Localization of oskar RNA regulates oskar translation and requires Oskar protein

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SUMMARY

The site of oskar RNA and protein localization within the oocyte determines where in the embryo primordial germ cells form and where the abdomen develops. Initiation of oskar RNA localization requires the activity of several genes. We show that ovaries mutant for any of these genes lack Oskar protein. Using various transgenic constructs we have determined that sequences required for oskar RNA localization and translational repression map to the oskar 3'UTR, while sequences involved in the correct temporal activation of translation reside outside the oskar 3'UTR. Upon localization of oskar RNA and protein at the posterior pole, Oskar protein is required to maintain localization of oskar RNA throughout oogenesis. Stable anchoring of a transgenic reporter RNA at the posterior pole is disrupted by oskar nonsense mutations. We propose that initially localization of oskar RNA permits translation into Oskar protein and that subsequently Oskar protein regulates its own RNA localization through a positive feedback mechanism.

Key words: oskar, mRNA localization, translational regulation, germ plasm, Drosophila

INTRODUCTION

In multicellular organisms, region-specific differentiation is facilitated by the spatial restriction of gene expression. One mechanism that spatially restricts gene expression is the localization of RNA and protein products to a specific site within a cell. The genetics of the localization process have been studied most extensively in Drosophila where differential localization of maternal RNAs and proteins in the growing oocyte play an important role in the determination of the body axes (reviewed by Gavis and Lehmann, 1994a).

At the posterior pole of the oocyte, localization of oskar (osk) RNA leads to the assembly of a highly specialized cytoplasmic region, the posterior pole plasm (Ephrussi et al., 1991; Kim-Ha et al., 1991; reviewed by Lehmann and Rongo, 1993). The posterior determinant nanos (nos), which is required for abdomen formation, is localized to the posterior pole plasm (Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991). The posterior pole plasm also contains unknown factors required for the formation of the pole cells, the germ cell precursors (Illmensee and Mahowald, 1974; Illmensee et al., 1996). Osk gene dosage experiments showed that osk is a limiting factor in determining the number of pole cells that form, the amount of nos RNA localized, and the amount of Nos protein synthesized (Ephrussi and Lehmann, 1992; Smith et al., 1992; Gavis and Lehmann, 1994b). At least eight genes (capucino (capu), spire (spir), staufen (stau), orb, mago nashi (mago), Notch (N), Delta (DI) and a maternal form of protein kinase A (PKA)) are required for the localization of osk RNA to the posterior pole (Manseau and Schüpbach, 1989; Schüpbach and Wieschaus, 1986; Lantz et al., 1994; Christerson and McKearin, 1994; Newmark and Boswell, 1994; Ruohola et al., 1991; Xu et al., 1992; Lane and Kalderon, 1994). Osk and four other genes (vasa (vas), pipsqueak (pip), tudor (tud), and valois (vls)) are required for the stepwise assembly of the posterior pole plasm (Schüpbach and Wieschaus, 1986; Siegel et al., 1993; Boswell and Mahowald, 1985).

The osk gene is a key component in pole plasm assembly. Mislocalization of osk RNA to the anterior pole by the replacement of the osk RNA localization signal with that of bcd leads to pole plasm assembly at the anterior pole (Ephrussi and Lehmann, 1992). At this ectopic site, germ cells form, nos RNA becomes localized and translated, and a second abdomen develops in mirror image to the posterior abdomen. Thus, osk is sufficient to initiate the assembly of germ plasm and the formation of an abdomen. Since these steps normally occur at the posterior pole, this experiment also suggests that the location of osk RNA within the oocyte defines polarity within the embryo. Therefore, to understand how polarity and pattern are established in Drosophila, it is essential to understand how osk RNA becomes localized to the posterior pole during oogenesis. Drosophila ovaries are composed of strings of maturing egg chambers. Each egg chamber consists of the oocyte and its 15 sister cells, the nurse cells, surrounded by somatic follicle cells (reviewed by Mahowald and Kambysellis, 1980). From stage 1 of oogenesis onward, osk RNA is enriched in the developing oocyte, which is positioned...
posterior to the nurse cells (Ephrussi et al., 1991; Kim-Ha et al., 1991). Osk RNA, initially transcribed in the nurse cells, is transported into the oocyte through large intracellular bridges, the ring canals, by a microtubule-dependent mechanism (Pokrywka and Stephenson, 1994; Clark et al., 1994). During stage 8 of oogenesis, osk RNA becomes concentrated at the posterior pole of the oocyte and slightly enriched at the anterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1991). By stage 9 of oogenesis, osk RNA is strictly localized to the posterior pole. Several results suggest that this initial localization is linked to the microtubule network. Mutations in capu, spir, PKA, N and DL disrupt microtubule organization as well as osk RNA localization in the oocyte (Theurkauf, 1994; Lane and Kalderon, 1994; Ruohola et al., 1991; Xu et al., 1992). Furthermore, a specific isoform of the motor protein dynein as well as a fusion protein between the motor protein kinesin and βgal become localized to the posterior pole during oogenesis, and localization of both motor proteins is dependent on gene functions (capu, spir, PKA, N and DL) that are also required for osk RNA localization (Li et al., 1994; Clark et al., 1994; Lane and Kalderon, 1994).

In contrast, mutations in the stau gene disrupt only osk RNA localization without disrupting the microtubule network (Ephrussi et al., 1991; Kim-Ha et al., 1991). Staußen protein colocalizes with osk RNA throughout oogenesis and Stau localization is affected by the same gene functions as osk (St Johnston et al., 1991). It has therefore been proposed that Stau protein and osk RNA are transported to the posterior pole as a complex. In stau mutants, osk RNA remains transiently at the anterior pole suggesting that stau may be specifically involved in the microtubule-dependent transfer of osk RNA from the anterior to the posterior pole of the oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991).

Both dynein and the kinesin-βgal fusion protein are released from the posterior pole of the oocyte after stage 10 of oogenesis, suggesting that additional functions are required to firmly anchor proteins and RNAs at the posterior pole throughout oogenesis and early embryogenesis (Li et al., 1994; Clark et al., 1994). Three alleles of osk, also referred to as the ‘delocalizing alleles,’ show a similar pattern for osk RNA and Stau protein localization (Ephrussi et al., 1991; Kim-Ha et al., 1991). Mutant oocytes possess localized osk RNA and Stau protein up to stage 10 of oogenesis. After this stage, osk RNA and Stau protein are not maintained at the posterior pole. Very little is known about the mechanisms involved in this ‘maintenance’ phase of the osk RNA localization process.

Here we describe experiments that analyze the mechanisms leading to the stable anchoring of osk RNA at the posterior pole. By examining the spatial and temporal distribution of the osk gene products, we have found that Osk protein is only detectable upon osk RNA localization and that genes that disrupt osk RNA localization affect osk protein levels. Transgenic constructs in which the Osk protein coding region was replaced with a heterologous sequence suggest that Osk protein levels are regulated by 3’UTR mediated translational repression. Furthermore, we show that Osk and Stau proteins function to maintain the localization of osk RNA at the posterior pole. These results have led us to propose the following stepwise model of osk localization: osk RNA and Stau protein initially accumulate in the growing oocyte and colocalize to the posterior pole in a stau dependent process. Upon localization, osk RNA is translated to produce Osk protein. Osk protein then stabilizes the initial localization by anchoring osk RNA and Stau protein at the posterior pole.

MATERIALS AND METHODS

Fly stocks

The following mutant alleles were used: osk^{54} (Lehmann and Nüsslein-Volhard, 1991); capu^{KK}, spir^{AB} (Manseau and Schüpbach, 1989); Df(2L)SD72^{p21}, stau^{b3}, stau^{c8} (Lehmann and Nüsslein-Volhard, 1991); mago^{1}, mago^{2} (Boswell et al., 1991); vas^{D}{(Schüpbach and Wieschaus, 1986); vas^{D}(Lehmann and Nüsslein-Volhard, 1991); vl^{AP}, vl^{AB}, tud^{BC8} (Schüpbach and Wieschaus, 1986); nos^{BN} (Wang et al., 1994).

Whole-mount in situ hybridization

Whole-mount in situ hybridization to embryos with digoxigenin-labeled riboprobes was performed according to the method of Gavis and Lehmann (1992). Whole-mount in situ hybridization to ovaries with digoxigenin-labeled DNA probes was performed according to the method of Ephrussi et al. (1991).

Riboprobes were prepared for nos detection using the plasmid pBluescript N5 containing a HindIII-Nol insert from the nos cDNA (Wang and Lehmann, 1991). Antisense RNA probes were synthesized using T7 polymerase and the Boehringer Mannheim ‘Genius’ 4 kit according to the method of Ephrussi and Lehmann (1992). DNA probes were prepared for osk detection using a 0.7 kb EcoRI fragment from the plasmid pBlueosk(−) containing a HindIII-Nol insert from the osk cDNA. DNA probes were synthesized with the Boehringer Mannheim ‘Genius’ kit according to the method of Ephrussi et al. (1991).

Embryos were mounted in LX112 medium (Ladd Research Industries, Inc.) according to the method of Ephrussi et al. (1991) or in 70% glycerol/PBS. Ovaries were mounted in 70% glycerol/phosphate buffered saline (PBS). Specimens were analyzed and photographed with a Zeiss Axioshot microscope and Nomarski optics.

Anti-Osk antiserum

To generate a bacterial fusion protein containing Osk, we inserted a 1.1 kb EcoRV-EcoRI fragment from pNBosk7 into the Smal site of pGEXXX after filling in the EcoRI site (Ephrussi et al., 1991; Smith and Johnston, 1988). This pGEXOsk1.1 vector was introduced into E. coli and inclusion bodies were isolated as described by Way et al. (1991). Inclusion bodies were boiled in sample buffer (0.06 M Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 5 M urea) and separated by SDS-PAGE. The gel was stained with Coomassie Brilliant Blue and the GST-Osk fusion protein was cut out and eluted from the gel slice. The fusion protein was dialyzed against PBS, emulsified with Freund’s adjuvant, and injected into rabbits.

We affinity-purified the antiserum against a bacterial fusion protein containing eleven amino acids of the T7 gene 10 protein and the carboxy-terminal half of Osk (pStyA; Ephrussi and Lehmann, 1992) as in the method of Smith and Fisher (1984) with several modifications. Immunoblots containing pStyA protein were incubated in PBS, 100 mg/ml BSA, and 0.5% (v/v) Tween-20. The blots were stained with Ponceau-S and the corresponding band was removed and incubated overnight with diluted sera in PBS and 0.5% (v/v) Tween-20. The strip was washed with PBS and 0.5% (v/v) Tween-20 and incubated 3× 30 seconds with 5 mM CAPS-NaOH (pH 12), 500 mM NaCl, 100 μg/ml BSA, and 0.5% (v/v) Tween-20. The elutions were pooled and neutralized with NaPO₄ (pH 6.8).

Whole-mount antibody staining

Antibody staining of embryos was performed with horseradish peroxidase (HRP) using biotinylated secondary antibodies and the Elite Kit (Vector Laboratories). Embryos were fixed for 20 minutes with

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gentle shaking in 4:1 heptane:4% paraformaldehyde in PBS and devitellinized as for in situ hybridization (Ephrussi et al., 1991). Embryos were rehydrated and the antibody staining was conducted as described by Eldon and Pirrotta (1991). Embryos were mounted as described above.

Antibody staining of ovaries was performed with a rat anti-Osk antibody (Ephrussi and Lehmann, 1992), a rabbit anti-Osk antibody (this report) and a rabbit anti-Stau antibody (gift of D. St Johnston; St Johnston et al., 1991). For secondary antibodies, fluorescein-conjugated goat anti-rabbit, fluorescein-conjugated goat anti-rat, or rhodamine-conjugated goat anti-rabbit (Jackson ImmunoResearch) antibodies were used. Ovaries were dissected from females in EB Ringer solution (Ephrussi et al., 1991) and fixed in 4% paraformaldehyde in PBS, 10% DMSO for 30 minutes. Ovaries were washed in PBT (0.1% Triton X-100 in PBS) and blocked in BBT (0.1% BSA in PBT). Samples were incubated with primary then secondary antibodies, washed in PBT overnight, and visualized with conventional fluorescence microscopy and confocal microscopy (Bio-Rad MRC600).

**Immunoblot analysis**

Ovaries from females of the indicated genotype were isolated as in the method of Theurkauf et al. (1992) and frozen in liquid N2. Samples were homogenized in boiling sample buffer (as above) and boiled for 5 minutes. Samples were then sonicated on ice for 10 minutes using a Branson 2400 sonicator. Supernatants were separated by SDS-PAGE and transferred to nitrocellulose. Protein was detected by Ponceau-S stain, and anti-Osk and anti-Vas antibodies were blocked in Blotto (5% NFD milk, 10 mM Tris-HCl, pH 7.5, 154 mM NaCl, 0.1% (v/v) Tween-20). The immunoblots were incubated with primary antisera in Blotto for 1 hour, washed in Blotto, and incubated in HRP-conjugated donkey anti-rabbit sera (Amersham) in Blotto. The immunoblots were washed and the HRP detected by ECL (Amersham).

Embryos were collected and anti-Nos immunoblots were performed as in the method of Gavis and Lehmann (1994b).

**Northern blot analysis**

Northern blotting was conducted as in the paper by Gavis and Lehmann (1992), using 10 μg of total RNA extracted from ovaries. 32P probes were synthesized from a 0.7 kb EcoRI fragment of pNBosk7 (Ephrussi et al., 1991) for osk or the 0.6 kb fragment from HR0.6 (O’Connell and Rosbash, 1984) for rp49.

**P element-mediated transformation**

The nos-osk3’UTR hybrid gene (pHSXn-o) was constructed by replacing the EcoRI fragment of pHSXnosbK containing the entire nos 5’UTR (Gavis and Lehmann, 1992) with the StyI-NotI fragment of pNBosk7 containing the osk 3’UTR (Ephrussi et al., 1991), after end-filling. The P element nos-osk3’UTR transgene was constructed by ligating a NotI fragment of pHSXn-o into the NotI site of the ry+ vector pDM30 (Misser and Rubin, 1987). Constructs were injected into ry+ embryos according to the method of Spradling (1986). P element plasmid DNA was injected at 0.5 mg/ml and pn25.7A2-3 helper plasmid DNA was coinjected at 0.1 mg/ml. Multiple independent lines were established for each construct.

**RESULTS**

**Osk protein is synthesized at the posterior pole**

In order to compare the spatial and temporal profile of Osk protein distribution with that of osk RNA and Stau protein, we analyzed oocytes and embryos using whole-mount in situ RNA hybridization and antibody staining. During the earliest stages of oogenesis (stage 1–stage 7), osk RNA and Stau protein accumulate in the oocyte, whereas Osk protein is not detectable (Fig. 1A,C,E). Osk protein is first detected during mid-oogenesis (stage 8 onward), coincident with the localization of osk RNA and Stau protein to the posterior pole (Fig. 1B,D,F). At this stage as well as during the later stages of oogenesis and early embryogenesis, the tight localization of Osk protein at the posterior pole closely resembles the distribution of osk RNA and Stau protein. Similarly, in the early embryo, Osk protein is present in the pole cells and is then rapidly lost from the pole cells at the cellular blastoderm stage (data not shown).

**Genes required for osk RNA localization affect Osk protein levels**

Since accumulation of Osk protein at the posterior pole occurs upon localization of osk RNA, we analyzed the expression of osk RNA and protein in extracts from wild-type ovaries and ovaries from mutants that fail to localize osk RNA. In wild-type oocyte extracts, the Osk antiserum detects two protein bands on immunoblots: a predominant protein of 54×10^3 M_r (p54Osk) and a less abundant form of 69×10^3 M_r (p69Osk) (Fig. 2). Both proteins are present in freshly laid eggs until four hours of development (Fig. 3). The two forms of the Osk protein may result from the differential use of two potential sites of translational initiation. Initiation at the first AUG, which poorly matches the Dro sophila consensus for initiation codons, should yield a 69×10^3 M_r protein (Ephrussi et al., 1991; Kim-Ha et al., 1991; Caven, 1987). Initiation at the second in-frame AUG, which shows an excellent match with the consensus, should yield a 54×10^3 M_r protein. Therefore, it
is likely that both polypeptides are alternatively translated forms of Osk. The specificity of the α-Osk antibody was demonstrated by its failure to detect either protein band in oocyte extracts from osk\textsuperscript{54} mutant females (Fig. 2). Sequence analysis of this allele predicts that osk\textsuperscript{54} should terminate translation prior to the carboxy-terminal region used as the antigen (Kim-Ha et al., 1991).

We examined protein extracts from mutant capu, spir, mago, and stau ovaries which fail to localize osk RNA. Neither form of Osk protein was detected on immunoblots of capu, spir, mago, or stau mutant ovaries (Fig. 2 and data not shown). However, wild-type quantities of osk RNA are present in ovaries from these genotypes (Fig. 2). This suggests that osk RNA localization is required whether for Osk protein synthesis or for Osk protein stability.

We also analyzed Osk protein in posterior group mutants that do not affect osk RNA localization but are required for germ plasm assembly. Both forms of Osk protein are present in extracts from vas, vls, and tud mutant ovaries, and ovaries mutant for osk missense alleles (Fig. 2 and data not shown). While the levels of both forms of Osk protein are unaffected in tud extracts, the overall levels are reduced in osk and vls mutant extracts. Vas mutant ovary extracts contain reduced levels of p54\textsuperscript{Osk} relative to wild type, while the levels of p69\textsuperscript{Osk} remain similar to wild-type extracts. This effect of vas on Osk protein levels is not mediated through the osk 3’UTR since vas mutant extracts that also carry a transgene in which the osk 3’UTR had been replaced by the bicoid 3’UTR (osk-bcd3’UTR) (Ephrussi and Lehmann, 1992) show similarly decreased Osk protein levels (Fig. 3, see below). These experiments show that gene functions that are not required for the localization of osk RNA nevertheless affect the stability or perhaps translational initiation of Osk protein.

**Identification of cis-acting sequences required for osk RNA translation**

Several mechanisms such as control of Osk protein stability or osk RNA translation could account for the absence of Osk protein in mutants that affect osk RNA localization. To distinguish between various modes of regulation we used two transgenes in which either the osk 3’UTR or the osk coding region and 5’UTR were replaced by heterologous sequences. First, a transgene in which the osk RNA localization signal had been replaced by the bicoid RNA localization signal (osk-bcd3’UTR) (Ephrussi and Lehmann, 1992) was analyzed. Previous experiments had shown that the functions of capu, spir, mago, and stau are dispensable for osk activity from the osk-bcd3’UTR transgene (Ephrussi and Lehmann, 1992). The α-Osk antibody detects both forms of the Osk protein in cell extracts prepared from osk-bcd3’UTR transgenic ovaries that are mutant either for osk\textsuperscript{54} or for stau (Fig. 3). Thus, the osk-bcd3’UTR hybrid RNA does not require stau function to produce wild-type levels of Osk protein. Since the osk-
null mutants that carried either the osk RNA localization regulates osk expression on a heterologous coding sequence and suggest that the translational regulation on a reporter, a second transgene was introduced into the germ line of flies by P element-mediated transformation. To determine whether osk translational sequences can confer translational regulation on a reporter, a second transgene was generated by fusing sequences encoding the nos promoter, the nos 5'UTR, and the nos open reading frame to osk 3'UTR sequences. This nos-osk 3'UTR transgene was introduced into the germ line of flies by P element-mediated transformation. We compared Nos protein and RNA in embryos from nos RNA null mutants that carried either the nos-osk 3'UTR or the nos-tub 3'UTR transgene, which produces a hybrid RNA containing the tubulin 3'UTR instead of the nos 3'UTR. Although embryos from nos-osk 3'UTR females contain comparable levels of transgenic RNA relative to embryos from nos-tub 3'UTR females, less Nos protein is synthesized from nos-osk 3'UTR RNA than nos-tub 3'UTR or wild-type nos RNA (Fig. 4A,B). These results demonstrate that the osk 3'UTR can confer translational repression on a heterologous coding sequence and suggest that osk RNA localization regulates osk translation.

**The osk 3' untranslated region is the RNA localization signal in oocytes and embryos**

Our results suggest that osk RNA localization and Osk translation are linked. Since the osk 3'UTR can confer translational repression, we wished to determine if it was also sufficient for RNA localization. Previously it had been shown that sequences necessary for osk RNA localization initiation are located in the 3' untranslated region (3'UTR) (Kim-Ha et al., 1993). Furthermore, a reporter construct that fused the β-galactosidase coding region to the osk 3' UTR shows localization of the reporter RNA to the posterior pole during the early stages of oogenesis; however, the reporter RNA fails to remain localized at the posterior pole after stage 10 of oogenesis. Since it was conceivable that poor expression resulted in the failure of this reporter RNA to faithfully recapitulate the localization pattern of the endogenous osk RNA, we reasoned that the nos-osk 3'UTR reporter construct might circumvent this technical problem since it is well expressed.

In order to distinguish the transgenic RNA from the endogenous osk or nos RNA, the distribution of the nos-osk 3'UTR RNA was examined in embryos and oocytes from nos RNA null mutant females using a nos probe. The pattern of localization of the nos-osk 3'UTR transgenic RNA is identical to that of endogenous osk RNA throughout all stages of development. Females carrying a single copy of the nos-osk 3'UTR transgene produce oocytes in which the hybrid RNA is localized to the posterior pole and slightly enriched at the anterior during stage 8 (data not shown). By stage 9 of oogenesis, the RNA is strictly localized to the posterior pole. The nos-osk 3'UTR RNA is present at the posterior pole of embryos and is no longer present after pole bud formation (Fig. 5A,B and data not shown). This pattern of localization is identical to that of osk RNA localization and strikingly different from that of nos RNA, which is localized at a late stage of oogenesis (stage 12) and whose RNA is present in the pole cells throughout embryogenesis (Wang and Lehmann, 1991; Wang et al., 1994). The fact that none of the features of nos RNA localization are conserved in the nos-osk 3'UTR fusion RNA is expected since sequences necessary and sufficient for nos RNA localization are located in the nos 3'UTR which was replaced in the fusion construct (Gavis and Lehmann, 1992, 1994b). In contrast, all aspects of the osk RNA localization pattern are recapitulated by the transgene, which suggests that the osk 3'UTR contains the sequences sufficient to localize and maintain osk RNA at the posterior pole of oocytes and embryos. We also generated a second hybrid reporter that fused bcd coding sequences to
the osk 3′UTR. This hybrid RNA behaves identically to osk and nos-osk3′UTR RNA (data not shown).

If the osk 3′UTR contains all sequences sufficient to localize osk RNA, then posterior group genes required for osk RNA localization should also be required for localization of the nos-osk3′UTR RNA. The nos-osk3′UTR transgene was therefore introduced into capu, spir, and stau mutants, and the distribution of the nos-osk3′UTR RNA was analyzed. Similar to the endogenous osk RNA, the transgenic RNA is not localized in these mutants (Fig. 5B and data not shown). Northern blot analysis of 0-2 hour mutant embryos revealed that like the endogenous osk RNA (see above) the nos-osk3′UTR is stable (data not shown). Mutations in vas, vls, and tud do not affect the localization of the endogenous osk RNA or that of the nos-osk3′UTR RNA (Fig. 5B and data not shown). These results demonstrate that the mechanism responsible for osk RNA localization operates through the osk 3′UTR and that the nos-osk3′UTR transgenic RNA behaves like osk RNA.

Osk protein functions to maintain the localization of osk RNA

We have shown that localization of osk RNA during early oogenesis coincides with the initiation of Osk translation. Such a link between RNA localization and translation would provide an efficient mechanism to ensure the restriction of Osk protein to the posterior pole. However, additional mechanisms must act to maintain osk RNA and protein localization at the posterior pole, since certain alleles of osk, the delocalizing alleles, affect osk RNA and Stau protein localization (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991). Mutants for any one of these alleles fail to maintain osk RNA and Stau protein localization after mid-oogenesis. The three delocalizing alleles are caused by nonsense mutations and lead to protein termination in the amino-terminal half of osk. In contrast, the twelve known missense mutations of osk do not affect osk RNA and Stau protein localization (Ephrussi et al., 1991; Kim-Ha et al., 1991; C.R. and R.L., unpublished results). Two models can, in principle, account for the phenotypes of the two classes of osk alleles. (1) In a positive feedback model, the delocalizing alleles affect a region of the Osk protein required to maintain the localization of osk RNA (Ephrussi et al., 1991; Kim-Ha et al., 1991). This model predicts that Osk protein conducts multiple functions, including assembling the germ plasm,
localizing the posterior determinant \textit{nanos}, and anchoring \textit{osk} RNA and Stau protein at the posterior pole (Ephrussi et al., 1991; Kim Ha et al., 1991). (2) In an alternate, co-translational model, the delocalizing effect of the nonsense alleles is not due to a special function of the Osk protein, but simply due to premature termination of Osk translation. This model predicts that completion of translation, and not necessarily the Osk protein itself, is required for \textit{osk} RNA localization (Kim-Ha et al., 1991).

To distinguish between these two models, we tested whether \textit{osk} function is required to maintain localization of \textit{nos-osk\textsuperscript{3}UTR} transgenic RNA. The \textit{nos-osk\textsuperscript{3}UTR} transgene was crossed into flies mutant for the delocalizing allele \textit{osk\textsuperscript{3}}. The distribution of the transgenic RNA is identical to that of the endogenous \textit{osk\textsuperscript{3}} RNA: \textit{nos-osk\textsuperscript{3}UTR} RNA is initially localized to the posterior pole of the early oocytes (Fig. 6A) but by mid-oogenesis (stage 10B) \textit{nos-osk\textsuperscript{3}UTR} RNA is no longer detected at the posterior pole (Fig. 6B.C). Northern blot analysis of 0-2 hour embryos shows that the transgenic RNA is present and thus stable throughout oogenesis and early embryogenesis (data not shown). These results demonstrate that full length Osk protein functions to maintain \textit{osk} RNA localization and therefore support a positive feedback model.

\textbf{\textit{stau} is required to maintain the localization of \textit{osk} RNA}

During all phases of \textit{osk} RNA localization, \textit{osk} RNA and Stau protein colocalize (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991). \textit{Stau} function is required for the initiation of \textit{osk} RNA localization and protein translation. It is thus conceivable that Stau protein initiates the localization of \textit{osk} RNA and that Osk protein maintains this localization independently of Stau. It is, however, equally likely that both the initiation and maintenance of \textit{osk} RNA localization require \textit{stau}. To examine the role of \textit{stau} in \textit{osk} RNA maintenance, we studied the distribution of \textit{osk} RNA in a temperature-sensitive allele of \textit{stau}, \textit{stau\textsuperscript{C8}} (referred to as \textit{stau\textsuperscript{a}}) (Lehmann and Nüsslein-Volhard, 1991). At the restrictive temperature, the majority of \textit{stau\textsuperscript{a}} egg chambers show a pattern of \textit{osk} RNA localization characteristic of \textit{stau} null alleles (Table 1). Initially, \textit{osk} RNA accumulates in \textit{stau\textsuperscript{a}} oocytes as in wild type. During stage 9 of oogenesis, when it normally becomes localized to the posterior, \textit{osk} RNA remains concentrated in an anterior ring in \textit{stau\textsuperscript{a}} oocytes. By stage 10, the RNA is uniformly distributed in \textit{stau\textsuperscript{a}} oocytes. This strong defect in RNA localization is accompanied by a strong embryonic phenotype: embryos from \textit{stau\textsuperscript{a}} females do not form germ cells and lack abdominal segmentation at the restrictive temperature (Lehmann and Nüsslein-Volhard, 1991). The phenotype at the permissive temperature (18°C) is considerably weaker: embryos do not form pole cells, but most develop the normal number of abdominal segments. This weaker phenotype is also reflected in the pattern of \textit{osk} RNA distribution. Many \textit{stau\textsuperscript{a}} stage 9 egg chambers (38%) show wild-type \textit{osk} RNA localization to the posterior. However, by stage 11, the number of egg chambers with localized \textit{osk} RNA decreases and by egg deposition, \textit{osk} RNA is unlocalized in all embryos. In wild-type egg chambers and embryos \textit{osk} RNA localization is unaffected at either temperature. We conclude that at the permissive temperature, \textit{osk} RNA localization can be initiated in \textit{stau\textsuperscript{a}} oocytes, but cannot be maintained. These results suggest a requirement for \textit{stau} not only during the initiation, but also throughout the maintenance phase of \textit{osk} RNA localization.

\section*{DISCUSSION}

\textbf{RNA localization and translation}

We have followed the distribution of Osk protein during oogenesis and early embryogenesis. We found that Osk protein is only detected after \textit{osk} RNA is localized and that Osk protein is absent in mutants that disrupt \textit{osk} RNA localization. A hybrid RNA containing wild-type \textit{osk} protein coding sequences fused to sequences encoding the \textit{bcd} 3'UTR produces Osk protein in mutants that disrupt \textit{osk} RNA localization. Furthermore, a hybrid RNA containing wild-type \textit{nos} protein coding sequences fused to sequences encoding the \textit{osk} 3'UTR is translationally repressed. These results strongly suggest that \textit{osk} RNA translation is regulated and that translational repression of \textit{osk} RNA is mediated through sequences located in the \textit{osk} 3'UTR. Furthermore, these experiments support the idea that translational activation or derepression of \textit{osk} requires components of the \textit{osk} RNA localization machinary. However, we cannot exclude the possibility that in addition to translational regulation, factors such as concentra-

\begin{table}[ht]
\centering
\caption{Temporal and spatial pattern of \textit{oskar} RNA localization in \textit{stau\textsuperscript{C8}/stau\textsuperscript{D3}}}
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
Genotype & Pattern of localization & \multicolumn{3}{c|}{Percentage per indicated stage at 29°C} & \multicolumn{3}{c|}{Percentage per indicated stage at 18°C} \\
\hline & & Stage 9 & Stage 10 & Stage 11 & Embryo & Stage 9 & Stage 10 & Stage 11 & Embryo \\
\hline Oregon R & Anterior ring & 1\% & - & - & 2\% & 3\% & - & - & - \\
& No localization & - & 1\% & - & 2\% & 4\% & 3\% & 16\% & - \\
& Posterior localization & 99\% & 99\% & 100\% & 98\% & 93\% & 97\% & 84\% & 100\% \\
& Total & 69 & 97 & 18 & 129 & 73 & 61 & 25 & 33 \\
\hline \textit{stau\textsuperscript{C8}/stau\textsuperscript{D3}} & Anterior ring & 97\% & 85\% & 29\% & - & 24\% & 5\% & 2\% & - \\
& No localization & 2\% & 14\% & 71\% & 100\% & 37\% & 58\% & 81\% & 100\% \\
& Posterior localization & 1\% & 1\% & - & - & 38\% & 37\% & 17\% & - \\
& Total & 94 & 106 & 35 & 157 & 180 & 167 & 53 & 27 \\
\hline
\end{tabular}
\end{table}

The temperature dependent effect of \textit{stau\textsuperscript{C8}} on the pattern of \textit{osk} RNA localization in oocytes and embryos is indicated. Anterior ring indicates that \textit{osk} RNA is trapped at the anterior of the oocyte. No localization indicates that \textit{osk} RNA is enriched in the oocyte or embryo, but not localized at either pole. Posterior localization indicates that \textit{osk} RNA is localized at the posterior pole. Total indicates the total number of egg chambers or embryos for the given stage. Embryo refers to cleavage stage embryos. Numbered stages refer to the indicated stage of oogenesis according to Mahowald and Kambysellis (1980). Columns are arranged by stage. The percentages in each column indicate the percentage of egg chambers or embryos displaying the indicated pattern of localization for the given genotype.
tion-dependent protein stability may contribute to the accumulation of Osk protein at the site of osk RNA localization.

A link between translation and localization of RNA has recently been demonstrated for the posterior determinant nos (Gavis and Lehmann, 1994b). Although several aspects of osk RNA translational regulation share striking similarities with those postulated for nos, regulation of the two RNAs differs in several respects. Potential regulatory factors that repress translation of unlocalized osk RNA must be present when osk RNA is deposited in the early oocyte; translational repression is relieved from localized RNA during mid-oogenesis. Nos protein, in contrast, is efficiently synthesized in the germarium and the nurse cells (Wang et al., 1994; Gavis and Lehmann, 1994b). The factors that repress nos translation must therefore be either quiescent or absent in the germarium and nurse cells, and active in the oocyte throughout oogenesis.

**Cis-acting RNA sequences**

For many RNA molecules localized in the *Drosophila* oocyte, it has been shown that sequences in the 3′UTR are not only necessary, but also sufficient for proper RNA localization (reviewed by Gavis and Lehmann 1994a). Kim-Ha et al. (1993) demonstrated that sequences required for the transport of *osk* RNA into the oocyte and its initial localization are present in the *osk* 3′UTR; however, sequences required for maintenance remained elusive. We show here that the *osk* 3′UTR can impose all aspects of osk RNA localization, including maintenance, onto a reporter RNA, demonstrating that all sequences required for osk RNA localization are located in the *osk* 3′UTR.

Unlocalized osk RNA is not translated. This finding suggests another level of RNA regulation involving the *osk* 3′UTR. Similar to recent observations with nos RNA, we have shown that the 3′UTR of *osk* is necessary for translational repression. Osk RNA lacking the *osk* 3′UTR is not regulated by the same gene functions that regulate endogenous *osk* RNA, indicating that sequences in the *osk* 3′UTR are necessary to link *osk* translation and localization.

Temporal regulatory sequences may also reside outside of the *osk* 3′UTR since Osk protein is synthesized from the *osk-bcd* 3′UTR RNA during oogenesis in a temporal pattern resembling that of the endogenous *osk* RNA, and not *bcd* RNA, which is inactive during oogenesis (Driever and Nüsslein-Volhard, 1988; Salles et al., 1994). These results suggest that translational regulation is achieved by the combined effect of cis-acting elements located outside the *osk* 3′UTR that are required for the temporal pattern of translational activation, and sequences within the *osk* 3′UTR that modulate the levels of translation and link translational activation to RNA localization.

**Positive feedback mechanisms**

Nonsense alleles of *osk* fail to maintain the localization of *osk* RNA (Kim-Ha et al., 1991). We showed that a reporter RNA that contains the *osk* 3′UTR, but not the *osk* open reading frame is properly localized and maintained at the posterior pole. When the reporter RNA is introduced into mutants lacking Osk protein, it is initially localized and translated; however, it fails to maintain its localization to the posterior pole. Therefore, Osk protein is required to anchor *osk* RNA at the posterior pole.

Missense mutations in *osk* do not affect *osk* RNA localization (Kim-Ha et al., 1991; C.R. and R.L., unpublished results). It is possible that the Osk protein might possess a single biochemical function, and that *osk* RNA maintenance requires the lowest threshold of activity for this function. Therefore, the known missense mutations in *osk* would provide enough activity to permit *osk* RNA maintenance, but not enough to direct germ plasm assembly or *nos* RNA localization and translation. Alternatively, separate domains of Osk protein may independently control germ plasm assembly, *nos* RNA localization, and the anchoring of *osk* RNA at the posterior pole. Mutations that specifically perturb *osk* RNA maintenance or germ plasm assembly may have escaped detection in the previous mutagenesis screens. Wherein, new *osk* alleles were identified based on the abdominal phenotype of the mutant progeny of females homozygous or transheterozygous for *osk*. Specific mutations in a putative ‘*osk* RNA localization maintenance domain’ may have a phenotype very similar to that of the *stau05* mutant described in the text. In such a mutant, *osk* RNA would be initially localized but subsequently not main-

![Diagram](https://example.com/diagram.png)

**Fig. 7.** A hypothetical model for *osk* RNA localization. (1) *osk* RNA and Stau protein are transported to the posterior pole. Initial localization is facilitated through the *osk* 3′UTR and requires *stau* function. Stau protein interaction with the *osk* 3′UTR is speculative. (2) Initiation of *osk* RNA posterior localization is completed and synthesis of Osk protein occurs. (3) *osk* RNA localization is maintained by *osk* and *stau* through the *osk* 3′UTR. Osk protein and Stau protein interactions with the *osk* 3′UTR are speculative.
tained at the posterior pole. As a consequence, sufficient levels of Osk protein would be synthesized to allow abdomen formation. Studies of the Drosophila virilis osk homologue support this hypothesis: when introduced into Drosophila melanogaster osk mutants, Drosophila virilis osk can complement the abdominal phenotype, but not the osk RNA maintenance phenotype (Webster et al., 1994). This suggests that abdomen formation does not require osk RNA maintenance, and that variations in the Oskar protein sequence can affect the maintenance function without affecting abdomen formation. A further genetic and molecular dissection of the Osk protein will be necessary to test this hypothesis.

Modulation of Osk protein expression through regulation of osk RNA localization helps to assure that the assembly of germ plasm occurs in a strictly defined position in the growing oocyte. A hypothetical model for osk RNA localization is presented in Fig. 7. Initially, when osk RNA is deposited in the oocyte, it is translationally inactive. Once the RNA is localized to the posterior, it is translated. Subsequently, Osk protein is synthesized and, in turn, acts to stabilize and maintain the localization of osk RNA. Osk RNA remains distributed throughout the oocyte during early oogenesis and during the localization process. Preventing translation of this unlocalized osk RNA ensures that Osk protein synthesis occurs only at the posterior pole. Since osk is sufficient to assemble the germ plasm and direct the localization of the posterior patterning signal, spurious osk expression would be detrimental to embryonic development. Thus, a link between osk RNA localization and osk translational regulation may be critical for germ plasm formation and establishment of the anterior-posterior axis.

We thank A. Williamson for the anti-Vas and Daniel St Johnston for the anti-Stau antisera. We thank Angelika Amon, Ewa Davison, Jen Mach, and Phil Zamore for critical comments on the manuscript. We are especially grateful to P. Zamore for advice on antibody purification and J. Mach for advice on confocal microscopy. This work was supported by a grant from the National Institute of Health. C.R. was supported by a National Science Foundation Predoctoral Fellowship and the NIH. E.R.G. was supported by fellowships from the Jane Coffin Childs Memorial Fund for Cancer Research and the Howard Hughes Medical Institute. R.L. is a Howard Hughes Medical Institute Associate Investigator.

REFERENCES


(accepted 25 May 1995)

Note added in proof

Kim-Ha et al. have recently reported the existence of elements in the *osk* 3'UTR that regulate *osk* translation (Kim-Ha, J., Kerr, K. and Macdonald, P. M. (1995). Translational regulation of *oskar* mRNA by Bruno, an Ovarian RNA-binding protein, is essential. *Cell* 81, 403-412).