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Ypsilon Schachtel, a *Drosophila* Y-box protein, acts antagonistically to Orb in the *oskar* mRNA localization and translation pathway

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SUMMARY

Subcellular localization of mRNAs within the *Drosophila* oocyte is an essential step in body patterning. Yps, a *Drosophila* Y-box protein, is a component of an ovarian ribonucleoprotein complex that also contains Exu, a protein that plays an essential role in mRNA localization. Y-box proteins are known translational regulators, suggesting that this complex might regulate translation as well as mRNA localization. Here we examine the role of the *yps* gene in these events. We show that *yps* interacts genetically with *orb*, a positive regulator of *oskar* mRNA localization and translation. The nature of the genetic interaction indicates that *yps* acts antagonistically to *orb*. We demonstrate that Orb protein is physically associated with both the Yps and Exu proteins, and that this interaction is mediated by RNA. We propose a model wherein Yps and Orb bind competitively to *oskar* mRNA with opposite effects on translation and RNA localization.

Key words: *yps*, Oogenesis, RNA localization, Translation, *Drosophila melanogaster*, Y-box protein

INTRODUCTION

Subcellular RNA localization is a strategy used by cells to generate cellular asymmetry. Examples include RNAs localized to the dendrites and axons of neurons, the processes of oligodendrocytes, the lamellopodia of mobile fibroblasts, the progeny of dividing yeast cells, and the eggs of many species [reviewed in Bashirullah et al., and Hazelrigg (Bashirullah et al., 1998; Hazelrigg, 1998)]. Localization to a particular subcellular domain allows the localized accumulation of the protein products of mRNAs, and in some cases restricts protein products from other cellular regions where their presence would be detrimental. Fine control over the spatial distribution of proteins within cells is often achieved by linking mRNA localization to translational regulation, such that only localized mRNA is translated [reviewed in Macdonald and Smibert, and Lipshitz and Smibert (Macdonald and Smibert, 1996; Lipshitz and Smibert, 2000)].

In *Drosophila* eggs, subcellular RNA localization is an essential step in anteroposterior and dorsoventral patterning of the embryo [reviewed in Cooperstock and Lipshitz (Cooperstock and Lipshitz, 2001)]. In the anteroposterior patterning system, transcripts that encode key developmental regulators are localized to the anterior or posterior poles of the developing oocyte. For example, *bicoid* (*bcd*) mRNA, which is localized to the anterior pole, encodes a transcription factor required for the development of embryonic head and thoracic structures. *oskar* (*osk*) mRNA, localized to the posterior pole, encodes a protein required for formation of the germ cells and the abdomen. Both transcripts are synthesized in the nurse cells, germline-derived cells attached to the developing oocyte, and subsequently transported into the oocyte through cytoplasmic bridges called ring canals. Within the nurse cells, *bcd* and *osk* mRNA share a common pattern of localization mid-way through oogenesis: both accumulate in apical patches within the nurse cell cytoplasm (St Johnston et al., 1989; Pokrywka and Stephenson, 1995; Karlin-Mcginness et al., 1996). This transient nurse cell localization may represent a common intermediate step in the localization pathway of these mRNAs. Following transport to the oocyte, the mRNAs are sorted, *bcd* to the anterior, and *osk* to the posterior, by a polarized microtubule network and microtubule-based motors (Theurkauf et al., 1992; Pokrywka and Stephenson, 1995; Brendza et al., 2000; Schnorrer et al., 2000). While Bcd protein is not expressed until early embryogenesis, when its mRNA undergoes cytoplasmic polyadenylation (Salles et al., 1994), *osk* mRNA is translated during oogenesis by a process of localization-dependent translation, which further restricts its protein product to the oocyte's posterior pole (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995).

The regulatory events underlying localization-dependent translation of mRNA are a current area of intense investigation. Several factors have been shown to mediate translational repression of unlocalized *osk* mRNA, including Bruno (Bru), Apontic (Apt) and Bicaudal C (BicC) (Kim-Ha et al., 1995; Webster et al., 1997; Saffman et al., 1998; Lie and Macdonald, 1999a). Several other proteins are implicated in the translational derepression of localized *osk* mRNA. These

include two proteins, p50 and p68, which bind the osk 5′ UTR. p50 also binds the *osk* 3′UTR and may mediate translational repression as well as derepression (Gunkel et al., 1998). Factors that enhance *osk* translation include Vasa (Vas), Aubergine (Aub), Staufen (Stau) and Oo18 RNA-Binding Protein (Orb) (Wilson et al., 1996; Markussen et al., 1997; Chang et al., 1999; Micklem et al., 2000). Stau and Orb are also key factors in the *osk* mRNA localization pathway, as is Osk protein itself (Ephrussi et al., 1991; Kim-Ha et al., 1991; Christerson and McKearin, 1994; Lantz et al., 1994; Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). Osk protein is autoregulatory in the sense that, while *osk* mRNA localization is required for Osk translation, Osk protein is required for maintaining the posterior localization of its own mRNA (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). The mechanisms by which the above factors regulate *osk* translation remain only partially understood.

The *Drosophila exuperantia (exu*) gene is required for localizing *bcd* mRNA to the anterior pole of the oocyte (Frohnhofer and Nusslein-Volhard, 1987), and also plays a secondary role in localizing *osk* mRNA (Wilhelm et al., 2000). A GFP-Exu fusion protein, which provides full *exu* function, associates with cytoplasmic particles that are transported along microtubules within the nurse cell-oocyte complex. The transport characteristics, and effects of *exu* mutations on RNA localization, suggest that these particles are transport vehicles that target RNAs from their sites of synthesis in the nurse cells to their final destinations in the oocyte (Wang and Hazelrigg, 1994; Theurkauf and Hazelrigg, 1998; Cha et al., 2001). GFP-tagged Exu provided a biochemical handle for the purification of several components of these particles, including at least seven proteins and RNA (Wilhelm et al., 2000). One of these proteins is the product of the *ypsilon schachtel* (*yps*) gene, previously identified in a degenerate PCR screen for *Drosophila* Y-box proteins (Thieringer et al., 1997; Wilhelm et al., 2000).

The presence of Yps, a Y-box protein, in the Exu RNP complex led us to rethink the role of this complex in the patterning of the *Drosophila* egg. Y-box proteins are a family of nucleic acid binding proteins, present in many invertebrate and vertebrate species [reviewed in Graumann and Marahiel, and Matsumoto and Wolffe (Graumann and Marahiel, 1998; Matsumoto and Wolffe, 1998)]. All members of this family contain a nucleic-acid binding domain, the cold shock domain, first identified in a set of bacterial proteins that are induced in response to low temperature [reviewed in Phadtare et al., (Phadtare et al., 1999)]. In *E. coli*, the major cold shock protein CspA binds to RNA, acts as an RNA chaperone to alter RNA secondary structure, and enhances translation of its target RNA (Jiang et al., 1997). In vertebrates, Y-box proteins play regulatory roles in both transcription and translation. Several Y-box proteins are highly expressed in germ cells. Examples include the *Xenopus* protein FRGY2, and the mouse proteins MSY2 and MSY4 [reviewed in Sommerville (Sommerville, 1999)]. Evidence from RNA injection experiments and in vitro assays indicates that FRGY2 is a translational repressor of stored maternal mRNAs (Richter and Smith, 1984; Crawford and Richter, 1987; Tafuri and Wolffe, 1993; Matsumoto et al., 1996). FRGY2 may also function in the nucleus, possibly linking transcriptional and translational regulation (Bouvet and Wolffe, 1994). MSY2 and MSY4 exist in an mRNP complex in mouse spermatocytes, and both proteins bind sequences within the 3′ UTR of *Prm1*, a translationally repressed mRNA (Davies et al., 2000).

Based on the involvement of vertebrate Y-box proteins in translational regulation of stored germ cell mRNAs, we hypothesized that Yps might play a similar role in *Drosophila* oogenesis, and that the Exu RNP complex might function both to localize mRNAs and to regulate their translation. We describe here a genetic analysis of the *yps* gene. Our results show that *yps* regulates the localization and translation of *osk* mRNA by antagonizing the function of Orb, a known enhancer of *osk* translation and mRNA localization (Christerson and McKearin, 1994; Lantz et al., 1994; Chang et al., 1999). Furthermore, we present evidence that both Yps and Exu interact with Orb in *Drosophila* ovaries, and that this interaction is mediated by RNA. Our work represents the first genetic analysis addressing the germline functions of a Y-box protein, and provides the first known link between Y-box proteins and events that underlie localization-linked translation of mRNAs.

MATERIALS AND METHODS

In situ hybridization

In situ hybridization was performed as described (Wilhelm et al., 2000).

Immunofluorescence

Immunofluorescence was performed essentially as described (Lantz et al., 1994) with minor modifications. Ovaries from 1- to 2-day-old females were dissected into PBT (1×PBS, 0.1% Triton X-100) and fixed for 9 minutes in 6:1 heptane:fixative (6% formaldehyde, 16.7 mM KPO_4, pH 6.8, 75 mM KCl, 25 mM NaCl, 3.3 mM $MgCl_2$). Ovaries were blocked and permeablized for 3-5 hours in 1% BSA in PBST (1×PBS, 0.1% Triton X-100, 0.05% Tween-80), washed 3×10 minutes in PBT, and incubated overnight at 4°C in primary antibody diluted in 0.1% BSA in PBST (1:20 for anti-Orb 6H4, or 1:500 for anti-OskC or 1:500 for anti-Yps). The ovaries were then washed 3×10 minutes in PBT, then incubated for 3-4 hours at room temperature (RT) in secondary antibody (fluorescein-conjugated anti-mouse or rhodamine-conjugated anti-rabbit $F(ab')_2$ fragments, Jackson Immunoresearch) in PBT. Following final 3×10 minute washes in PBT, the ovaries were mounted in Fluoromount-G (Southern Biotechnology Associates).

Deletion mapping

The *TM3-GFP* balancer chromosome, designated TKG4, expresses green fluorescent protein (GFP) from a *Kruppel* promoter, and has been described (Casso et al., 2000). 0-2 hour embryos from *Df(3L)BK9*/*TM3-GFP* parents were collected and aged 7 hours at 25°C. The embryos were scored for GFP expression and placed individually in Eppendorf tubes. Single embryo DNA samples were prepared by homogenizing each embryo in 50 μl buffer (10 mM Tris, pH 8.2, 1 mM EDTA, 25 mM NaCl, 200 μg/ml proteinase K (Roche)) followed by incubation at 37°C for 30 minutes and at 95°C for 2 minutes. 25 μl samples for polymerase chain reaction (PCR) (5 μl DNA sample, 4.5 units AmpliTaq polymerase (Perkin Elmer), 1× Perkin-Elmer PCR buffer, 1.8 mM $MgCl_2$, 10 mM of each dNTP, and 20 pmol of each primer) were subjected to 30 cycles of 1 minute at 94°C, 2 minutes at 67°C and 3 minutes at 72°C. The primers used amplified a fragment including both the start codon and cold shock domain (CSD) of *yps*, and include appended restriction sites. The following are the primer sequences: b, 5′GGG GGA TCC ATG GCC GAT GCC GCG GAG AGT A3′ (Fig. 1); d, 5′GCG AAG CTT GAA

TTC CTA CTT GCG GCG ATT CTT CTT CAT C3′ (Fig. 1). To confirm genotypes, each DNA sample was subject to PCR with GFP primers. NGF-1: 5′CTG GTA ACC ATG AGT AAA GGA GAA3′ and GFP-XhoI: 5′CTC GAG TTT GTA TAG TTC ATC CAT3′. PCR reactions with the GFP primers were done with 1.5 mM $MgCl_2$ and an annealing temperature of 55°C.

Generation of *yps* mutations

The EP(3)3078 P-insertion line was obtained from the Berkeley *Drosophila* Genome Project (BDGP, unpublished data) (Rorth, 1996; Rorth et al., 1998) Excisions of the EP(3)3078 P-element were generated by standard procedures (Hamilton and Zinn, 1994). y,*w; EP(3)3078/TM3* virgin females were crossed en masse to *w; SbΔ2-3/TM2UbxΔ2-3* males. Dysgenic *w; EP(3)3078/SbΔ2-3* males were crossed to *w; CxD/TM3* virgin females, and male progeny bearing the *CxD* balancer and white eyes (indicating excision of the EP element) were collected. These males were crossed individually to *w; CxD/TM3* virgin females to establish stocks. Each excision line was crossed to *Df(3L)BK9* and those viable as hemizygotes were tested by PCR for a deletion in *yps*. (Two hemizygous lethal lines were also obtained and balanced with the *TM3-GFP* balancer (see above), and non-GFP embryos were collected for PCR. However, by PCR analysis the *yps* gene appeared intact in these two cases, and since further analysis of a *yps* null allele showed that *yps* is not required for viability, these two lines probably contain second-site lethal mutations.) DNA samples for PCR were made from single flies or embryos following the protocol described above for single embryo PCR. Primers for the initial screening were b and d (Fig. 1). Deletions were confirmed by Southern blot (Sambrook et al., 1989) using a *yps* cDNA probe. For amplification of the CSD, the primers used were: c, 5′ATC AAC CGC AAC GAC ACC AGA GAG3′; and d (Fig. 1), with an annealing temperature of 55°C. As a positive control for the presence of template DNA in the PCR reaction, an unrelated region of the second chromosome was amplified. The sequences of the control primers are as follows: 5′EBP10-1: 5′CAC GAA TTC GAG CTG ATT CTG GAG GAG C3′, and 3′EBP10-1: 5′CAT CTC GAG CTT CTG TGC AGA TCT CTG3′. Breakpoints of the yps^{JM2} deletion were determined by PCR amplification of the surrounding DNA using primer a, 5′GCT CTA GAC GCG TAA CAG CAC ACT GGA G3′; and primer d (Fig. 1), as described above except with an annealing temperature of 54°C. The amplified fragment was subcloned into the *Xba*I and *Eco*RI sites of PBS-SK and sequenced.

Northern analysis

Ovary poly(A^+)RNA was prepared as follows. 50-100 hand-dissected ovaries from w^{1118} or $yps^{JM2}/Df(3L)BK9$ females were homogenized with a pestle in 1 ml homogenization buffer (50 mM Tris, pH 7.5, 60 mM NaCl, 20 mM EDTA, pH 8.0, 1% SDS, 100 μg/ml proteinase K), incubated for 1 hour at 37°C, and phenol-chloroform extracted and ethanol-precipitated by standard procedures (Sambrook et al., 1989). The pellet was redissolved in 250 μl 10 mM EDTA, pH 8.0, and RNA was precipitated overnight at 4°C by addition of 125 μl 10 M LiCl. The RNA was pelleted by centrifugation, washed in 70% ethanol, and redissolved in 0.5 ml 10 mM EDTA, pH 8.0. The solution was heated to 70°C for 10 minutes, cooled on ice for 5 minutes, then mixed with 100 μl 10 M LiCl and added to 0.2 g pretreated oligo-dT cellulose (Stratagene) in 1 ml LB (0.5 M LiCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.1% SDS). The oligo-dT cellulose was pretreated by hydrating in 1 ml EB (2 mM EDTA, 0.1% SDS) and washing 3× in LB. The RNA was allowed to bind the oligo-dT cellulose by incubation at RT for 15 minutes, and loaded onto a BioRad poly-prep chromatography column. The flow-through solution was reloaded once, the column was washed with 1 ml LB, and poly(A^+)RNA was eluted from the column with 1 ml EB. The poly(A^+)RNA was ethanol precipitated twice and redissolved in diethyl pyrocarbonate (DEPC)-H_2O at a final concentration of 0.5 μg/μl.

1 μg poly(A^+)RNA per lane was electrophoresed on a formaldehyde gel (1% agarose, 20 mM Mops, 0.5 mM sodium acetate, 0.1 mM EDTA, 6.5% formaldehyde), transferred to a Magna NT nylon membrane (MSI) on a vacuum blotter (LKB) following the manufacturer's instructions, and crosslinked by UV light in a Stratalinker.

The blot was probed with the *yps* cDNA LD01538 (BDGP) insert, excised from pBluescript by digestion with *Xba*I and *Kpn*I, and ^{32}P-labeled with the RadPrime random-primer labeling kit (BRL) according to the manufacturer's instructions.

Western analysis

Ovary protein was prepared by dissecting ovaries from 1-2 day old w^{1118} control and $yps^{JM2}/Df(3L)BK9$ females. For each mg ovary tissue, 10 μl homogenization buffer (100 mM benzamidine HCl, 1 mg/ml Pepstatin A, 100 mM PMSF, 1 mg/ml phenanthroline) was added, and the tissue was homogenized with a pestle. The concentration of protein was determined by a BioRad Protein Assay according to the manufacturer's instructions. The remaining homogenate was diluted 1:1 in 2× Laemmli buffer (80 mM Tris, pH 6.8, 15.4 mg/ml dithiothreitol (DTT), 2% SDS, 10% glycerol, 0.006% Bromophenol Blue), boiled for 5 minutes, and passed 5× through a 26-gauge syringe needle. The protein preparations were frozen in liquid N_2 and stored at –80°C until use. Prior to loading on a gel, each protein preparation was boiled for 5 minutes.

Approximately 0.03 mg ovary protein per lane was run on a 12% polyacrylamide gel (Sambrook et al., 1989). One set of samples was stained with Coomassie Blue (Sambrook et al., 1989), and the remainder of the gel was transferred to a Magna NT nylon filter (MSI) with a semi-dry electrophoretic transfer cell (BioRad) for 25 minutes at 15 V. After blocking overnight at 4°C in 4% BSA in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20), and washing 3×10 minutes in TBST at RT, the blot was incubated for 1 hour at room temperature at a 1:5000 dilution in TBST of an antibody raised to the N-terminal portion of Yps, including the CSD (Wilhelm et al., 2000). Blots were washed for 3×10 minutes in TBST, then incubated with an alkaline-phosphatase conjugated anti-rabbit secondary antibody (Jackson Immunoresearch) at 1:5000 in TBST for 30 minutes. The blot was washed 3×10 minutes in TBST, then incubated in staining solution (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.66 mg/ml NBT (Roche), 0.165 mg/ml BCIP (Roche)) until bands developed, before being washed and dried.

Generation of recombinants

yps^{JM2}, orb^{mel} recombinants were recovered using PCR to identify the yps^{JM2} and orb^{mel} mutations. Virgin $yps^{JM2}/TM3,Sb$ females were mated *en masse* to $orb^{mel},ry/TM3,Sb,ry$ males, to generate $yps^{JM2}/orb^{mel},ry$ female progeny. These females were crossed to *TM2,ry/MKRS,ry,Sb* males, and single *Sb,ry* males among the progeny were crossed individually to *CxD/TM3,Sb* females to generate individual lines of each potential recombinant. After mating for several days, each male was removed from the cross and subjected to single-fly PCR as described above for single embryos. Primers 5′-1: 5′CCA GTA TCA GAG CAT CGA TAG CAC C3′ and CSD-HE (see above) amplify a novel 100 bp band in yps^{JM2} mutants, and were therefore used to identify single males carrying the yps^{JM2} allele. Annealing conditions for these primers were 55°C and 1.8 mM $MgCl_2$. To detect the orb^{mel} mutation, single fly-PCR was performed on the same DNA with the primers orb 5′: 5′TGC TGA TAC GGC CAC ATC TGC3′ and orb 3′: 5′TGA AGC GGA TTG TCA GGC AGG3′, with an annealing temperature of 63°C and 2 mM $MgCl_2$. These primers span the orb^{mel} deletion and amplify a fragment that is approximately 500 bp smaller in the orb^{mel} allele than in the corresponding wild-type DNA fragment. Recombinants between yps^{JM2} and the orb^{F303} and orb^{F343} mutations were generated with a similar strategy as for orb^{mel}, except that recombinant lines mutant for *orb* were detected by backcrossing to either orb^{F303} or orb^{F343} and assaying for early oogenesis arrest associated with these alleles (Lantz et al., 1994).

Fertility assays

The genotypes tested for female fertility were: w^{1118}, yps^{JM2}/yps^{JM2}, orb^{mel}/orb^{mel}, $yps^{JM2}orb^{mel}/yps^{JM2}orb^{mel}$, orb^{mel}/orb^{F303}, $yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}$, orb^{mel}/orb^{F343} and yps^{JM2} orb^{mel}/yps^{JM2} yps^{JM2} orb^{F343}. Virgin females of these genotypes were collected and crossed to w^{1118} males. After allowing the flies to mate for at least 48 hours, eggs were collected on apple juice plates at 4 hour intervals and aged 25 hours at 25°C, and the percentage of eggs hatched was scored.

Immunoprecipitation

Immunoprecipitations and RNAse treatments were as described previously using whole fly extracts (Wilhelm et al., 2000). Orb immunoblots used the anti-orb 6H4 mouse monoclonal antibody (Developmental Studies Hybridoma Bank) at a concentration of 1:20 using conditions described previously (Lantz et al., 1994).

RESULTS

yps maps to 68F3-F6

We mapped *yps* to polytene interval 68F3-F7 by chromosome in situ hybridization, using a *yps* cDNA probe (Fig. 1). The smallest published deficiency that removes this region is *Df(3L)BK9* (Leicht and Bonner, 1988). To confirm that this deficiency deletes *yps* we constructed a *Df(3L)BK9*/*TM3-GFP* stock. The *TM3-GFP* balancer chromosome expresses GFP in the *kruppel* expression pattern (Casso et al., 2000). Embryos were collected from *Df(3L)BK9*/*TM3-GFP* parents, and scored for GFP expression commencing at the germ-band extension stage. Those not expressing GFP (homozygous deficiency embryos) were selected for single-embryo PCR using *yps* primers. Embryonic genotypes were confirmed by PCR amplification of a fragment of the *gfp* gene (see Materials and Methods). We were unable to amplify *yps* DNA from homozygous *Df(3L)BK9* embryos, indicating that the deletion uncovers *yps*. In support of this result, we found by the same method that the larger 68F deficiencies *Df(3L)vin4* and *Df(3L)vin7* also uncover *yps* (Fig. 1). *Df(3L)BK9* was used in our subsequent genetic analysis of *yps*.

yps expression in ovaries

Northern blot analysis, using a *yps* cDNA to probe poly(A$^+$)RNA isolated from hand-dissected ovaries, revealed the presence of two species of transcripts: a broad mRNA band migrating at approximately 1.7 kb, which may contain several transcripts of similar size, and a minor 2.3 kb transcript (Fig. 2A, lane WT). Alignment of previously published *yps* cDNA sequences (Thieringer et al., 1997; Wilhelm et al., 2000) with genomic DNA reveals the gene structure shown in Fig. 1. The exon-prediction computer programs FGENE and FEX (see Materials and Methods) both give high scores to an alternate fourth exon, which could account for the size difference of approximately 600 bp between the larger and smaller transcripts. In addition, this predicted exon contains multiple consensus polyadenylation signals, which could produce the broad 1.7 kb band observed on northern blots. If used, this exon would replace the last 15 amino acids of the known protein sequence with 23 alternative amino acids.

We also examined *yps* expression in ovaries by whole-mount in situ hybridization (Fig. 2B). *yps* is transcribed throughout oogenesis, and its transcripts are evenly distributed in nurse cells, and at lower levels in the oocyte. A low level of expression is also detected in the follicle cells.

Generation of an internally deleted *yps* allele

The P-element insertion EP(3)3078 was obtained from the Berkeley *Drosophila* Genome Project. The original EP(3)3078 insertion chromosome is homozygous lethal, but this lethality proved to be due to a second-site mutation, which we removed by recombination. The insertion site of EP(3)3078 lies 259 bp upstream of the *yps* translation start. In order to generate a null allele, we screened for imprecise excisions of the EP(3)3078 element that delete portions of the *yps* coding region (see Materials and Methods for details of the screen). We identified ten imprecise excisions from which we could not PCR-amplify a DNA fragment extending from the *yps* translation start site to a site downstream of the cold shock domain (CSD) (using primers b and d in Fig. 1). One excision, yps^{JM2}, additionally

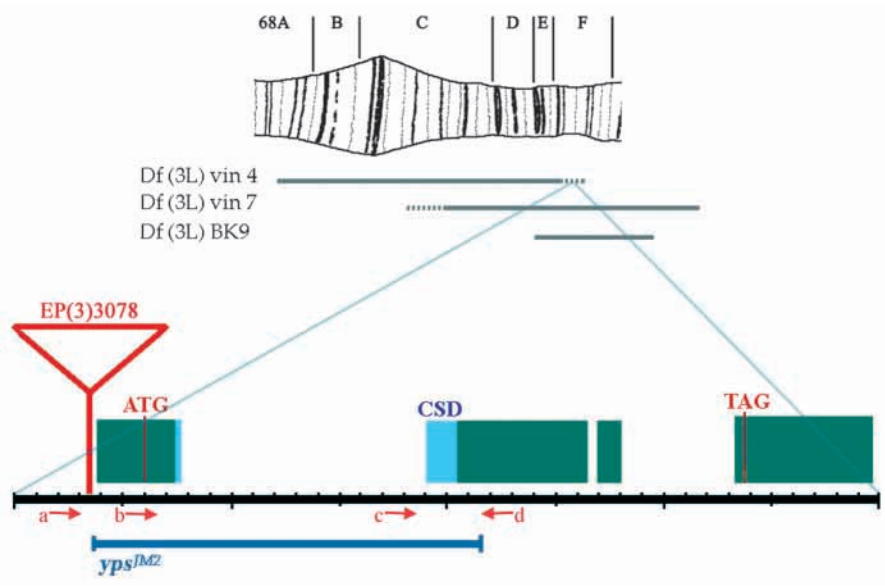

Fig. 1. A physical map of the *yps* gene, and the yps^{JM2} deletion mutation. *yps* was mapped to polytene region 68F3-F7 by chromosome in situ hybridization. Three deletions that uncover *yps* are indicated: *Df(3L)vin4*, *Df(3L)vin7* and *Df(3L)BK9*. Alignment of known *yps* cDNAs with genomic sequence predicts the exon structure of *yps* shown in the diagram. Each division of the black line represents 100 bp of genomic DNA, while the thick green and blue boxes represent exons. The precise locations of the 5′ end of the transcripts have not been mapped. The cold shock domain (CSD) is shown in light blue, and the start (ATG) and stop (TAG) codons are marked. The EP(3)3078 insertion (represented by a red triangle) was mapped 259 bp upstream of the start codon (BDGP). The limits of the yps^{JM2} deletion are represented by the blue line at the bottom. The positions of PCR primers used for the excision screen (see text) are indicated by the red arrows labeled a-d. The chromosome drawing is reproduced after the drawings of Bridges (Bridges, 1941).

Fig. 2. *yps* expression in ovaries. (A) A northern blot of ovary poly(A$^+$)RNA probed with a *yps* cDNA. Two forms of the *yps* transcript are detected in wild-type ovaries (lane WT): a minor band of approximately 2.3 kb and a major broad band of approximately 1.7 kb, which may actually represent several transcripts of similar size. The positions of RNA molecular mass markers (M) are indicated on the right. In ovary poly(A$^+$) mRNA from *yps^{JM2}/Df(3L)BK9* females, the *yps* mRNAs are truncated and present at greatly reduced levels (lane yps^{JM2}). *rp49* serves as a loading control for both samples. (B) Whole-mount in situ hybridization of a *yps* cDNA to wild-type ovaries. *yps* is expressed in the germ cells from early oogenesis on, and its transcripts are distributed evenly throughout the nurse cells and can be detected at a low level in the oocyte. *yps* is also expressed at a low level in the follicle cells. (C) A western blot of ovary protein, probed with an antibody to the N terminus of Yps (a region that includes the CSD) reveals a single protein isoform migrating at approximately 50 kDa (lane WT). In protein extracted from *yps^{JM2}/Df(3L)BK9* ovaries (lane yps^{JM2}), Yps is not detected.

failed to PCR-amplify with primers spanning the majority of the CSD (primers c and d in Fig. 1), as well as the initial primer pair used for screening. Using a primer upstream of the original EP(3)3078 insertion, coupled with a primer downstream of the CSD (primers a and d in Fig. 1), a novel DNA fragment was amplified from *yps^{JM2}* DNA and sequenced. Analysis revealed that *yps^{JM2}* contains a deletion of 1.8 kb of genomic DNA (Fig. 1), which removes both the translation start codon and the entire CSD.

The *yps^{JM2}* allele produces truncated transcripts that are present at significantly reduced levels in ovaries (Fig. 2A, lane yps^{JM2}), and no protein is detected on western blots (Fig. 2C, lane yps^{JM2}). By immunocytochemistry, using antibodies against the amino terminus (including the CSD) or against the full-length Yps protein, we detected no Yps protein in *yps^{JM2}* homozygous and hemizygous ovaries (not shown).

The *yps^{JM2}* mutation has no effect on zygotic viability: *yps^{JM2}/yps^{JM2}* and *yps^{JM2}/Df(3L)BK9* flies survive at expected frequencies. Both homozygous and hemizygous *yps^{JM2}* females are fertile, and although there is a slight reduction in the hatching rates of their embryos, these rates are comparable to *Oregon R* and *w^{1118}* control females (Table 1).

Yps and Exu proteins localize independently of each other

Previously, we showed that Yps and Exu proteins associate in vivo as well as in in vitro binding assays, and colocalize throughout oogenesis (Wilhelm et al., 2000). Both proteins accumulate in the oocytes of young egg chambers, associate with particles in the nurse cell cytoplasm, and localize to both the anterior and posterior poles of mid-stage oocytes (Fig. 3A,C). It therefore seemed reasonable that one protein could be required for the localization of the other. However, we found this not to be the case. In ovaries of *yps^{JM2}/Df(3L)BK9* females expressing a *gfp-exu* transgene, GFP-Exu was localized

Table 1. The maternal effect lethal phenotype of hypomorphic *orb* alleles is suppressed by *yps^{JM2}*

Genotype	Eggs hatched/total	% hatched
(A)		
Oregon R	2876/3180	90
yps^{JM2}/yps^{JM2}	1195/1419	84
orb^{mel}/orb^{mel}	231/416	44
yps^{JM2} orb^{mel}/yps^{JM2} orb^{mel}	1529/1838	83
(B)		
w^{1118}	1888/2083	94
yps^{JM2}/yps^{JM2}	1616/1890	86
orb^{mel}/orb^{F303}	156/2554	6
yps^{JM2} orb^{mel}/yps^{JM2} orb^{F303}	507/1265	40

Females of the indicated genotypes were mated with wild-type males. Their eggs were collected and allowed to develop for 25 hours at 25°C, and scored for embryo hatching.

(A) The maternal effect lethal phenotype of *orb^{mel}/orb^{mel}* is completely rescued by *yps^{JM2}*. There is a slight reduction in the hatching rate of eggs from *yps^{JM2}/yps^{JM2}* mothers compared to wild-type controls, in this case Oregon R. (Similar hatching rates were also observed in eggs from hemizygous *yps^{JM2}/Df (3L)BK9* females; data not shown.) Eggs from *orb^{mel}/orb^{mel}* females have a pronounced reduction in hatching, while in the double mutant, *yps^{JM2}orb^{mel}/yps^{JM2}orb^{mel}*, the hatching rate is rescued to that of *yps^{JM2}/yps^{JM2}* single mutants.

To rule out the possibility of breakdown or contamination of stocks, we confirmed the genotypes of all of the individual *orb^{mel}/orb^{mel}* and *yps^{JM2}orb^{mel}/yps^{JM2}orb^{mel}* females in these assays by PCR, using primers that detect unique DNA fragments for both the *orb^{mel}* and *yps^{JM2}* alleles (see Materials and Methods).

Note: our hatching rate for embryos from *orb^{mel}/orb^{mel}* mothers is higher than previous reports (Christerson and McKearin, 1994). While we are not sure of the reason for this discrepancy, it may simply reflect greater accuracy due to the large number of embryos scored in the present study.

(B) The maternal effect lethal phenotype of *orb^{mel}/orb^{F303}* is partially rescued by *yps^{JM2}*. While hatching of eggs from *orb^{mel}/orb^{F303}* mothers is extremely low, significant rescue is observed when females are doubly mutant, *yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}*.

In this and subsequent assays, a *w^{1118}* stock was used as the control.

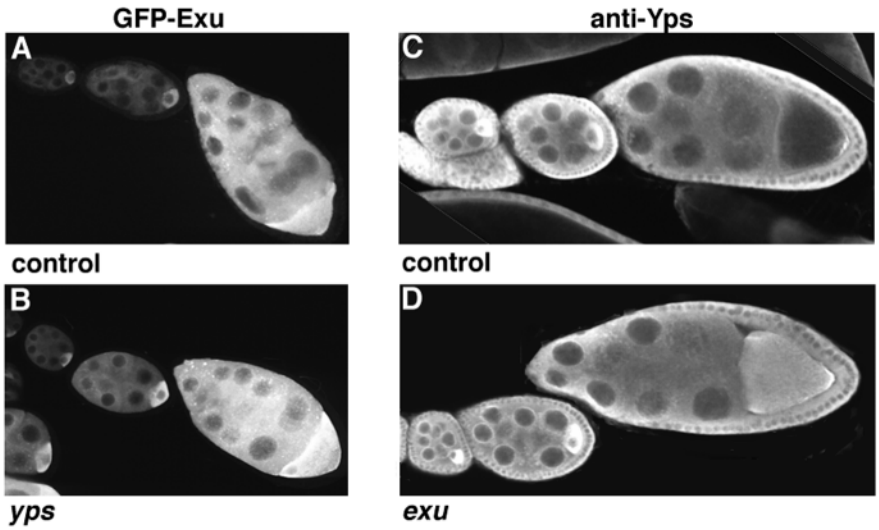

Fig. 3. Yps and Exu proteins are localized independently of one another. Ovaries in A and B are from female flies carrying a *gfp*-exu transgene. (A) GFP-Exu in control egg chambers illustrates the wild-type localization state of the protein. Exu accumulates in the oocyte from early oogenesis, and during mid-oogenesis becomes concentrated at the anterior and posterior poles of the oocyte. Within the nurse cells, Exu is localized to cytoplasmic particles. (B) GFP-Exu is expressed and localized normally in yps^{JM2}/*Df(3L)BK9* ovaries. (C) Control ovaries labeled with an antibody to Yps reveal that, like Exu, Yps accumulates early in the oocyte, becomes concentrated at the anterior and posterior poles of the oocyte during mid-oogenesis, and is associated with particles in the nurse cell cytoplasm. Yps is also expressed in the follicle cells, unlike Exu. (D) In exu^{SC02}/exu^{SC02} ovaries, Yps is expressed and localized normally.

normally throughout oogenesis (Fig. 3B). Conversely, using an antibody against Yps, we found no defects in Yps localization in exu^{SCO2} (a null allele) ovaries (Fig. 3D).

Yps is not required for *osk* mRNA localization in the oocyte

Yps is a member of an ovarian ribonucleoprotein (RNP) complex hypothesized to function in mRNA localization and/or translational regulation (Wilhelm et al., 2000). Therefore, we expected that *yps* mutants could be defective in one of these processes. Because *osk* mRNA is present in the Exu-Yps complex, we asked whether *yps* might play a role in *osk* mRNA localization or translation. By whole-mount in situ hybridization to ovaries, we found that *osk* mRNA localization is normal in yps^{JM2} oocytes (Fig. 4B). Also, *osk* mRNA remains subject to localization-dependent translation in yps^{JM2} ovaries: Osk protein is detected only at the posterior pole of the oocyte, and appears with normal developmental timing (see below, and Fig. 5B).

yps interacts genetically with *orb*

We reasoned that if Yps normally functions to regulate *osk* RNA localization or translation, we might detect genetic interactions with known repressors or activators of these events. In fact, we detected a robust genetic interaction between yps^{JM2} and mutations in the *oo18-RNA binding protein (orb)* gene. Orb is an RNA binding protein with strong similarity to CPEB, a protein required for cytoplasmic polyadenylation of mRNAs in *Xenopus* oocytes (Lantz et al., 1992; Hake and Richter, 1994; Stebbins-Boaz et al., 1996; Chang et al., 1999). The phenotypes of *orb* mutants indicate that Orb is required for *osk* mRNA localization and translation, and that Orb may directly regulate cytoplasmic polyadenylation of *osk* mRNA (Christerson and McKearin, 1994; Lantz et al., 1994; Chang et al., 1999). Strong *orb* alleles are female-sterile and cause oogenesis to arrest at very early stages, indicating an early requirement for the protein. Weaker allelic combinations allow oogenesis to be completed, but are maternal-effect lethal. In these combinations, the localization

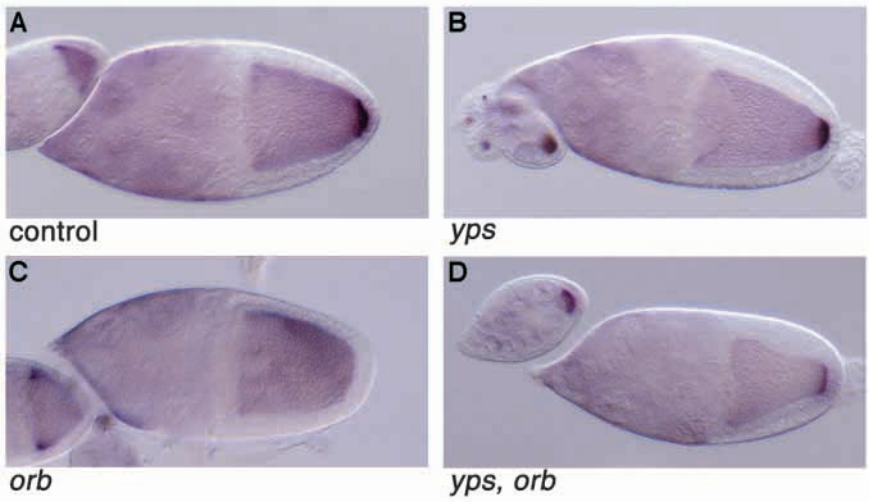

Fig. 4. yps^{JM2} rescues the *osk* mRNA localization defects of orb^{mel}/orb^{F303} females. In wild-type ovaries, *osk* mRNA accumulates in the oocytes of young egg chambers (stages 1-7), is transiently localized to the anterior of the oocyte in stage-8 to early stage-9 egg chambers, and then becomes localized to the posterior pole of the oocyte by late 9. (A) Egg chambers from control (w^{1118}) ovaries. In the stage-10 egg chamber on the right, *osk* mRNA is localized at the posterior of the oocyte. (B) yps^{JM2}/yps^{JM2}egg chambers. *osk* mRNA accumulates normally in the oocytes of young-stage egg chambers on the left. In the stage-10 egg chamber in the center of the panel, *osk* mRNA is localized normally to the posterior pole of the oocyte. (C) orb^{mel}/orb^{F303} egg chambers. In the stage-10 egg chamber in the center of the panel, *osk* mRNA is not localized at the posterior pole of the oocyte. (D) $yps^{JM2}orb^{mel}$/$yps^{JM2}orb^{F303}$egg chambers. In the stage-10 egg chamber on the right, *osk* mRNA is localized at the posterior pole of the oocyte, although its level is reduced compared to wild type.

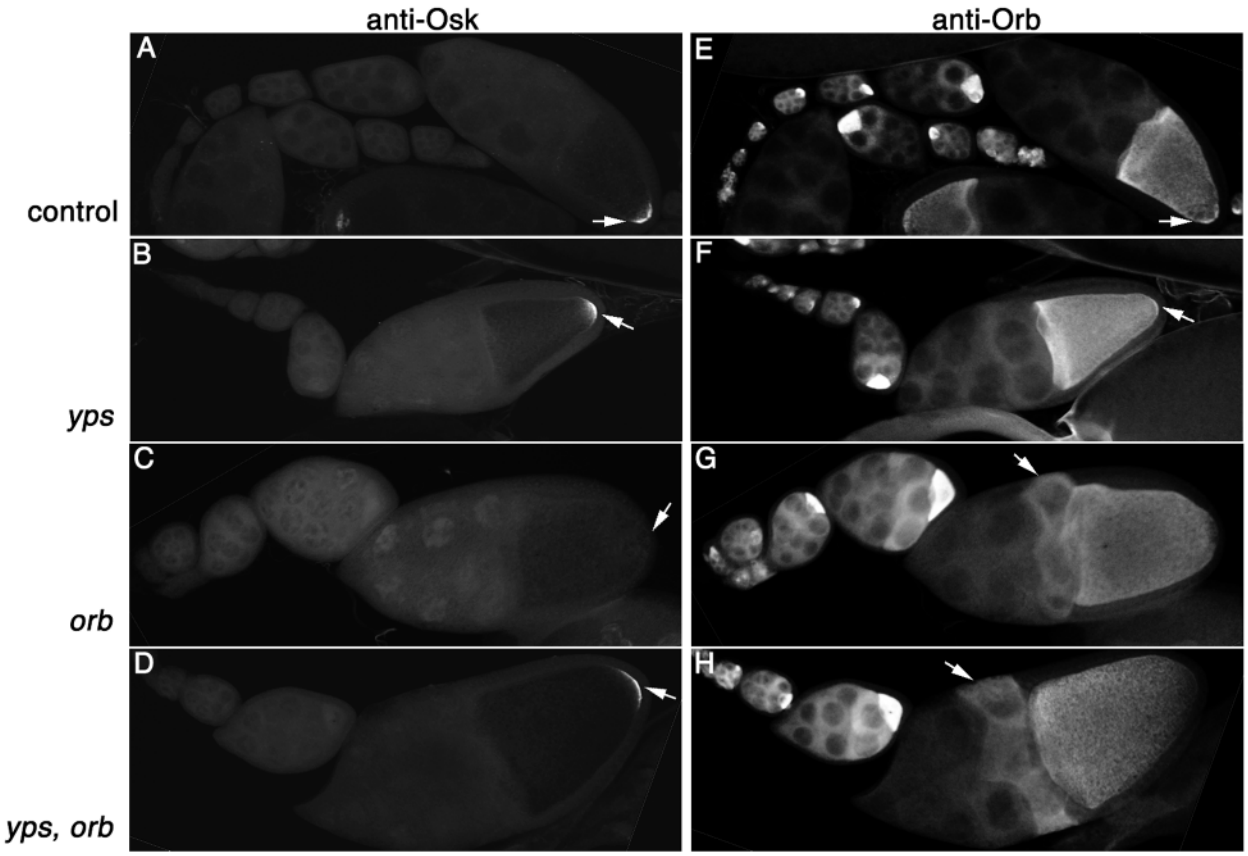

Fig. 5. yps^{JM2} rescues *orb*-associated defects in *osk* protein expression, but does not alter the expression or localization of Orb protein. The ovaries in A-D were labeled with an antibody to the Osk protein. Arrows in A-D indicate the posterior poles of stage-10 oocytes. (A) w^{1118} (control) egg chambers. Osk protein is present at the posterior pole of the stage-10 oocyte. (B) An identical pattern is observed in yps^{JM2}/yps^{JM2} egg chambers. (C) In orb^{mel}/orb^{F303} mutant egg chambers, Osk protein is not detected at the posterior pole of the oocyte. (D) In many $yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}$ doubly mutant egg chambers, Osk protein is present, localized at the posterior pole. The ovaries shown in A-D were also labeled with an antibody to the Orb protein (E-H). (E) In control egg chambers, Orb concentrates in the oocyte from early oogenesis, and is present at lower levels in the nurse cells. During mid-oogenesis the protein accumulates at the anterior and posterior (arrow) poles of the oocyte. (F) An identical pattern is observed in yps^{JM2}/yps^{JM2} egg chambers. (G) In orb^{mel}/orb^{F303} egg chambers, Orb accumulates in the nurse cells to a higher level than in wild type. During mid-oogenesis, Orb is particularly concentrated in the nurse cells that border the oocyte (arrow). Orb rarely accumulates at the anterior or posterior poles of the oocyte. (H) In $yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}$ ovaries, the distribution of Orb is identical to orb^{mel}/orb^{F303} ovaries.

of both *grk* and *osk* mRNA is disrupted at mid-oogenesis, and Osk protein is frequently not translated. These aberrations lead to embryonic defects in both anteroposterior and dorsoventral patterning (Christerson and McKearin, 1994; Lantz et al., 1994; Roth and Schupbach, 1994; Chang et al., 1999).

Four alleles of *orb* have been described, three of which were used in our study. orb^{mel}, a hypomorphic allele, is associated with a 500 bp deletion within the gene that is predicted to yield a transcript in which the second exon, which encodes a portion of the *orb* 5′UTR, is missing, and exons 1 and 3 are spliced directly together (Christerson and McKearin, 1994). Orb protein is expressed in orb^{mel} homozygous ovaries, although at reduced levels, and with abnormal distribution **(**Christerson and McKearin, 1994; Chang et al., 1999). Homozygous females complete oogenesis, but many of their progeny die with defects in both anteroposterior and dorsoventral patterning. orb^{F343} is a protein null; in homozygous females, oogenesis arrests during cyst formation, so that egg chambers are not formed (Lantz et al., 1994). A strong allele, orb^{F303}, is a point mutation that expresses only one of the two Orb protein isoforms (Lantz et al., 1994), and displays an intermediate phenotype. Homozygous orb^{F303} females are completely sterile, and oogenesis arrests shortly after egg chambers have budded from the germarium (Lantz et al., 1994). The *orb* allelic combinations we used in this study are expected to provide varying levels of functional Orb protein, from least to most, in the following order: $orb^{F343}/orb^{F343} < orb^{F303}/orb^{F303} < orb^{mel}/orb^{F343} < orb^{mel}/orb^{F303} < orb^{mel}/orb^{mel}$.

We constructed yps^{JM2} orb^{mel}, yps^{JM2} orb^{F303} and yps^{JM2} orb^{F343} doubly mutant chromosomes (see Materials and Methods), and tested the fertility of these females. We found that yps^{JM2} rescues the embryonic hatching defects of the weakest *orb* allelic combination, orb^{mel}/orb^{mel}. While the hatching rate of eggs laid by orb^{mel}/orb^{mel} females was 44% in our assay, this rate increased to 83% in yps^{JM2} orb^{mel}/yps^{JM2} orb^{mel} females, the same as the hatching rate of eggs from yps^{JM2} single mutants (Table 1A). We confirmed the genotypes of all orb^{mel}/orb^{mel} and yps^{JM2} orb^{mel}/yps^{JM2} orb^{mel} females

used in our assay by single-fly PCR to detect the unique DNA fragments associated with these alleles (see Materials and Methods). In the case of orb^{mel}/orb^{F303}, only 6% of eggs laid by mutant females hatched (Table 1B). Additional loss of *yps* increased the hatching rate to 40%. As previously reported, we found that no eggs hatch from orb^{mel}/orb^{F343} mothers (Christerson and McKearin, 1994). We observed a very weak rescue of this phenotype in yps^{JM2} orb^{mel}/yps^{JM2} orb^{F343} mothers, such that rare escapers complete embryogenesis and hatch. yps^{JM2} did not rescue the female sterile phenotype of the strongest allelic combinations, orb^{F303}/orb^{F303} or orb^{F343}/orb^{F343}. Thus, rescue of the *orb* maternal effect lethal phenotype by yps^{JM2} requires the presence of some functional Orb protein, and the degree of rescue reflects the level of Orb supplied by each mutant combination.

yps^{JM2} rescues *orb*-associated defects in *osk* mRNA localization and translation

Defects in *osk* mRNA localization have been described for orb^{mel}/orb^{F343} ovaries (Christerson and McKearin, 1994), and we found an identical phenotype in orb^{mel}/orb^{F303} ovaries. In these females, *osk* mRNA accumulates normally in the oocyte during previtellogenic stages. During mid-oogenesis, however, *osk* mRNA fails to localize to the posterior pole of the oocyte (Fig. 4C). As shown in Fig. 4D, this defect is strikingly rescued in $yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}$ egg chambers. The amount and distribution of *osk* mRNA is not completely normal, however; its signal is reduced, and it is often less tightly localized to the posterior pole than in wild type. We analyzed this further by comparing *osk* mRNA localization in early stage-9, late stage-9 and stage-10 oocytes [for staging, see Spradling (Spradling, 1993)]. We divided stage 9 into early and late stages on the basis of follicle cell migration: egg chambers in which the columnar follicle cells had migrated less than half of the distance between the anterior tip of the egg chamber and the anterior margin of the oocyte were considered early stage 9. Chambers in which the columnar follicle cells had migrated more than half of this distance were considered late stage 9. During early stage 9, *osk* mRNA is in the process of being relocalized from the anterior to the posterior of the oocyte, leading to variability between samples that made precise quantitation at this stage difficult. By late stage 9, however, the *osk* mRNA localization process is complete: 100% of late stage-9 control (w^{1118}) oocytes had posteriorly localized *osk* mRNA, and localization was maintained in stage 10 (Table 2A). In yps^{JM2} ovaries, *osk* mRNA localization was comparable to control oocytes at both stages. In orb^{mel}/orb^{F303}, there was a striking reduction in the percentage of oocytes with localized *osk* mRNA at both late stage 9 (22%) and stage 10 (9%). This defect was substantially rescued at both stages in $yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}$ ovaries: the percentage of double mutant egg chambers with posteriorly localized *osk* mRNA peaked at late stage 9 at 66%, and fell to 45% at stage 10. The percentage of egg chambers with correct RNA localization at stage 10 (45%) closely parallels the degree of rescue of the maternal effect lethal phenotype (40% hatching; Table 1B).

In wild-type egg chambers, Osk protein is translated at the posterior pole of the oocyte following localization of its mRNA (Rongo et al., 1995) (Fig. 5A). The same is true of yps^{JM2} mutant egg chambers (Fig. 5B). We found that Osk protein is absent, or greatly reduced, at the posterior pole of orb^{mel}/orb^{F303} oocytes (Fig. 5C), as has been reported for orb^{mel}/orb^{F343} egg chambers (Christerson and McKearin, 1994). In contrast, Osk protein is present at the oocyte posterior in many $yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}$ egg chambers (Fig. 5D). We examined the timing of appearance of Osk protein to determine if this time-course was altered in mutants; these data are presented in Table 2B. By late stage 9, all of the w^{1118} control oocytes had detectable Oskar protein at their posterior poles, and the protein was retained at the posterior during subsequent stages of oogenesis. The same was observed in yps^{JM2} mutant oocytes. In contrast, Osk protein was first reliably detected at the posterior of $yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}$ oocytes at stage 10. Thus, while there is substantial rescue of the Oskar protein defect in the $yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}$ double mutant, there is a temporal delay in detecting the protein.

Table 2. *osk* mRNA localization and protein accumulation in mutant ovaries

Genotype	Late stage 9	Stage 10
(A) Egg chambers with *osk* mRNA at the posterior pole of the oocyte		
w^{1118}	55/55 (100%)	51/51 (100%)
yps^{JM2}/yps^{JM2}	58/61 (95%)	65/68 (96%)
orb^{mel}/orb^{F303}	15/68 (22%)	7/78 (9%)
yps^{JM2} $orb^{mel}/yps^{JM2}orb^{F303}$	42/63 (66%)	54/121 (45%)
(B) Egg chambers with Osk protein at the posterior pole of the oocyte		
w^{1118}	70/70 (100%)	105/105 (100%)
yps^{JM2}/yps^{JM2}	31/31 (100%)	58/58 (100%)
orb^{mel}/orb^{F303}	1/38 (3%)	2/87 (2%)
yps^{JM2} orb^{mel}/yps^{JM2} orb^{F303}	3/46 (7%)	40/93 (43%)

(A) *osk* mRNA localization in the oocytes of late stage-9 and stage-10 egg chambers. Whole-mount in situ hybridization was performed on the indicated genotypes. Shown are the fractions of egg chambers with *osk* mRNA localized to the posterior pole of the oocyte. yps^{JM2} and control ovaries show similar patterns of *osk* mRNA localization. In orb^{mel}/orb^{F303} mutants, the percentage of egg chambers with localized *osk* mRNA is greatly reduced. In $yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}$ mutants, significant rescue of *osk* mRNA localization is apparent.

(B) Osk protein accumulation in late stage-9 and stage-10 oocytes. Ovaries of the indicated genotypes were labeled with an antibody to the Osk protein. Shown are the fractions of oocytes with posterior accumulation of Osk protein. The control and yps^{JM2}/yps^{JM2} ovaries exhibit an identical pattern of Osk protein accumulation: by late stage 9, and continuing through stage 10, Osk protein is present at the posterior pole of every oocyte. In contrast, in orb^{mel}/orb^{F303} oocytes Osk protein is rarely detected. In $yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}$ double mutant oocytes, however, Osk protein is frequently present, although with a timing lag.

yps^{JM2} does not rescue *orb*-associated defects in Orb protein distribution or *grk* mRNA localization

The distribution of Orb protein in wild-type egg chambers has been described previously (Lantz et al., 1994; Chang et al., 1999) and is shown in Fig. 5E. Orb is concentrated in the oocyte cytoplasm of young egg chambers (stages 1-6), and is also detected in the nurse cell cytoplasm, particularly in the nurse cells closest to the oocyte. By mid-oogenesis (stages 8-10), Orb is localized to the cortex of the oocyte, with peak concentrations at the anterior margin and the posterior pole (Fig. 5E, arrow). In yps^{JM2} mutant egg chambers, the amount and distribution of Orb appears identical to control egg chambers (Fig. 5F). In orb^{mel}/orb^{F303} mutant egg chambers (Fig. 5G), the amount and distribution of Orb is abnormal. In

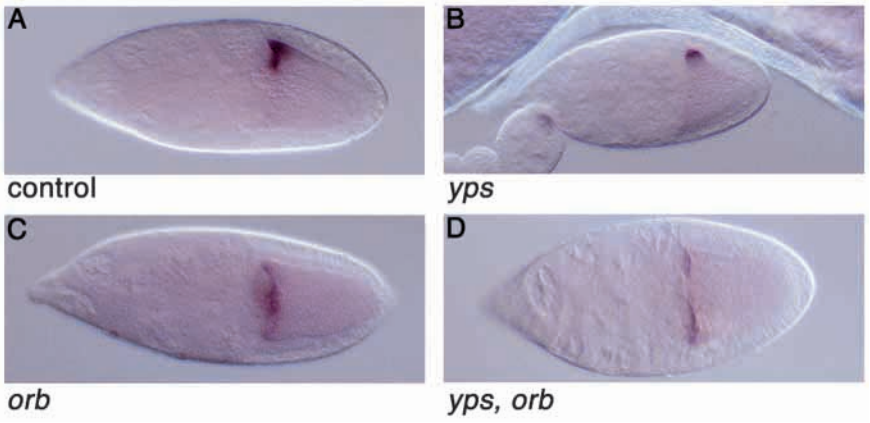

Fig. 6. *yps*^{JM2} does not rescue *orb*-associated defects in *grk* mRNA localization. (A) In *w*^{1118} (control) mid-stage egg chambers, *grk* mRNA is localized to the dorsal anterior corner of the oocyte. (B) An identical pattern is observed in *yps*^{JM2}/*yps*^{JM2} egg chambers. (C) In *orb*^{mel}/*orb*^{F303} egg chambers, *grk* mRNA is spread along the entire anterior margin of the oocyte. (D) In *yps*^{JM2}*orb*^{mel}/*yps*^{JM2}*orb*^{F303} doubly mutant egg chambers, *grk* mRNA localization is identical to *orb*^{mel}/*orb*^{F303} egg chambers.

mutant stage 8-10 egg chambers, Orb frequently persists in the posteriormost nurse cells that border the oocyte at higher levels than in wild-type (Fig. 5G, arrow). Within the oocyte, the protein rarely localizes to the anterior or posterior poles. The expression pattern of Orb in *yps*^{JM2}*orb*^{mel}/*yps*^{JM2}*orb*^{F303} double mutants is identical to *orb*^{mel}/*orb*^{F303} mutants (Fig. 5H). Therefore, *yps*^{JM2} does not rescue the *orb*^{mel}/*orb*^{F303} phenotype by increasing the level, or altering the localization of Orb protein itself.

Embryos from *orb* mutant mothers have dorsoventral polarity defects in their chorions, usually fused and/or reduced dorsal appendages (Christerson and McKearin, 1994; Roth and Schupbach, 1994). In mid-oogenesis in wild-type ovaries, *grk* mRNA becomes localized to the dorsal-anterior corner of the oocyte (Fig. 6A). This RNA localization pattern is normal in *yps*^{JM2} egg chambers (Fig. 6B), and no chorionic patterning defects were observed in *yps*^{JM2} mutant eggs. In *orb*^{mel}/*orb*^{F343} egg chambers, *grk* mRNA is mislocalized: instead of localizing to the anterior dorsal corner of the oocyte, *grk* mRNA is spread out along the entire anterior margin of the oocyte (Christerson and McKearin, 1994). The same is true of many mid-stage *orb*^{mel}/*orb*^{F303} oocytes (Fig. 6C). We found that the *orb*-associated dorsoventral patterning defects are not rescued in the *yps*^{JM2}*orb*^{mel}/*yps*^{JM2}*orb*^{F303} double mutant: eggs laid by *yps*^{JM2}*orb*^{mel}/*yps*^{JM2}*orb*^{F303} mothers had fused, reduced, or absent dorsal appendages, and *grk* mRNA was diffused along the anterior margin of the oocyte in many mid-stage egg chambers (Fig. 6D). While we cannot rule out a role for *yps* in dorsoventral patterning, the rescued phenotypes we have observed in *yps*^{JM2}*orb*^{mel}/*yps*^{JM2}*orb*^{F303} double mutants are specific for the anteroposterior patterning defects of *orb*^{mel}/*orb*^{F303}mutants.

Orb is part of an RNP complex containing Yps and Exu

Since *yps* mutants rescue the *osk* mRNA localization and translation defects associated with *orb* mutants, we investigated whether or not Yps and Orb are part of the same biochemical complex. We immunoprecipitated Yps from *Drosophila* extracts and assayed for the presence of Orb by immunoblot. We found that Orb specifically coimmunoprecipitates with Yps (Fig. 7A). Because we previously demonstrated that Exu and Yps bind directly to each other (Wilhelm et al., 2000), we also wished to determine if Exu protein was in a complex with Orb. Immunoblots of GFP-Exu immunoprecipitations showed that Exu and Orb are also part of the same biochemical complex (Fig. 7A). We previously showed that Exu and Yps do not require the presence of RNA for their physical interaction (Wilhelm et al., 2000), so we asked whether RNA was necessary for interactions between Orb and Yps or Orb and Exu. For this, we assayed the ability of Orb to coimmunoprecipitate with Exu or Yps in extracts that had been treated with RNAse A (Fig. 7B). Immunoblots of these immunoprecipitates demonstrated that Orb's ability to coimmunoprecipitate with Exu and Yps is dependent on the presence of RNA. Together these results suggest that Orb is part of a ribonucleoprotein complex containing Yps and/or Exu and that RNA is an essential component of the complex.

A small proportion of *yps*^{JM2}*orb*^{mel}/*yps*^{JM2}*orb*^{F303} egg chambers exhibit a bipolar phenotype

We observed a novel phenotype in *yps*^{JM2}*orb*^{mel}/*yps*^{JM2}*orb*^{F303} egg chambers, never seen in either single mutant alone. During all stages of oogenesis, we observed bipolar egg chambers, meaning that the oocyte lies in the middle of the egg chamber with nurse cells on either side of it (not shown). Females bearing strong alleles of *orb* produce egg chambers in which the oocyte is mispositioned; however, these alleles cause egg chambers to arrest in the germarium or shortly after budding

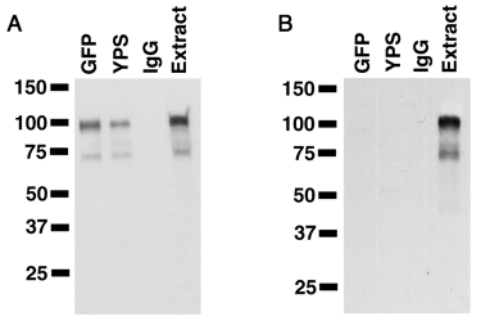

Fig. 7. Orb coimmunoprecipitates with Exu and Yps in an RNAse-sensitive manner. (A) Immunoblot for the presence of Orb in Exu and Yps immunoprecipitates from GFP-Exu extracts using anti-GFP (GFP), anti-Yps (YPS) or rabbit IgG antibodies. Orb protein specifically coimmunoprecipitates with both Exu and Yps. (B) Immunoblot for the presence of Orb in Exu and Yps immunoprecipitates from RNAse-treated GFP-Exu extracts using anti-GFP (GFP), anti-Yps (YPS) or rabbit IgG antibodies. Orb protein does not coimmunoprecipitate with either Exu or Yps when RNAse-treated extracts are used, indicating that RNA is required for Orb to associate with Exu and Yps. In both panels, the right lane shows ovary extract prior to immunoprecipitation.

from it (Lantz et al., 1994). In contrast, the bipolar egg chambers we observed in $yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}$ females included mid- and late-stage egg chambers. The frequency of bipolar egg chambers was low: in one sample of stage-9 or older egg chambers, 5% (12/219) were bipolar. This result suggests that *yps* may also serve an as-yet-uncharacterized function in early oogenesis (see Discussion).

DISCUSSION

We previously showed that Ypsilon Schachtel (Yps), a *Drosophila* Y-box protein, is a subunit of a cytoplasmic RNA-protein complex hypothesized to regulate subcellular RNA localization in *Drosophila* ovaries (Wilhelm et al., 2000). In the present study, we generated a loss-of-function allele of the *yps* gene, and analyzed its effects on the localization and translation of *oskar* (*osk*) mRNA, a known component of this RNP complex.

Y-box proteins are present in a diverse set of species, including worms, flies, frogs, mice and humans [reviewed in Graumann and Marahiel, and Matsumoto and Wolffe (Graumann and Marahiel, 1998; Matsumoto and Wolffe, 1998)]. All members of this family of proteins contain a well-defined nucleic acid binding domain, the cold shock domain (Schindelin et al., 1993; Schindelin et al., 1994; Schroder et al., 1995). In vertebrate germ cells, Y-box proteins are major cellular components [reviewed in Matsumoto and Wolffe (Matsumoto and Wolffe, 1998)]. Biochemical evidence indicates that some of these proteins bind mRNA and regulate translation. For example, FRGY2 in *Xenopus*, and MSY2 and MSY4 in the mouse, repress translation of mRNAs in oocytes and spermatocytes (Richter and Smith, 1984; Ranjan et al., 1993; Bouvet and Wolffe, 1994; Fajardo et al., 1994; Fajardo et al., 1997; Gu et al., 1998; Davies et al., 2000). In somatic cells, Y-box proteins are also implicated in translational regulation. The p50/YB-1 proteins are highly conserved across vertebrate species (Matsumoto and Wolffe, 1998) and can enhance or mask translation in a concentration-dependent manner [reviewed in Evdokimova and Ovchinnikov (Evdokimova and Ovchinnikov, 1999)]. The mechanism by which such translational masking and enhancement is accomplished is not well understood, although it appears to involve modulation of mRNA secondary structure (Evdokimova and Ovchinnikov, 1999).

Prior to this work, only one member of the Y-box family had been genetically characterized, the *C. elegans* heterochronic gene *lin-28*, which functions in the development of somatic cell lineages (Moss et al., 1997). Targets of Lin-28 activity have not yet been reported, and while it is predicted to be an RNA-binding protein, its mode of action is not known [reviewed in Ambros (Ambros, 2000)]. The null allele of *yps* that we generated, yps^{JM2}, produced by excising a P-element, contains an internal deletion in the gene that removes the start codon and the N-terminal half of the protein, including the entire cold shock domain. Several lines of evidence indicate that *osk* mRNA, which encodes a primary organizer of the germ plasm, is a target of Yps' activity. First, we have previously shown that *osk* mRNA is coimmunoprecipitated with both Yps and Exu proteins from ovary extracts (Wilhelm et al., 2000). Second, *osk* mRNA colocalizes with Yps and Exu throughout oogenesis. Third, we have demonstrated a robust genetic interaction between *yps* and *oo18 RNA-binding protein* (*orb*), a known regulator of *osk* mRNA translation and localization: our *yps* null allele rescues *orb*-associated defects in *osk* mRNA localization and translation (Table 1, Table 2, Fig. 4, Fig. 5).

In intermediate allelic combinations of *orb*, *osk* mRNA fails to localize to the posterior pole of the oocyte, and Osk protein is not translated (Christerson and McKearin, 1994; Lantz et al., 1994; Chang et al., 1999). The localization and translation of *osk* mRNA is subject to a complex autoregulatory loop, whereby *osk* mRNA must first be localized to the posterior pole of the oocyte to be translated, and subsequently Osk protein is required to maintain the localization of its own mRNA (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). Because the localization and translation processes are so entwined, it can be difficult to establish which process a regulatory factor affects. In the case of Orb, however, evidence suggests that its primary function may be translational regulation of *osk* (Chang et al., 1999). In *Xenopus*, CPEB, which is virtually identical to Orb in its RNA-binding domain, regulates translation of stored maternal mRNAs by binding a U-rich region of 3′UTRs (the Cytoplasmic Polyadenylation Element) and promoting cytoplasmic polyadenylation [reviewed in Richter (Richter, 1999)]. The role of *osk*'s poly(A) tail in translation is controversial. Results from in vitro systems developed to study translation in *Drosophila* ovaries suggest that the length of *osk*'s poly(A) tail is not critical for regulating its translation (Lie and Macdonald, 1999b; Castagnetti et al., 2000). However, in vivo studies of *osk* mRNA suggest that poly(A) tail length does affect its translation (Chang et al., 1999). These latter results indicate that polyadenylation of the *osk* transcript is dependent on the function of *orb*, as is accumulation of Osk protein, suggesting that Orb serves a similar function to that of CPEB. In addition, Orb binds specifically to the *osk* 3′UTR (Chang et al., 1999). Given this evidence, it appears that Orb may function as a translational enhancer of Osk, although a direct role in *osk* mRNA localization cannot be ruled out.

The *orb* genotypes that are rescued in double mutant combinations with yps^{JM2} all include the orb^{mel} mutation, a hypomorphic allele that produces some functional Orb protein (Christerson and McKearin, 1994; Chang et al., 1999). In contrast, females homozygous for a null allele (orb^{F343}) or a strong allele (orb^{F303}) show no rescue by the yps^{JM2} mutation. These results indicate that rescue by yps^{JM2} requires the presence of some functional Orb protein, and that Yps may normally act antagonistically to Orb. In the presence of Yps, the low amount of functional Orb protein present in orb^{mel} mutants is not capable of promoting normal *osk* mRNA localization and translation, whereas in the absence of Yps, the reduced Orb protein is sufficient.

Our data indicate that *yps* is unlikely to regulate the expression or localization of Orb protein itself. First, the distribution and levels of Orb produced by hypomorphic *orb* alleles are not altered in a yps^{JM2} background. In addition, our genetic analysis of *yps*, *orb* double mutants shows that yps^{JM2} specifically rescues defects in *osk* mRNA localization and translation, but not *orb*-associated defects in dorsoventral chorion patterning or *grk* mRNA localization. Taken together, these results indicate a specific effect of *yps* on *orb*'s function in localizing and/or translating *osk* mRNA.

Previous work has shown that, in the minority of orb^{mel} egg chambers in which Osk protein is detectable, Orb protein can be detected at the posterior pole as well (Chang et al., 1999). This correlation has been interpreted as evidence of a requirement for Orb for the on-site expression of Osk (Chang et al., 1999). We find that, when ovaries are doubly mutant for *yps* and *orb*, this correlation disappears. While Orb can rarely be detected at the posterior pole of the oocyte in *yps, orb* mutants, Osk protein is frequently present even in the absence of detectable Orb (Fig. 5). However, our results also show that loss of Yps cannot eliminate the requirement for Orb in Osk expression. It is possible that, in the absence of Yps, a very low concentration of Orb, which is undetectable by immunocytochemistry, is sufficient to localize or enhance the translation of *osk* mRNA at the posterior pole. Alternatively, in the absence of Yps, the function of Orb might be accomplished at regions other than the posterior, since in *yps, orb* double mutants Orb protein is present throughout the oocyte.

Although *osk* translation is significantly rescued in *yps, orb* ovaries, the amount of Osk present at the posterior appears reduced compared to wild type. In addition, as shown in Table 2B, Osk is not reliably detected in *yps, orb* egg chambers until stage 10. In wild-type ovaries, however, Osk can be detected in stage-9 oocytes, and sometimes as early as stage 8 (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995) (Table 2B). We believe that the temporal delay in detecting Osk is due simply to a reduction in Osk expression in *yps, orb* egg chambers during all stages of oogenesis, such that accumulation of the protein to levels detectable by our method does not occur until stage 10. We also hypothesize that, due to this reduction in the accumulation of Osk protein in *yps, orb* ovaries, *osk* mRNA localization is not efficiently maintained. As shown in Table 2A, in late stage 9, 66% of *yps, orb* oocytes displayed localized *osk* mRNA, while in stage 10 the percentage fell to 45%. This number closely parallels the percentage of *yps, orb* stage-10 oocytes with detectable Osk protein (43%) and the number of eggs (40%) that hatched from mutant mothers (Table 2B, Table 1B).

Biochemically we have detected an association between Yps and Orb. Orb protein was found to coimmunoprecipitate with Yps (Fig. 7). This association is mediated by RNA, since their coimmunoprecipitation is RNAse-sensitive. Similarly, Orb coimmunoprecipitates with Exu, in an RNA-dependent manner (Fig. 7). Exu and Yps also coimmunoprecipitate, but independently of RNA, and bind each other in vitro, indicating that their interaction is probably direct (Wilhelm et al., 2000). Despite their direct association, Yps is localized normally in *exu* null ovaries, and Exu protein is localized normally in *yps* null ovaries. Thus Yps and Exu appear to be recruited to this ovarian complex independently. Do the associations detected by immunoprecipitation reflect biologically significant interactions that occur in vivo? Several other lines of evidence suggest that these proteins interact in vivo, and that *osk* mRNA is part of this complex. First, all three proteins, and *osk* mRNA, colocalize throughout oogenesis. Second, *osk* mRNA associates with both Exu and Yps (Wilhelm et al., 2000), and Orb binds directly to *osk* mRNA (Chang et al., 1999). And finally, the genetic results presented in this work are strong evidence for a biologically significant interaction of Yps and Orb in *Drosophila* ovaries.

Doubly mutant $yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}$ ovaries display a novel phenotype, not observed in yps^{JM2} or orb^{mel}/orb^{F303} females: a small proportion (5%) of mid- and late-stage egg chambers are bipolar. Strong allelic combinations of *orb* also generate a high proportion of egg chambers with the oocyte mispositioned (Lantz et al., 1994), but these egg chambers arrest oogenesis before budding from the germarium, or shortly thereafter. The low frequency of late-stage bipolar egg chambers observed in $yps^{JM2}orb^{mel}/yps^{JM2}orb^{F303}$ females may result from partial rescue of egg chambers that would normally have arrested at very early stages in orb^{mel}/orb^{F303} ovaries, with a phenotype similar to orb^{F303}/orb^{F303} egg chambers. Alternatively, this may be a novel phenotype resulting from the additive loss of both *yps* and *orb*. In either case, this phenotype suggests an earlier, as yet uncharacterized function of *yps*. In support of this idea, *yps* is expressed in the germarium (Wilhelm et al., 2000).

In addition to its expression in female germ cells, *yps* is also expressed in the testes and in somatic cells (Thieringer et al., 1997; Wilhelm et al., 2000) (J. M. and T. H., unpublished data). We therefore believe it unlikely that *osk* mRNA is the only target of Yps. In fact, most Y-box proteins are thought to be general factors that regulate the translation of large classes of mRNAs [reviewed in Matsumoto and Wolffe (Matsumoto and Wolffe, 1998)]. FRGY2, for example, is thought to be a general masking factor for stored maternal mRNAs in the oocyte (Tafuri and Wolffe, 1993). However, we have no evidence to suggest that Yps serves such a general role in translational repression. The fact that Yps is localized to specific subcellular sites in the nurse cells and the oocyte argues against it being a general regulator of translation. We favor the hypothesis that Yps is specific to a limited number of target mRNAs. Perhaps Exu, which has been shown to coimmunoprecipitate with both Yps protein and *osk* mRNA, or another protein in the complex, confers sequence specificity on Yps' RNA-binding activity.

An interesting possibility is suggested by studies of the p50/YB1 Y-box proteins. p50/YB1 is nearly completely conserved across vertebrate species, including human, mouse, rabbit and chick, and is expressed in both germline and somatic cells [reviewed in Evdokimova and Ovchinnikov (Evdokimova and Ovchinnikov, 1999)]. The most abundant protein in mRNP particles in a variety of cells types, p50/YB1 has been shown to act as a translational repressor at high concentrations and a translational enhancer at low concentrations. Recent work has shown that, at low concentrations (i.e. when mRNAs are unmasked) p50/YB1 also binds actin, and at higher p50/YB1: mRNA ratios (conditions under which translation is repressed) p50/YB1 and actin do not bind (Ruzanov et al., 1999). This suggests a mechanism by which unmasked mRNAs may be anchored to regions of the cytoplasm. This is particularly intriguing in the light of evidence that *osk* mRNA is anchored by an actin-dependent mechanism to the posterior pole of the oocyte, where it is translated (Erdelyi et al., 1995; Tetzlaff et al., 1996; Glotzer et al., 1997; Lantz et al., 1999). While Yps cannot be an essential component of a posterior RNA anchor, it may play a role in the association of *osk* mRNA with the actin cytoskeleton, which may in turn affect translation.

One model supported by our data is that Yps and Orb both bind to *osk* mRNA, and have opposite effects on translation: Yps represses, and Orb activates translation. Our immunoprecipitation experiments (Fig. 7) show that both proteins are present in an RNP complex and that their

association is mediated by RNA, suggesting that both proteins simultaneously bind a common RNA target. This target is likely to be *osk* mRNA. *osk* mRNA is a member of this RNP complex (Wilhelm et al., 2000), Orb is known to bind *osk* mRNA (Chang et al., 1999), and our genetics results show that a *yps* loss-of-function mutation suppresses the defects in *osk* mRNA localization and translation associated with reduced function *orb* alleles (Fig. 4, Fig. 5). Yps could prevent translation by preventing Orb from promoting cytoplasmic polyadenylation. At the posterior of the oocyte, where Orb and Yps both concentrate during mid-oogenesis, and where *osk* mRNA is localized and translated, concentration differences between the two proteins could push the complex from being a negative to a positive regulator of translation. Additional factors at the posterior could also interact with either Orb or Yps to modify their functions, as might association with the actin cytoskeleton. This model accounts for why our *yps* mutation cannot eliminate the requirement for Orb, but can reduce the amount of Orb required for sufficient *osk* translation. In the rescued genotypes, there may be enough Orb at the oocyte posterior to allow for on-site cytoplasmic polyadenylation of *osk* mRNA, in the absence of negative regulation by Yps. It is also possible that, in the absence of Yps, Orb can stimulate polyadenylation of *osk* mRNA before it becomes localized, although it remains subject to translational repression by other factors, such as Apontic and Bruno, until it reaches the posterior pole. Future studies will test this model by determining if Yps and Orb bind competitively to *osk* mRNA, and if so, how their combined binding affects the translation of *osk* mRNA, and its polyadenylation state. These studies should contribute not only to our understanding of localization-dependent mRNA translation in *Drosophila*, but also to a better understanding of the biological roles of the widespread family of Y-box proteins.

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