Isolation of a Ribonucleoprotein Complex Involved in mRNA Localization in Drosophila Oocytes

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Abstract. Localization of bicoid (bcd) mRNA to the anterior and oskar (osk) mRNA to the posterior of the Drosophila oocyte is critical for embryonic patterning. Previous genetic studies implicated exuperantia (exu) in bcd mRNA localization, but its role in this process is not understood. We have biochemically isolated Exu and show that it is part of a large RNA-sensitive complex that contains at least seven other proteins. One of these proteins was identified as the cold shock domain RNA-binding protein Ypsilon Schachtel (Yps), which we show binds directly to Exu and colocalizes with Exu in both the oocyte and nurse cells of the Drosophila egg chamber. Surprisingly, the Exu-Yps complex contains osk mRNA. This biochemical result led us to reexamine the role of Exu in the localization of osk mRNA. We discovered that exu-null mutants are defective in osk mRNA localization in both nurse cells and the oocyte. Furthermore, both Exu/Yps particles and osk mRNA follow a similar temporal pattern of localization in which they transiently accumulate at the oocyte anterior and subsequently localize to the posterior pole. We propose that Exu is a core component of a large protein complex involved in localizing mRNA both within nurse cells and the developing oocyte.

Key words: oogenesis • nurse cells • cold shock domain • oskar mRNA • exuperantia

Introduction

Localization of mRNA s is used by many polarized cells as a means of restricting the distribution of a protein to a particular cytoplasmic domain (Bashirullah et al., 1998; Hazelrigg, 1998; St Johnston, 1995). Whereas the types of localized transcripts vary, mRNA localization in all systems shares several common features (Wilhelm and Vale, 1993; Mcdonald and Smibert, 1996; Bassel and Singer, 1997; Gavis, 1997; Oleynikov and Singer, 1998). First, the cis-acting mRNA localization elements generally reside within the 3' untranslated region (UTR) (Kislauskis and Singer, 1992; Bashirullah et al., 1998). Second, the transport of localized messages from the nucleus to their final destinations occurs along either actin filaments or microtubule tracks. Third, transcripts are anchored at their sites of localization through attachments to cytoskeletal elements and then activated for translation. Although these phenomena have been well-documented, their molecular bases remain poorly understood.

One of the most extensively characterized systems for studying mRNA localization is the Drosophila oocyte. In the Drosophila egg chamber, an oocyte is linked to 15 nurse cells by a network of cytoplasmic bridges called ring canals (Spradling, 1993). The nurse cells synthesize various mRNAs that are required for early embryogenesis, such as the bicoid (bcd) and oskar (osk) transcripts, and transport them in a microtubule-dependent manner to discrete locations within the oocyte (Pokrywka and Stephen-son, 1991, 1995). bcd mRNA is localized to the anterior of the oocyte (Berleth et al., 1988), and the resulting anterior-posterior gradient of the Bcd homeodomain protein initiates a series of concentration-dependent transcriptional programs that establish the anterior pattern of the embryo (St Johnston and Nusslein-Volhard, 1992). In contrast, osk mRNA is transported from the nurse cells to the anterior of the oocyte, but is ultimately localized to the posterior of the oocyte where it becomes stably anchored...
Oregon R (Ore) was the wild-type stock used for antibody staining and generation of wild-type Drosophila extracts. Females from NG5/NG5; Sco/SM1, which contains an X-linked insertion of a P[CasNGE] gfp-exu transgene (Wang and Hazelrigg, 1994), were used for double labeling of Ypsilon Schachtel (Y ps) and Exu. For biochemical analysis, flies bearing a gfp-exu-his transgene (16b-16) were used.

To construct the gfp-exu-his transgene, the 5.5-kb Smal-EcorI exu genomic fragment was subcloned into pBlueScript II (SK) (Stratagene), to create pB8S-exu5.5; pB8S-exu5.5 was used as the template for in vitro mutagenesis to introduce BstEII and SphI restriction sites immediately upstream of exu’s translation start codon (using the Bioread Muta-Gene kit). Simultaneously, an 18-bp insertion encoding six histidine codons was introduced just upstream of exu’s stop codon. Correctly mutagenized plasmids were identified by restriction analysis for the presence of the BstEII and SphI sites, and DNA sequence analysis for the presence of the 18-bp his-encoding insertion. A 700-bp BstEII-SphI gfp fragment was excised from pB8S, gel-purified (Wang and Hazelrigg, 1994), and subcloned into the BstEII and SphI sites of this mutagenized exu fragment. The resulting 6.2-kb gfp-exu-his fragment was then excised by Smal and EcorI digestion and inserted into pCasPeR4 through its StuI and EcoRI cloning sites to create pWg16b. Peptide transformation followed standard isolation protocols (Spradling and Rubin, 1982), with transposase activity provided by the recipient strain (y w; Sb-2/TM6). A transformant line bearing an insertion on the X chromosome was established (w, 16b-16), and genetic crosses with an exu^{ScO2}CyO strain determined that the gfp-exu-his transgene rescued the female sterility associated with the exu^{ScO2} mutation.

Extract Preparation

Extracts were prepared by flash freezing 50–60 ml of flies in liquid N2. The frozen flies were ground to a fine powder with a precooled mortar and pestle, with regular additions of liquid N2 to keep the sample frozen. The fly powder was degassed for 5–10 min on a 50-m1 Falcon tube and mixed either 1:1 or 1:2 with Drosophila extract buffer (DXB: 25 mM Hepes, pH 6.8, 50 mM KCl, 1 mM MgCl2, 1 mM DTT, 250 mM sucrose) containing 10 µg/ml aprotinin, leupeptin, pepstatin, and 1 mM PM SF. The extracts were homogenized with 10 strokes of the Dounce in a 50-m1 dounce homogenizer, followed by 10 strokes of the A dounce, and then centrifuged at 10,000 g for 15 min at 4°C in the Beckman TL X ultracentrifuge. The supernatant was collected and centrifuged a second time. The supernatant from the second spin was collected, aliquoted, frozen in liquid N2, and stored at −80°C. H and dissected extracts were prepared by dissecting 50 ovaries into 0.7 ml of modified Drosophila extract buffer (mDXB: 25 mM Hepes, pH 6.8, 50 mM KC1, 1 mM MgCl2, 1 mM DTT, 125 mM sucrose) containing 10 µg/ml aprotinin, leupeptin, and 1 mM PM SF. The sample was then homogenized with 15 strokes in a 2-m1 dounce homogenizer (Wheaton, Inc.) at 4°C. The extract was centrifuged at 10,000 g for 10 min at 4°C to collect the supernatant.

Sucrose Density Gradient Analysis of Exu Complex

Extract from hand dissected ovaries (250 µl) was loaded on a 5-m1 5–40% sucrose gradient made with DXB containing 10 µg/ml leupeptin, pepstatin, and aprotinin. For frozen Drosophila extracts, 150 µl of ~10 mg/ml extract was diluted 1:1 with sucrose-free DXB containing 10 µg/ml pepstatin, leupeptin, aprotinin, and 1 mM PM SF. The diluted extract was centrifuged at 10,000 g for 5 min in a microcentrifuge and the supernatant was loaded on a 5-m1 5–40% sucrose gradient. The gradients were centrifuged at 237,000 g for 4 h in a Beckman SW-55 rotor. 300-µl fractions were collected from the gradient and precipitated with 1/10 volume TCA using 20 µg aprotinin as a carrier. The precipitate was resuspended in 30 µl sample buffer and 15 µl was loaded onto SDS-PAGE gel for immunoblot analysis. For R Nase shift experiments, the extract (250 µl) was either treated with 2.5 µg of R Nase A for 10 min at 4°C, followed by addition of 2,000 U of R Q1 RNasin (Promega), or treated for 10 min at 4°C with 2.5 µg of R Nase A that had been preincubated with 2,000 U of R Q1 RNasin. These samples were loaded onto a 5-m1 10–40% sucrose gradient and centrifuged as above.

Two-Step Purification of GFP-Exu-His6

5 ml of ~10 mg/ml Drosophila extract (made with 5 mM β-mercaptoethanol instead of 1 mM DTT) were incubated with 0.5 ml of Ni-NiTA resin in the presence of 0.1% Triton X-100 for 1 h at 4°C. The resin was washed once in batch with 10 ml of DXB150 (DXB with 150 mM KCl) supplemented with 0.1% Triton X-100 (wash 1) followed by a 10-m1 column wash (wash 2). The column was eluted with 250 mM imidazole in
DXB150, and 0.5-ml fractions collected. The peak fractions were pooled and immunoprecipitated as described.

**Antibody Generation**

The full-length coding region and the first 160 amino acids of Yps (Yps160) were each cloned into pGEX-2T and expressed as a COOH-terminal fusion protein in Escherichia coli. GST-YpsYps160 were purified on a glutathione affinity column, eluted with glutathione, and injected into rabbits (antiseraum production by BABCO). GST-Yps did not express as a full-length protein in E. coli, but rather yielded a protein product fractionated using a Vydac microbore C8 column (The Separations Group), and individual peptides subjected to Edman degradation with a protein sequencer, Model 492 (Perkin Elmer Biosystems). BLAST searches with these peptides identified p57 as Yps. Six expressed sequence tags (ESTs) corresponding to yps (GM1405, LD01826, GM03816, LD10988, GM02353, LD01338) were obtained (Berkeley Drosophila Genome Project/Howard Hughes Medical Institute EST Project) and sequenced.

**Immunoprecipitations for Reverse Transcribed-PCR**

Immunoprecipitations were carried out as above, but the antibody was not cross-linked to the protein A agarose beads (GIBCO BRL). Beads were washed four times with DXB200 (DXB, with 200 mM KCl) containing 10 μg/ml leupeptin, pepstatin, and 1 mM PM SF. Beads were then resuspended in 200 μl buffer (100 mM HEPES, pH 6.8, 150 mM NaCl, 12.5 mM EDTA, 1% SDS) and heated to 65°C for 10 min. The beads were pelleted and the supernatant from two immunoprecipitations was phenol/chloroform-extracted followed by an ethanol precipitation using 20 μg glycogen as carrier. The pellet was resuspended in 50 mM HEPES, pH 6.8, 1 mM MgCl2, and treated with 10 U RNasin (Promega) at 30°C for 30 min. The sample was then phenol/chloroform-extracted, and ethanol-puriﬁed at a 2:1 ratio. The RNA sample was reverse transcribed (RT) using the Superscript preampliﬁcation system (GIBCO BRL), and 2 μg of 10-, 100-, and 1,000-fold dilutions of the RT product were ampliﬁed in a 50-μl PCR reaction (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 1 mM dNTPs, 2.5 U Taq polymerase, 15 pmol primer). Cycling conditions were 5 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 55°C, and then 1 min at 72°C. Primers were tested by using the same RT-PCR conditions as above, except 5 μg of total RNA from extracts was used in the RT reaction and only 30 cycles of PCR were performed. The primers used were: bcd2761, 5'-gtcgaactctgctgacagcagaattc-3'; bcd2738, 5'-gtcgaactctgctgacagcagaattc-3'; osk2880, 5'-gtcgaactctgctgacagcagaattc-3'; osk2881, 5'-gtcgaactctgctgacagcagaattc-3'; RT-PCR primers were designed for osk, nos (nos), and bcd to span an intron to easily separate RT-PCR products from products that were ampliﬁed due to genomic contamination. Products for osk, nos, and bcd were cloned and their identity conﬁrmed by DNA sequencing.

**Immunofluorescence**

0-1-d-old females were collected and fed for 1-2 d with dried baker’s yeast, in the presence of males. Ovaries were dissected into PBS and ﬁxed in 600 μl of heptane and 100 μl of ﬁxative (6% formaldehyde, 16.7 mM KPO4, pH 6.8, 7.5 mM KCl, 1.3 mM MgCl2) rocking at room temperature for 9 min. The ovaries were then washed several times in PBS, for a total of 15 min. Ovaries were permeabilized in PBS for 5 h, blocked in 0.5% BSA in PBS for 30 min, and incubated in a 1:500 dilution of the primary antibody in PBS overnight at 4°C. The secondary antibody (rhodamine-conjugated goat anti-rabbit; Fab fragments; Jackson Immunoresearch Laboratory, Inc.) was simultaneously preabsorbed to ﬁxed ovaries at a dilution of 1:500 in PBS overnight at 4°C. A ﬂat removal of the primary antibody, the ovaries were washed in PBS (ﬁve times for 5 min) at room temperature, and incubated in the preabsorbed secondary antibody for 3 h at room temperature. The ovaries were then washed in PBS (ﬁve times for 5 min), and either mounted in 20 μl Fluoromount-G (Southern Biotechnology Associates, Inc.) or incubated overnight in 60% glycerol in PBS at 4°C, then mounted in 40 μl of 60% glycerol. Imaging was performed on a Biorad MRC600 laser confocal unit attached to a Zeiss A xiplanar microscope.

**Binding Assays for In Vitro Translated Exu and Yps**

Site-directed mutagenesis and PCR were used to create unique restriction sites in order to attach NH2-terminal epitope tags in frame to exu and yps cDNA s. DNA encoding the 3x myc tag was obtained from T7myg-6-plw vector (gift of Erin O’Shea, University of California, San Francisco, San Francisco, CA) and joined to the 5’ end of yps cDNA encoding the 3x he-magglutinin (HA) tag was obtained from the pGTEP vector (gift of) oachim Li, U niversity of California, San Francisco) and joined to the 5’ end of exu.

**Microsequence Analysis of p57/Yps**

p57 was immunoprecipitated as described, separated by SDS-PAGE, and then stained with Coomassie blue. A ﬂat removing the protein band, an in-gel digestion with Endoproteinase Lys-C (Roche Diagnostics) was carried out (Desbrown described [Hellman et al., 1993]). Gel-extracted peptides were fractionated using a Vydac microbore C8 column (The Separations Group), and individual peptides subjected to Edman degradation with a protein sequencer, Model 492 (Perkin Elmer Biosystems). BLAST searches with these peptides identiﬁed p57 as Yps. Six expressed sequence tags (ESTs) corresponding to yps (GM1405, LD01826, GM03816, LD10988, GM02353, LD01338) were obtained (Berkeley Drosophila Genome Project/Howard Hughes Medical Institute EST Project) and sequenced.
Each of the two fusions was then subcloned into the Ncol and EcoRl sites of the pSPBplI transcription vector (gift of Visher Lingappa, University of California, San Francisco); this vector was derived from pSP64 and includes a 5’S UTR and strong Kozak consensus region from Xenopus globin cDNA.

A coupled transcription-translation system (Promega) was used to in vitro translate tagged Exu and Yps. 600 ng of plasmid DNA was added to each 50-μl translation-mix. The myc-Yps translation-transcription mix contained [35S]methionine (Translabel; ICN), whereas the HA – Exu mix did not. A 30-min incubation at 30°C, 0.5 μl of 400 nM 7-methylguanosine 5-monophosphate (CAP Analogue) and 5 μl of 10 mg/ml RNase A were added to each reaction. 5 μl of radiolabeled myc-Yps ps was either incubated alone or mixed with cold HA – Exu and coincubated for 30 min at room temperature. Afterwards, 490 μl of DX BT250 (DXB containing 250 mM KCl and 0.1% Triton X-100) was added to the coincubation mixtures before the immunoprecipitations.

For immunoprecipitations, 0.5 μl of anti-HA (16512 monoclonal; gift of Dave Morgan) was added to translations and set on a rotator at 4°C for 60 min. A 30-μl slurry containing 10 μl of protein A beads (Life Technologies, Inc.) was added to the translation-antibody mix and incubated for another 60 min at 4°C on a rotator. The beads were then pelleted in a low-speed microfuge for 2 min and the supernatant was removed. A 1-μl washing with DX BT250 (three times in 1 ml), the beads were resuspended in SD S sample buffer and boiled for 5 min. 7.5 μl was loaded on 4-12% SD S-PAGE gels and processed for autoradiography.

**osk In Situ Hybridization**

Whole mount in situ hybridization to ovaries was performed as described previously (Tautz and Pfeifle, 1989) with several modifications. Ovaries from well-fed, 2–3-d-old females raised at 18°C previously (Tautz and Pfeifle, 1989) with several modifications. Ovaries were then washed several times in PBT and equilibrated overnight at 4°C in 60% glycerol. Ovaries were mounted in 60% glycerol and visualized on a Nikon Eclipse E800 microscope with a Nikon Plan Fluor 20× objective, under Nomarski optics. Photographs were taken with a Nikon FX-35WA camera on Kodak E-ite Chrome slide film, and scanned into A dobe Photoshop with a Polaroid SprintScan 35 slide scanner.

Probes were prepared by labeling a plasmid containing the osk cDNA. PNBosk7 (courtesy of R. Lehmann, Skirball Institute, New York University Medical Center, New York, NY) with dig-dUTP using D1G-Nick Translation Mix (Boehringer Mannheim). A 1.8-μl ethanol precipitation, the probe was resuspended in 100 μl HB. Just before use, the probe was denatured by boiling for 5 min, and cooled on ice.

**Results**

**Exu Exists in a Large RNase-sensitive Complex**

To define the biochemical properties of Exu, we determined its size in Drosophila extracts using sucrose density gradients. In extracts made from either hand dissected ovaries (Fig. 1 A) or whole flies that were frozen in liquid N2 (data not shown), Exu migrated in a very broad distribution with a significant fraction of Exu migrating larger than 20 S (Fig. 1 A). In contrast, in vitro–translated Exu sedimented at 4.5 S, the value expected of a monomeric 58-kD protein (data not shown). Therefore, the large size of Exu in Drosophila extracts must be due to its association with additional proteins and/or RNAs. To test whether the exu complex contains RNA, the extract was treated with RNase A before sedimentation analysis. This treatment caused the complex to migrate as a 7-S species; this shift was not due to proteinolysis, since a control reaction in which RNase A was premixed with RNase inhibitor did not affect Exu migration (Fig. 1 B). These results demonstrate that Exu is associated with a large RNA-containing complex.

![Figure 1. RNase-sensitive sedimentation of Exu in Drosophila extracts. (A) Sedimentation of Exu from an extract made from hand-dissected ovaries through a 5-40% sucrose gradient (see Materials and Methods). This immunoblot shows a broad distribution of Exu signal with the majority sedimenting >20 S. (B) Sedimentation of Exu in extracts treated with RNase A (10 μg/ml, 10 min at 4°C) followed by addition of pancreatic RNase inhibitor (8,000 U/ml) (upper panel). A as a control, RNase A was premixed with the inhibitor before addition to the extract (lower panel). When RNase A is added first, the Exu immunoblot signal shifts from a large broad distribution to a tight 7-S peak. A though there is some variability in the sedimentation profile of Exu from extract to extract, there is always a significant fraction of Exu sedimenting at 20 S or greater.](image-url)
**The RNA-binding Protein Yps Copurifies with Exu**

To identify the protein components of the Exu complex, GFP-Exu was immunoprecipitated from whole fly extracts prepared from a GFP-Exu-expressing fly line using an anti-GFP antibody. The GFP tag does not impair Exu protein function, since the gfp-exu transgene fully complements a null allele of exu (exu^SCO2) (Wang and Hazelrigg, 1994). Fig. 2 shows that seven polypeptides of 57, 74, 76, 78, 82, 88, and 147 kD coimmunoprecipitated specifically with GFP-Exu, but not with control IgG-coated beads. A similar set of polypeptides coimmunoprecipitated with GFP-Exu from an extract made from hand dissected ovaries, indicating that this complex is present within the female germ line (data not shown). This same set of polypeptides also coimmunoprecipitated with Exu from wild-type fly extracts using an antibody directed against a COOH-terminal peptide of Exu (data not shown; see Materials and Methods). Of the coimmunoprecipitated proteins, the 57-, 74-, 76-, 78-, and 82-kD proteins were present in amounts comparable to that of GFP-Exu, whereas the 88- and 147-kD proteins were clearly substoichiometric. When the extract was extensively treated with RNase A, only the 57-kD protein remained associated with GFP-Exu (Fig. 2). Taken together with our gradient analysis (Fig. 1B), these results suggest that Exu and p57 are components of a 7-S RNase-resistant core complex; the other polypeptides (p74, p76, p78, p82, p88, and p147) all require the presence of RNA in order to associate with Exu.

To confirm that the 57-kD polypeptide was a bona fide Exu-associated protein, we used a different purification strategy to isolate Exu complexes. Using flies that express Exu with an NH2-terminal GFP tag and a COOH-terminal His6 tag, extracts were subjected to a two-step purification consisting of binding to an Ni-NTA column, elution with imidazole, and then immunoprecipitation with the anti-GFP antibody (Fig. 3). The 57-kD protein consistently copurified stoichiometrically with GFP-Exu-His6 through this two-step affinity purification, confirming that it is a true Exu-associated polypeptide. The other polypeptides in the 74–82 kD range were identified in some of our preparations, but their presence and amount was highly variable, possibly due to RNA degradation during the procedure or instability of the complex during imidazole elution from the column.

To identify the 57-kD Exu-associated protein, we mi-
microsequenced three tryptic peptides from the purified protein. The sequence from two of the three peptides matched a previously identified protein, the product of theyps gene (Fig. 4A). Yps is a member of the cold shock family of RNA-binding domain proteins and was identified as part of a degenerate PCR screen to identify cold shock domain containing genes from Drosophila melanogaster (Thieringer et al., 1997). However, the third peptide only matched the yp sequence in a reading frame other than the published open reading frame. To rule out the possibility thatyps expression is subject to ribosomal frameshifting or RNA editing, we obtained and sequenced six independentyps ESTs (Berkeley Drosophila Genome Project/Howard Hughes Medical Institute EST Project, unpublished). Our sequence revealed that the original yp sequence contained several sequencing errors and that the correct open reading frame contains all three microsequenced peptides (Fig. 4A). The cold shock domain of Yps shows extensive sequence identity to other cold shock domain proteins (Fig. 4B). This domain has been shown in several studies to bind RNA, although its ability to recognize specific substrates remains uncertain (Murray, 1994; Bouvet et al., 1995; Matsumoto et al., 1996). Beyond the cold shock domain, Yps exhibited no significant homology to any other protein except YB-1, a cold shock domain protein from Drosophila silvestris (AAC06034).

Since the YB-1 protein is 70% identical to Yps across the entire length of the protein, it is likely to be a true ortholog of Yps. No function was assigned to either YB-1 or Yps in these prior studies.

**Yps Is a Component of the Exu Complex**

To further characterize Yps, we prepared affinity purified antibodies against bacterially expressed Yps (amino acids 1–160). These antibodies recognized the 57-kD Yps protein in crude extracts by immunoblot (Fig. 5A). Using the Yps antibody, we found that Yps comigrates with Exu in sucrose gradients and is distributed broadly in the 20–60 S size range (Fig. 5B). To rule out the possibility thatExu and Yps are components of distinct complexes of similar size, GFP-Exu was immunoprecipitated from individual gradient fractions and immunoblotted with the Yps antibody (Fig. 5C). This experiment showed that Exu and Yps coimmunoprecipitate together across the gradient, arguing strongly that Exu and Yps are part of the same complex.

To provide further evidence for an Exu–Yps complex, we immunoprecipitated GFP-Exu extracts with our anti-Yps polyclonal antibody. In agreement with our previous immunoprecipitation results, immunobots showed that GFP-Exu specifically coimmunoprecipitated with Yps (Fig. 5D). However, immunobots showed only a weak GFP-Exu band in the Yps immunoprecipitate. The inefficient coimmunoprecipitation of GFP-Exu with Yps is probably due to the fact that our anti-Yps antibody may displaceExu from the complex, since it was raised against the Exubinding region of Yps (discussed below; Fig. 6). The Yps immunoprecipitates also contained the same six proteins (p74, p76, p78, p82, p88, and p147) that strongly coimmunoprecipitated with GFP-Exu and were present in similar stoichiometries (Fig. 5E). The coimmunoprecipitation of these six proteins with Yps was diminished by RNase treatment, as was observed in our previous Exu immunoprecipitation experiments (Fig. 5E). The ability of Yps and Exu antibodies to coimmunoprecipitate the same set of polypeptides argues that these proteins are bona fide components of the Exu–Yps complex.

**Exuperantia Binds Directly to the NH$_2$-Terminal Region of Yps in the Absence of RNA or Other Proteins**

The coimmunoprecipitation of Exu and Yps after RNase treatment suggested, but did not prove, that Exu and Yps bound directly to each other. To test this idea, we examined the Exu–Yps interaction in an in vitro translation reaction (Fig. 6). Myc-tagged Yps was in vitro translated in the presence of [$^{35}$S]methionine and then added to an unlabeled in vitro translation of HA-tagged Exu. Before mixing, each translation reaction was treated with RNase A to eliminate any residual RNA from the translation reaction. When HA–Exu was immunoprecipitated from the combined mixture with the anti-HA antibody, the $^{35}$S-labeledmyc-Yps protein was coimmunoprecipitated. The amount ofmyc-Yps that coimmunoprecipitated with HA–Exu was approximately half of the amount ofmyc-Yps that was immunoprecipitated directly with the anti-myc antibody, showing that Yps is predominantly bound to Exu under these experimental conditions. These results demonstrate that the additional RNA and protein components of the

**Figure 4.** Sequence of p57/Yps, a cold shock domain RNA-binding protein. (A) The predicted protein sequence of Yps determined from sequencing of six ESTs from the Berkeley Drosophila Genome Project. Solid lines highlight the peptides obtained by microsequencing of p57. Mismatches between the translated sequence and the microsequenced peptides are in lower case. The dashed line delineates the cold shock domain. Amino acid residue number are shown on the right. (B) Sequence alignment of the cold shock domain of Yps with other cold shock domains from human (DBPA), Xenopus (YB1), goldfish (YB2), and Aplysia (YB1) proteins. Residues that are identical or similar in four of the five proteins are shown in upper case and less-well conserved residues are in lower case.
native Exu complex are not required for Exu and Yps to associate stably with each other (Fig. 6).

To determine which region of Yps is important for binding to Exu, deletions of myc-Yps were assayed for their ability to bind HA–Exu in vitro. The NH₂-terminal region (1–160 amino acids) of Yps, which contains the cold shock domain, bound to Exu at the same efficiency as the full-length protein (Fig. 6). However, the minimal cold shock domain (56–151 amino acids) did not bind to Exu in this assay (Fig. 6), suggesting that the sequences flanking the cold shock domain are likely to contribute to the Exu-binding site. The proline-rich COOH terminus of Yps presumably is not sufficient for binding. However, this could not be assessed experimentally, since this region did not stably express either in vitro or in bacteria.

**osk mRNA Is Present in the Exu–Yps Complex**

The shift in the size of the Exu–Yps complex after RNase treatment suggested that it might be directly involved in mRNA localization. To test this idea, we immunoprecipitated either Exu or Yps from extracts and analyzed the pellet for the presence of various localized (bcd, osk, nos) messages and a housekeeping message (phosphoglycerokinase [pgk]). We analyzed these four transcripts because each gene appears in the EST database (Berkeley Drosophila Genome Project/Howard Hughes Medical Institute EST Project, unpublished) at roughly equivalent frequencies, indicating that these messages are likely to be present in comparable amounts. Furthermore, bcd, osk, and nos are localized differently during oogenesis. bcd mRNA is localized to the anterior of the oocyte beginning at stage 7 of oogenesis; osk mRNA is localized transiently to the anterior of the oocyte during stages 8/9 and is exclusively localized to the posterior by the end of stage 9; nos mRNA is localized to the posterior of the oocyte during late oogenesis. Using a RT-PCR assay, we amplified bcd, osk, nos, and pgk transcripts from total RNA from extracts (Fig. 7 A). When GFP–Exu immunoprecipitates were analyzed by RT-PCR, osk transcript (Fig. 7 B), but none of the others (data not shown) was amplified by RT-PCR. The identical result was obtained when RT-PCR was performed on immunoprecipitations with the anti-Yps antibody, whereas control IgG immunoprecipitations yielded no osk RT-PCR signal (Fig. 7 B). To rule out artifacts due to signal saturation, we serially diluted the RT
product from our immunoprecipitations before conducting the PCR. osk mRNA was consistently detected in the 10-, 100-, and 1,000-fold dilutions of the RT product, and the signal decreased with increasing dilution (Fig. 7B). This result indicates that the PCR reaction is approximately in the linear range and that signal saturation is not a factor in our assay. Therefore, the RT-PCR assay demonstrates that both Yps and Exu are associated with a complex that contains osk mRNA. This was an unanticipated result, since previous genetic studies only reported a role for exu in the localization of bcd mRNA to the anterior of the oocyte. We cannot rule out that the Exu-Yps complex contains bcd mRNA, since our negative result could reflect technical difficulties, such as poor bcd mRNA stability. However, our RT-PCR results suggested the unanticipated possibility that exu is involved in posterior mRNA localization.

Yps Colocalizes with Exu in Oocytes and Nurse Cells and Accumulates at the Posterior Pole during Mid-Oogenesis

To learn more about the in vivo role of Yps in RNA localization, Drosophila ovaries were labeled with affinity purified antibodies to the NH$_2$ terminus (1-160 amino acids) of Yps. Immunofluorescence staining revealed a strong Yps signal in both the germ cells and follicle cells of developing egg chambers (Fig. 8). In contrast, labeling with secondary antibody alone produced a much weaker background signal (data not shown). This result, together with the specificity of our anti-Yps antibody by immunoblot (Fig. 5A), argues that the staining we observe is specific for Yps. Examination of different stage egg chambers (for staging see Spradling, 1993) revealed that Yps accumulates in the oocyte during stages 1-7. This signal was stronger at the posterior of the oocyte in early stages, although it was also present throughout much of the oocyte cytoplasm (Fig. 8A). In both early and midstage egg chambers, Yps exhibited a particulate staining that was frequently concentrated around the nurse cell nuclei (Fig. 8B). At stages 8 and 9, faint anterior localization was sometimes apparent in the oocyte (Fig. 8C), and during stages 9 and 10 Yps accumulated at the posterior of the oocyte (Fig. 8C and D).

The localization of Yps during oogenesis was very similar to the previously observed distribution of GFP-Exu (Wang and Hazelrigg, 1994). To compare the distributions of Exu and Yps directly, we immunostained Yps in egg chambers expressing GFP-Exu. Individual particles containing both proteins were detected in the nurse cells (Fig. 8E and F). The early accumulation of Yps in the oocyte (stages 1-7), its accumulation at the oocyte anterior (stages 8 and 9), and its later localization to the posterior pole (stages 9 and 10) all coincide with the localization of Exu protein and osk mRNA (Ephrussi et al., 1991; Kim-Ha et al., 1991; Wang and Hazelrigg, 1994). The agreement between our biochemical and in vivo localization studies further supports a role for the Exu-Yps complex in the localization of osk mRNA. This hypothesis was tested directly by genetic studies, as described below.

exu Mutants Have Defects in osk mRNA Localization

Whole mount in situ hybridization was performed on ovaries from wild-type (Oregon R) and exu null (exu$^{CO2}$/ Df[2R]MK1) females to determine whether exu is re-
required for any aspect of osk mRNA localization. In ovaries from wild-type females, osk mRNA is often concentrated in apical patches in the nurse cells of stage 9 and 10 egg chambers (Pokrywka and Stephenson, 1995) (Fig. 9 A). In contrast, osk mRNA was dispersed in the nurse cells of exu egg chambers of the same stages (Fig. 9 B). Thus, exu is required for osk mRNA to be correctly localized within nurse cells. Interestingly, bcd mRNA is also localized to apical patches in the nurse cells in an exu-dependent manner (St Johnston et al., 1989). Although the functional significance of this nurse cell localization is unknown, the fact that exu mutants disrupt this localization for both osk and bcd mRNA’s indicates that at least some of the components required for this localization are common to both transcripts.

exu mutants also caused a partial defect in osk mRNA localization to the posterior pole. In ovaries from wild-type females, a strong osk mRNA signal was detected at the posterior pole in 90% (n = 90) of wild-type stage 9 and stage 10 oocytes (Fig. 9 C); the remainder (10%) showed reduced (9%) or barely detectable (1%) posterior osk mRNA signal. In contrast, only 64% of stage 9 and stage 10 egg chambers from exu mutants (n = 210) showed an osk mRNA signal at the posterior of the oocyte, and this signal was consistently weaker than that observed in the wild-type egg chambers; the remainder (36%) showed reduced (23%), barely detectable (8%) (Fig. 9 D), or no (5%) posterior osk mRNA signal. Thus, mutations in exu cause a defect in the amount of osk mRNA that is localized to the posterior pole during stages 9 and 10 of oogenesis.

Discussion

Although mRNA localization in Drosophila has been the subject of extensive genetic analysis, only a few attempts have been made to characterize biochemically the proteins

Figure 8. Yps accumulates at the posterior pole during mid-oogenesis and colocalizes with Exu within the ovary. Wild-type (Oregon R) ovaries were labeled with anti-Yps antibody (see Materials and Methods). (A) In previtellogenic stages, Yps is most concentrated in the oocyte (arrows). (B) In both early and midstage egg chambers, Yps is present in particles that cluster around the nurse cell nuclei (arrow). (C) At stage 9, Yps continues to be concentrated in particles in the nurse cell cytoplasm, while it begins to accumulate at the posterior pole and anterior margin (arrows) of the oocyte. (D) During stage 9, Yps is restricted to the posterior pole of the oocyte (arrow). Yps is also highly expressed in the follicle cells, the somatic cells that surround each egg chamber, during all stages of oogenesis, although this is not visible in all images due to the plane of focus. For colocalization studies (E and F), ovaries from flies carrying a gfp-exu transgene were labeled with an anti-Yps antibody detected with a rhodamine-tagged secondary antibody. (E) In the nurse cells of a stage 10 egg chamber, Yps is localized to perinuclear particles that colocalize with (F) GFP-Exu particles.
associated with localized messages. In this study, we have demonstrated that E\textsubscript{exu}, a protein shown by genetic studies to be involved in mRNA localization, is part of a large RNA-sensitive complex. Through a combination of affinity chromatography and immunoprecipitation experiments, we have uncovered seven proteins associated with the E\textsubscript{exu} complex, one of which is a new RNA-binding protein, Y\textsubscript{ps}.

To our knowledge, this is the first isolation of a native RNP complex involved in mRNA localization. There are several pieces of evidence that argue against the E\textsubscript{exu}-containing complex being a biochemical artifact. First, the complex was identified using three different antibodies for immunoprecipitation. Second, the complex that we isolated from total fly homogenates is not an artifact of mixing proteins from different tissues, since it is also present in extracts made from hand dissected ovaries. Third, E\textsubscript{exu} and Y\textsubscript{ps} are also copurified after metal affinity chromatography, and the two proteins interact in vitro in the absence of RNA. Fourth, E\textsubscript{exu} and Y\textsubscript{ps} colocalize by immunofluorescence indicating that they form a complex in vivo. Fifth, E\textsubscript{exu}, Y\textsubscript{ps}, and osk mRNA all show a transitory accumulation at the anterior in mid-oogenesis and then colocalize at the posterior pole. Finally, the relevance of the biochemical association with osk mRNA is supported by genetic experiments showing a mislocalization of osk mRNA in exu-null mutants. Although further genetic analysis of Y\textsubscript{ps} and the other proteins in the complex will provide additional insights into this problem, the current combination of biochemical cofractionation, in vivo colocalization, and genetic analysis argues for a direct role of E\textsubscript{exu} and its associated proteins in mRNA localization.

The Role of the Exu Complex in mRNA Localization

Previous genetic work implicated exu in mRNA localization, but did not address whether E\textsubscript{exu} plays a direct or indirect role in the localization process (Berleth et al., 1988; St Johnston et al., 1989; Mcdonald et al., 1991; Macey et al., 1991). This study supports a direct involvement for E\textsubscript{exu}, since we show that E\textsubscript{exu} is part of a large RNA-sensitive complex and interacts directly with an RNA-binding protein. Furthermore, the association of osk mRNA with the E\textsubscript{exu} complex establishes a physical connection between E\textsubscript{exu} and a localized mRNA. The biochemical characteristics of our large E\textsubscript{exu} complex also likely reflect the properties of E\textsubscript{exu} in vivo. E\textsubscript{exu} has been shown to be part of large particles by both GFP fluorescence (Wang and Hazelrigg, 1994) and immunoelectron microscopy (Wilsch-Brauninger et al., 1997). Moreover, E\textsubscript{exu} and Y\textsubscript{ps}, which are associated components in our biochemically isolated complex, also colocalize in particles within the nurse cells and oocytes.

The identification of osk mRNA in the E\textsubscript{exu}-Y\textsubscript{ps} complex was surprising, given the pronounced anterior patterning defects associated with exu mutants (Schupbach and Wieschaus, 1986). However, a role for E\textsubscript{exu} in osk mRNA localization is consistent with several other findings. First, previous work has shown that E\textsubscript{exu} accumulates at both the anterior and posterior poles of the oocyte (Macey et al., 1991; Wang and Hazelrigg, 1994). Second, osk mRNA transiently accumulates at the anterior pole along with bcd mRNA before its transport to the posterior (Ephrussi et al., 1991; Kim-Ha et al., 1991). Finally, one of the effects of exu mutants was to disrupt the localization of osk mRNA to apical patches within nurse cells. This defect is identical to the nurse cell localization defect described previously for bcd mRNA in exu mutants (St Johnston et al., 1989) and suggests that this step in the localization pathway may be common to both transcripts.

One of the reasons that exu mutants have not been examined previously for defects in osk mRNA localization is that only a small percentage of embryos from exu mothers display posterior patterning defects (Schupbach and Wieschaus, 1986). Our examination of exu mutants revealed that the amount of osk mRNA localized to the posterior pole is decreased in these mutants, suggesting that exu plays a role in localizing osk RNA within oocytes. However, as this defect is only partially penetrant, E\textsubscript{exu}-dependent posterior localization within the oocyte may be redundant with other localization mechanisms. In addition, the posterior patterning defects associated with the decrease in osk mRNA localization in exu mutants during stages 9 and 10 of oogenesis may be rescued by localization of osk mRNA during cytoplasmic streaming later in oogenesis. In support of this idea, Glotzer et al. (1997) have shown that injected, fluorescently labeled osk mRNA can be localized to the posterior at the time when cytoplasmic streaming occurs. Such localization most likely occurs by random motion during cytoplasmic streaming and specific anchoring of osk mRNAs that come in contact with the posterior pole. These multiple mechanisms of localization osk mRNA account for the fact that exu mutants do not display pronounced defects in abdominal patterning.

Figure 9. osk mRNA localization is disrupted in exu\textsuperscript{SCO2} egg chambers. Whole mount in situ hybridization was performed on egg chambers from wild-type (Oregon R) and exu\textsuperscript{SCO2} flies raised at 18°C to examine the distribution of osk mRNA. (A) In the nurse cells of wild-type stage 10 egg chambers, osk mRNA is often concentrated in patches within the nurse cell cytoplasm. (B) In nurse cells from stage 10 exu\textsuperscript{SCO2}/Df(2R)M-K1 egg chambers, the osk mRNA signal within the nurse cells is frequently dispersed. (C) In oocytes from wild-type stage 10 egg chambers, osk mRNA is highly concentrated at the posterior pole. (D) In oocytes from stage 10 exu\textsuperscript{SCO2}/Df(2R)M-K1 egg chambers, the osk mRNA signal is often reduced at the posterior pole. This particular egg chamber is an example of an oocyte with barely detectable osk mRNA (see text).
Previous work has shown that exu is necessary for normal bcd mRNA localization (Berleth et al., 1988; St Johnston et al., 1989; Pokrywka and Stephenson, 1991; Macdonald and Kerr, 1997), although these studies did not establish a direct connection between Exu and bcd mRNA. Nevertheless, it was surprising that we could not detect bcd mRNA in our Exu complex. This negative result should not be taken to indicate that bcd mRNA is not in the complex, since it could reflect poor stability of bcd mRNA, a weaker association of bcd mRNA with the Exu complex, or difficulty in extracting and/or purifying RNP particles containing bcd mRNA. Alternatively, it is possible that exu's role in bcd localization is indirect. We are currently trying to address these issues by using other approaches to detect specific Exu-bcd mRNA interactions.

**The Role of Yps in mRNA Localization**

Our biochemical studies linking Yps to Exu and osk mRNA suggest that this RNA-binding protein plays a role in posterior mRNA localization. This assertion is further supported by our immunofluorescence studies showing that Yps and osk mRNA have strikingly similar localization patterns throughout oogenesis: both accumulate in the early oocyte, transiently localize to the oocyte anterior during stages 8 and 9, and then assume their final positions at the posterior pole during stages 9 and 10 (Fig. 8) (Ephrussi et al., 1991; Kim-Ha et al., 1991). What role might Yps play in the localization complex? Yps belongs to the cold shock domain family of RNA-binding proteins that have been implicated previously in regulating translation and mRNA secondary structure (Jiang et al., 1997; Sommerville, 1999). A notable example is FRY2, which is complexed with mRNAs in the Xenopus oocyte and is thought to be important for translational silencing (Tafuri and Wolffe, 1993; Y urkova and M urray, 1997). Yps may serve a similar role, since osk mRNA is translationally repressed until it reaches the posterior pole (Kim-Ha et al., 1995; Macdonald and Smibert, 1996; Gunkel et al., 1998). Interestingly, Yps must also serve a function without Exu, since yps is expressed broadly, whereas exu expression is limited to the germ line (Macdonald et al., 1991; Marcey et al., 1991; Thieringer et al., 1997). It is possible that Yps is a component of the mRNA localization machinery outside the germ line, since other components of the oocyte mRNA localization machinery, such as Staufen, are also used for mRNA localization in somatic tissues (Li et al., 1997; Broadus et al., 1998). Determining Yps's precise involvement in transport and/or translational regulation in the oocyte and other tissues will be resolved in the future by mutational studies.

**A Model for mRNA Transport by the Exu–Yps Complex**

The pathways by which anterior- and posterior-localized mRNA s arrive at their destinations are poorly understood, although it is generally believed that these RNAs are recognized by different proteins and utilize distinct transport machineries. However, we propose that anterior- and posterior-localized mRNAs begin their localization process in the nurse cells using a similar complex, with Exu serving as a common core component (Fig. 10). In our model, one of Exu's functions is as a component of an mRNA transport complex, since GFP-Exu particles have been observed to move in a microtubule-dependent manner (Theurkauf and Hazelrigg, 1998). Consistent with this idea, both osk and bcd mRNA accumulate in apical patches within nurse cells, and exu mutants disrupt this localization pattern for both mRNAs (St Johnston et al., 1989; Pokrywka and Stephenson, 1995) (Fig. 9 B). We also propose that the Exu complex transports mRNAs from the nurse cells to the oocyte, as well as within the oocyte (Fig. 10), although these transport steps also can be achieved through other redundant mechanisms, such as nurse cell dumping and cytoplasmic streaming (Spradling, 1993). Although the above model places Exu as part of a transport complex, it...
should be noted that our present data also could be explained if Exu contributes to the establishment of anchoring once mRNA s reach their final destination.

After arriving in the oocyte, bcd- and osk-containing RNPs must be sorted so that bcd becomes anchored at the anterior pole, whereas osk is transported to the posterior pole. Since Yps, Exu, bcd mRNA, and osk mRNA all first colocalize at the anterior (Fig. 8) (Ephrussi et al., 1991; Kim-Ha et al., 1991; Wang and Hazelrigg, 1994; Theurkauf and Hazelrigg, 1998), we propose that this sorting decision occurs at the anterior of the oocyte (Fig. 10). Evidence for this anterior sorting model comes from genetic studies of staufen (staupropomyosin II (TmII), which show that these transport proteins do not interfere with anterior localization but rather block the release and transport of osk transcripts to the posterior (Ephrussi et al., 1991; Kim-Ha et al., 1991; Erdelyi et al., 1995; Tetzlaff et al., 1996). The molecular basis for this sorting decision is unclear, but may involve modifications to the transport machinery or the recruitment of additional factors.

**Biochemistry of the Exu Complex: Relationship to In Vivo Function**

Biochemical isolation of a native RNP complex provides an opportunity to identify new proteins involved in mRNA localization, which may have been missed by genetic analyses due to lethality or redundant functions. However, a significant caveat of our approach is that our isolated RNP particles may be heterogeneous, since our purification strategy begins with a crude extract containing multiple Exu particles which contain distinct mRNA in the nurse cells to the anchoring of the complexes at the posterior pole. In addition to temporal changes in the composition of the Exu complex, it is also possible that multiple Exu particles exist which contain distinct mRNA cargos.

To resolve these important issues, it will be important to determine the molecular identity of the six RNAse-sensitive polypeptides in our Exu complex and determine if they are required selectively for the localization of bcd mRNA or osk mRNA. In addition, simultaneous observation and colocalization of GFP-tagged mRNA s (Bertrand et al., 1998) and blue fluorescent protein-tagged components of the Exu complex will reveal which mRNA s and polypeptides are contained within the same transport complex in vivo. This approach will also allow direct observation of mRNA transport in Drosophila for the first time and provide a method for analyzing the changes in RNP components during the movement of RNA s from the nurse cell to their final destination in the oocyte.

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