogenic chambers lack the Orb protein cap at the posterior, and there is a marked reduction in the amount of protein associated with the oocyte cortex. The *orb* mRNA localization pattern in the germarium and early egg chambers of orb^{343}/orb^{mel} mutant ovaries is, like the protein, generally indistinguishable from the wild-type control. High concentrations of *orb* mRNA are found in the oocyte and the message accumulates in a posterior cap. However, beginning at stages 5-6 a range of abnormalities in *orb* mRNA localization are observed. In many stage 5-6 chambers, *orb* mRNA is distributed uniformly in the oocyte instead of being concentration at the posterior. These abnormalities in mRNA localization appear to precede the appearance of *readily* detectable defects in Orb protein accumulation which are usually first evident in the mutant ovaries slightly later. Even more severe defects in mRNA localization are found after the onset of vitellogenesis. Unlike wild-type vitellogenic chambers, there is typically no anterior ring of *orb* mRNA. Instead the message is present throughout the oocyte-nurse cell complex (see Fig. 5A). In some orb^{343}/orb^{mel} chambers, the overall level of *orb* mRNA also appears to be reduced. While the *orb* mRNA distribution in most vitellogenic chambers is aberrant, we occasionally observe chambers in which the localization pattern resembles wild type. (Similar results have been obtained by L. Christerson and D. McKearin, personal communication.)

Although these results demonstrate that the *orb* mRNA localization pathway is disrupted in orb^{343}/orb^{mel} ovaries, there is a potential complication. The failure to properly localize *orb* mRNA could be due to a defect in some key *cis*-acting element in the *orb* mRNAs produced by the mutant orb^{343} or orb^{mel} genes, rather than to a lack of sufficient quantities of functional Orb protein. To address this problem, we examined the localization of mRNA expressed by the *HD lacZ orb* 3′ UTR transgene. The *orb* 3′ UTR sequences in the HD fragment are sufficient to direct the accumulation of *lacZ* mRNA in wild-type ovaries in a pattern that closely resembles that of the endogenous mRNA (Lantz and Schedl, 1994; see also the $orb^{343}P/+$ ovaries in Fig. 5B). If Orb functions in the localization of this heterologous mRNA, then its localization pattern should be altered in the orb^{343}/orb^{mel} ovaries. As shown in Fig. 5B, this is the case. Like the endogenous *orb* message, mRNA expressed by the *HD* transgene is not properly localized after stages 5-6 in the mutant ovaries. Typically the *HD* mRNA is distributed throughout the egg chamber instead of being concentrated at specific sites within the oocyte. These findings provide additional evidence that *orb* function is required for the proper localization of *orb* mRNA. Moreover, as was the case for translation, Orb mediates localization through *cis*-acting sequences in the 3′ UTR of the *orb* message.

Orb protein associates with the 3′ UTR of *orb* mRNAs in vivo

The results described above suggest that Orb autoregulates both the translation and localization of *orb* mRNA through sequences in the 3′ UTR of the message. Since Orb is predicted to be an RNA binding protein, it could carry out these functions by interacting with *orb* mRNA. To test this possibility, RNA isolated from ovary extracts immunoprecipitated with Orb antibody was reverse transcribed with an ‘anchored’ oligo(dT) primer (see Materials and Methods) and the reverse transcription products were then PCR amplified using an upstream primer specific for the *orb* 3′ UTR and a downstream primer corresponding to the ‘anchor’ sequence. The PCR products were then detected with a ^{32}P-labeled *orb* cDNA probe. To control for non-specific association of *orb* mRNA with antibody-bound beads, we reverse transcribed and PCR amplified (using the same primers) RNA immunoprecipitated from ovary extracts using Dorsal antibody. *orb* mRNA is absent in the Dorsal immunoprecipitates while it can be readily detected in the Orb immunoprecipitates (Fig. 6).

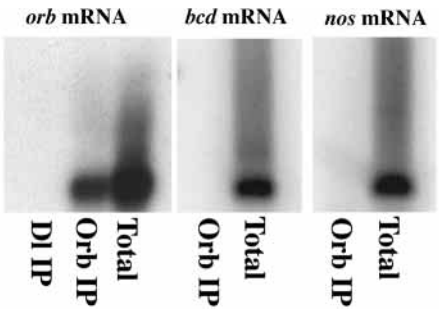

Fig. 6. *orb* mRNA in ovaries is associated with Orb protein in an immunoprecipitable complex. Anti-Orb or anti-Dorsal antibody was used for immunoprecipitation of wild-type ovary extracts. RNA isolated from the immunoprecipitates (IP) and from the total extracts was reverse transcribed with an anchored 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 *orb*, *bcd* or *nos*. The amplification products were displayed by electrophoresis and blotting to nitrocellulose and the filters were then hybridized with probes derived from the 3′ UTRs of *orb*, *bcd* and *nos*. *orb* 3′ UTR 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 (left panel). In both the Orb IP and the total samples, the Orb probe hybridizes to a prominent band and an upward smear. The prominent band corresponds in size to a PCR amplification product extending from the Orb 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 *bcd* PCR products (middle panel) or *nos* 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.

To assess the specificity of the *orb* mRNA:Orb ribonucleoprotein complex, we RT-PCR amplified RNA isolated from the Orb and Dorsal immunoprecipitates using the downstream anchored primers and upstream primers for two localized mRNAs, *bicoid* and *nanos*, which are not *orb* regulatory targets (Christerson and McKearin, 1994). These mRNAs are not present in the Orb immunoprecipitates (Fig. 6). We also used a RT-PCR primer pair derived from the protein coding region of the *orb* message. In contrast, to the anchored oligo(dT) 3′ UTR primer pair, we did not detect *orb* amplification products with these upstream primers (data not shown). Since RNAs are partially hydrolyzed during the immunoprecipitation procedure, this finding suggests that Orb is complexed with sequences in the *orb* 3′ UTR.

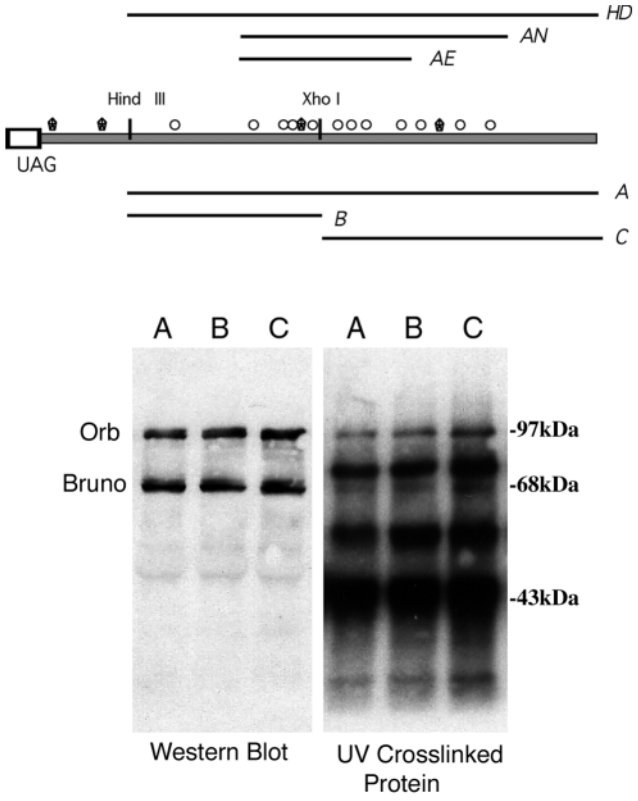

Fig. 7. Orb protein in ovaries extracts can be cross-linked to the 3′UTR of *orb* mRNA. (Top) The diagram shows the distribution of potential Orb protein binding sites in the 3′ UTR of the *orb* mRNA. Also shown is the approximate extent of the 3′ UTR sequences in the *HD*, *AN* and *AE* transgenes and the three *orb* 3′ UTR fragments, A, B and C, used to generate ^{32}P-labeled RNA probes. The stars indicate sequences in the 3′ UTR that correspond to the consensus CPEB sequence, while circles indicate U(A) rich sequences.
(Bottom) Protein samples from the UV cross-linking reaction were analyzed by SDS-PAGE gel electrophoresis and blotting to nitrocellulose. Autoradiography was used to detect proteins transferred to the nitrocellulose filter that were labeled by the ^{32}P RNA probes. There is a prominent doublet of approx. 40 kDa, a strong band at approx. 60 kDa, a weak band at approx. 70 kDa, a stronger band at approx. 75 kDa and a band of approx. 100 kDa. Position of molecular weight markers is indicated. After autoradiography, the nitrocellulose filter was probed with Orb antibody, and the Orb protein visualized by chemilluminesence. The filter was then re-probed with Bruno antibody (a gift from P. Macdonald) and the Bruno protein visualized by chemilluminesence. In the reprobe shown on the left, both Bruno and the residual Orb protein can be seen. Orb protein typically migrates as a doublet (Lantz et al., 1994), and the mobility of both bands in the doublet can be increased by phosphatase treatment. By overlaying the film of the western blot and the autoradiogram, it is possible to simultaneously align the upper band of the Orb protein doublet with the ^{32}P-labeled approx. 100 kDa protein and the Bruno protein band with the weak ^{32}P-labeled band of approx. 70 kDa. Note also that the ‘bubble’ that interrupts mostly the lower band of the Orb protein doublet is also evident at the lower edge of the approx. 100 kDa ^{32}P-labeled protein. Finally, the filter was re-probed with Squid antibody and the Squid protein visualized by chemilluminence (not shown). The Squid band co-migrates with the lower band of the approx. 40 kDa doublet. Again all three proteins visualized in the western blot can be simultaneously aligned with the corresponding ^{32}P-labeled species by overlaying the western and the autoradiograph. The identity of the ^{32}P-labeled ‘Squid’ and ‘Bruno’ bands in cross linking experiments with *orb* probes was further confirmed by UV cross linking experiments (run on the same gel) using two known RNA substrates for these proteins, *grk* (Norvell et al., 1999) and *osk* (Kim-Ha et al., 1995). Note also that there is an air bubble from the transfer that overlaps one edge of both the approx. 100 kDa ^{32}P-labeled band in the autoradiograph and the Orb protein doublet in the western blot. This air bubble (as well as several others) provides an independent marker for aligning the bands in the two films.

Protein co-migrating with Orb can be cross-linked to *orb* mRNA

The *Xenopus* CPEB protein binds to a U rich sequence (UUUUAU, UUUUAAU and variants thereof) that is present in several masked mRNAs. Since Orb has RRM and zinc finger domains that are closely related to the CPEB protein, it would be reasonable to suppose that Orb recognizes similar RNA sequences. Inspection of the UTR reveals that there are approximately 16 U rich sequences, which resemble the binding sites for the *Xenopus* protein (see Fig. 7). Four of these have the consensus CPE sequence UUUUAU, while the remaining twelve are variants. Two of the UUUUAU sequences, and twelve of the variants are included in the 3′UTR of the *HD* construct. The other two consensus CPE-sequences are in UTR sequences located just upstream of the HD construct in a region of the UTR present in both female and male mRNAs (see Lantz et al., 1992).

We used UV cross-linking to test if Orb protein in ovary extracts can interact with exogenous *orb* 3′ UTR RNA. Ovarian proteins cross-linked to RNA were displayed by gel electrophoresis, blotted to nitrocellulose and visualized by autoradiography. At least 6 distinct ^{32}P-labeled bands were detected (Fig. 7). Three of these, a 100 kDa band, a weak 70 kDa band, and the lower band in the 40 kDa doublet, correspond closely in size to known ovarian proteins, Orb, Bruno, and Squid respectively (Kelley, 1993; Kim-Ha et al., 1995; Lantz et al., 1994). Since *nos* mRNA is absent in Orb immunoprecipitates, we used a probe from the *nos* 3′ UTR for UV cross-linking. As expected, the approx. 100 kDa band was not labeled by the *nos* probe, though other protein species (including the approx. 70 kDa and approx. 40 kDa bands) were observed (not shown).

To determine if the 100, 70 and 40 kDa ^{32}P-labeled bands co-migrate with Orb, Bruno and Squid proteins, we first probed the nitrocellulose blot with Orb antibody. After visualizing Orb, we re-probed the same blot with Bruno antibody and subsequently Squid. In the Bruno re-probe, both Bruno and Orb are observed (Fig. 7), while all three proteins are observed in the Squid reprobe (not shown). We then overlayed the films to ascertain if the three bands in the western blot could be simultaneously aligned with the ^{32}P-labeled protein species seen in the autoradiograph. As illustrated for Orb and Bruno in Fig. 7, we found that the ^{32}P-labeled 100 kDa band co-migrates with the upper band of the Orb protein doublet, while the weaker ^{32}P-labeled 70 kDa band co-migrates with Bruno. We also found that the lower band in the 40 kDa doublet co-migrates with Squid (not shown). We subdivided the *orb* 3′ UTR into two smaller probes, B and C. Since each of these smaller probes is predicted to contain one of the consensus CPEB sites, plus 5 non-consensus sites, both would be

expected to cross-link Orb protein. Both smaller probes label protein species that co-migrate with Orb. While these results are consistent with the suggestion that the ^{32}P-labeled, 100 kDa, band is Orb, we cannot exclude the possibility that some other, unknown protein of the same size as Orb binds to the *orb* transcript in our cross-linking experiments.

DISCUSSION

The RRM-type RNA binding protein Orb plays a central role in the establishment of polarity in the *Drosophila* egg and embryo (Lantz et al., 1994; Christerson and McKearin, 1994). In addition to its functions early in oogenesis in the formation and initial differentiation of the egg chamber, *orb* is required for the establishment of the DV and AP axes. In DV axis formation, Orb protein is required to localize *grk* mRNA to the dorsal anterior part of the oocyte and promote translation. In AP axis formation, the translation of *osk* mRNA localized at the posterior pole of the oocyte also depends upon Orb. Critical to its functions in these polarity pathways, Orb protein must be properly localized within the developing oocyte. In the studies reported here we have investigated the mechanisms directing the expression of Orb protein in the appropriate pattern. Our results suggest that autoregulation plays a critical role in promoting the on site accumulation of Orb protein. In this model, translationally repressed *orb* mRNA synthesized in nurse cells would be transported into and targeted to specific sites within the oocyte. Orb protein already present at these sites would bind to the *orb* mRNA when it arrives, anchoring the message to the cortex of the oocyte and activate its translation. Newly synthesized Orb protein would then be available to interact with incoming localized mRNAs and activate their translation. Once initiated, this *orb* autoregulatory activity would ensure the accumulation of high levels of Orb protein at sites in the oocyte containing localized *orb* mRNAs.

Orb autoregulates its own expression

The autoregulatory model was initially suggested by the dominant negative activity of transgenes expressing *lacZ* mRNAs which have the *orb* 3′ UTR. As in classical antimorphic mutations, the phenotypic effects of the *lacZ orb* 3′ UTR transgenes can be exacerbated by increasing the transgene dose relative to the endogenous *orb* gene. Conversely, it is possible to suppress the phenotypic effects of the *orb* 3′ UTR transgenes by increasing the relative dose of the endogenous gene. Since β-galactosidase has no adverse effects on *orb* function, the antimorphic activity of these transgenes can be attributed to the *orb* 3′ UTR sequences in the transgene mRNAs. To have an antimorphic activity, these RNA sequences must interfere with the functioning of the endogenous gene. The most plausible mechanism is that the transgene 3′ UTR sequences compete with mRNA from the endogenous gene for some limiting factor that is essential for the expression of sufficient quantities of Orb protein. Since *orb* is haploinsufficient, the obvious candidate for this limiting factor is the Orb protein itself.

One prediction of the autoregulatory model is that the expression of β-galactosidase from the *lacZ orb* 3′ UTR mRNA should depend upon *orb* function. This is case. We found that Orb is required for the translation of the *lacZ orb* 3′UTR mRNA, and that the synthesis of β-galactosidase from the *lacZ orb* 3′ UTR message is substantially reduced in *orb* mutant ovaries. In contrast, *orb* mutations have no effect on the translation of *lacZ* mRNAs that have unrelated 3′ UTR sequences. A second prediction of the autoregulatory model is that the *lacZ orb* 3′UTR mRNA should compete with the endogenous message for *orb* function. Consistent with this prediction, increasing the relative dose of the transgene *lacZ orb* 3′ UTR mRNA down regulates Orb protein expression from the endogenous gene. Taken together with the dominant negative activity of the *lacZ orb*3′UTR transgene evident in genetic assays these two lines of evidence provide strong support for the autoregulatory model.

Since Orb is an RRM type RNA binding protein, the simplest hypothesis is that Orb activates translation by interacting with *orb* mRNA rather than indirectly by controlling the synthesis or functioning of some other protein that binds to the 3′ UTR. Two lines of evidence support the idea that Orb associates with *orb* mRNA in vivo. First, the defects in *orb* mRNA localization evident in *orb* mutant ovaries. Both the endogenous *orb* mRNA and the *lacZ orb* 3′ UTR transgene mRNA are not properly localized in the absence of wild-type *orb* activity. The abnormalities in localization, particularly the loss of the anterior ring along the nurse cell-oocyte margin, are consistent with a failure to properly anchor *orb* message to the oocyte cortex. Second, *orb* mRNA is associated with Orb protein in an immunoprecipitable complex in vivo. This complex appears to be specific as neither *bcd* nor *nos* can be detected in the immunoprecipitates. We have also assayed the Orb immunoprecipitates for 3′ UTR sequences from four mRNAs, *osk*, *K(10)*, *grk* and *Bic-D* that exhibit defects in localization and translation in *orb* mutant ovaries (Lantz et al., 1994; Christerson and McKearin, 1994; unpublished data), and could potentially be targets for *orb* regulation in vivo. Of these four, *osk*, *K(10)* and *Bic-D* are present in Orb immunoprecipitates, while *grk* is not (Chang et al., 1999; L. Tan, unpublished data).

An obvious question is whether Orb binds directly to *orb* or any of the other mRNAs found in the immunoprecipitates. Orb homologs (the CPEB proteins) in other species have been shown to recognize a U-rich 'CPE' sequences in the 3′ UTRs of masked mRNAs (Fox et al., 1989; Paris and Richter, 1990; Hake and Richter, 1994; Gebauer and Richter, 1996; Hake et al., 1998). It is interesting to note that CPE-like sequences are found in the *orb* 3′ UTR and also in the UTRs of *osk*, *K(10)*, and *Bic-D*. In contrast, CPE-like sequences are not found in *grk nos* or *bcd* mRNAs. Also favoring a direct interaction is the finding that a protein species which co-migrates with Orb can be UV cross-linked to ^{32}P-labeled sequences from the *orb* 3′ UTR RNA. However, even if Orb recognizes the CPE-like sequences in the *orb* 3′UTR, we cannot rule out the possibility that autoregulation is nevertheless indirect, and, for example, depends on activating the expression of some other protein which also binds to *orb* mRNA.

Mechanism of autoregulation

Assuming that Orb plays a direct role in autoregulation, two different mechanisms could potentially account for the autoregulatory activity. In the first, efficient translation of *orb*

mRNA would depend upon localization to the oocyte cortex and Orb protein would be required because it functions as an anchor. In this model, the defects in the expression of β-galactosidase from the *lacZ orb* 3′ UTR mRNAs in the absence of wild-type Orb would be explained by a failure in localization. Similarly, the antimorphic effects of the *lacZ orb* 3′ UTR transgenes would be explained by the displacement of the endogenous mRNA from the cortex by the transgene mRNA. Since *lacZ* mRNAs lacking the *orb* 3′ UTR are not localized in the oocyte, but are translated even in *orb* mutants, this postulated requirement for cortical association would have to be a special feature of mRNAs containing the *orb* 3′ UTR. For example, a translational repressor might be displaced from the *orb* 3′ UTR when the message is associated with the cortex.

In the second, Orb protein would not only anchor *orb* mRNA to the cortex, but also actively promote its translation. In this case, cortical localization would not in itself be sufficient for the translation of either the *orb* or *lacZ orb* 3′ UTR mRNAs. Arguing in favor of a more active role is the fact that the Orb homologs of *Xenopus* and other species, the CPEB proteins, function in the translational regulation of masked maternal mRNAs. These CPEB proteins are thought to bind to target sequences in the 3′ UTRs of masked maternal messages, initially helping to ensure that the mRNAs remain translationally silent (de Moor and Richter, 1999; Minshal et al., 1999). In response to an oocyte maturation signal, the proteins then activate translation of the masked mRNAs by promoting cytoplasmic polyadenylation (Fox et al., 1989; Paris et al., 1991; Hake and Richter, 1994; Stebbins-Boaz et al., 1996; Sheets et al., 1994, Sheets et al., 1995).

It would be reasonable to suppose that the regulatory activities of Orb resemble the CPEB proteins of other organisms. In this case Orb would positively autoregulate its own expression by activating the polyadenylation of localized *orb* mRNAs. However, experiments aimed at demonstrating this point have been inconclusive. Using the anchored-dT RT-PCR procedure of Salles et al. (Salles et al., 1994), we found that *orb* mRNAs isolated from the two strong loss-of-function *orb* mutants have much shorter poly(A) tails than wild type. The caveat here is that the apparent reduction in poly(A) tail length may be only an indirect consequence of the defect in *orb* function. For example, orb^{343} is an apparent protein null and the level of *orb* mRNA is quite low. The short poly(A) tails in this mutant could be due to the fact that the message is targeted for degradation and deadenylated by an Orb independent mechanism. For the hypomorphic allele orb^{mel}, the average poly(A) length appeared to be only slightly shorter than wild type. The presence of *orb* mRNAs with extended poly(A) tails is not surprising as substantial quantities of Orb protein are expressed in orb^{mel} mutant ovaries, especially in the pre-vitellogenic stages. However, this finding leaves open the possibility that Orb activates the translation of *orb* mRNAs by some other mechanism.

The autoregulatory cycle

While a positive autoregulatory feedback loop would provide a mechanism for ensuring that Orb accumulates at sites of localized *orb* mRNA, a number of important questions remain. Some of these can be illustrated by comparing *orb* autoregulation with the autoregulatory cycle of the *Sex-lethal* gene (*Sxl*) (Cline and Meyer, 1996). For *Sxl*, autoregulation is crucial to its function as a binary switch gene, *on* in females and *off* in males. When the gene is *on*, Sxl proteins promote their own synthesis by directing the splicing of *Sxl* pre-mRNAs in the productive female pattern. When the gene is *off* and no Sxl proteins are present, splicing is in the default male pattern and the resulting mRNAs do not encode functional proteins. At this point it seems unlikely that *orb* autoregulation is used as an *on/off* switch; rather we suspect that it serves to augment the on site accumulation of Orb protein. In this view, *orb* mRNAs would be translated at a low level in the absence of Orb, while translation would be upregulated in its presence. Consistent with this suggestion we have found that β-galactosidase is expressed from the *lacZ orb* 3′UTR transgene in both orb^{343} and orb^{303} ovaries even though these mutants have little or no functional Orb. (There is, however, an important caveat that the *lacZ orb* 3′ UTR mRNA does not have the long 5′ UTR (which contains several short Orfs) and consequently may not fully reproduce the translational regulation of *orb* mRNA.)

A second question relates to the initiation mechanism. In the case of *Sxl*, productive splicing can only occur in the presence of Sxl proteins. Consequently, activation of the autoregulatory cycle depends upon a special initiation pathway which bypasses this requirement (Cline and Meyer, 1996). If *orb* mRNAs can be translated at a reduced level in the absence of Orb protein, a special bypass mechanism would be unnecessary. However, there remains the problem of generating the proper spatial pattern of Orb protein accumulation within the egg chamber. For example, if the autoregulatory cycle is activated inappropriately in nurse cells, it would promote the accumulation of Orb protein in these cells instead of at the proper sites in the oocyte. One possibility would be to link autoregulation to Orb protein localization – for example, only Orb protein associated with the oocyte cortex would be able to activate *orb* mRNA translation. In this model, the binding of free Orb protein to *orb* mRNAs in transit in the nurse cells or in the oocyte would either have no effect on translation or (given the activities of CPEB proteins in other species) might actually repress translation.

Another issue is the mechanism that limits the positive autoregulatory feedback loop, preventing the over expression of Orb protein. In the case of *Sxl*, several factors appear to be responsible for limiting protein accumulation. One is the comparatively low level of *Sxl* transcription, while another is the instability of the Sxl protein (Bell et al., 1991). In addition, in females Sxl proteins negatively regulate their own translation by binding to target sites in the 3′ UTR of the *Sxl* mRNAs (Yanowitz et al., 1999). For *orb*, there must be mechanisms that turn off the autoregulatory cycle once sufficient quantities of Orb protein have been synthesized at particular sites. Since the localization of *orb* mRNA changes during oogenesis, one mechanism might be turnover (or re-localization) of *orb* mRNA. Another possibility is that high levels of Orb protein inhibit, instead of promote, translation. Finally, if positive autoregulation is coupled to the binding of Orb protein to the cortex, then the cycle might be inactivated once all Orb protein target sites are occupied. Further studies will be required to resolve these questions.

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