Translational control of *oskar* generates Short OSK, the isoform that induces pole plasm assembly

Finn-Hugo Markussen, Anne-Marie Michon, Wolfgang Breitwieser and Anne Ephrussi*

Differentiation Program, European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

*Author for correspondence (e-mail: ephrussi@embl-heidelberg.de)

SUMMARY

At the posterior pole of the *Drosophila* oocyte, *oskar* induces a tightly localized assembly of pole plasm. This spatial restriction of *oskar* activity has been thought to be achieved by the localization of *oskar* mRNA, since mislocalization of the RNA to the anterior induces anterior pole plasm. However, ectopic pole plasm does not form in mutant ovaries where *oskar* mRNA is not localized, suggesting that the unlocalized mRNA is inactive. As a first step towards understanding how *oskar* activity is restricted to the posterior pole, we analyzed *oskar* translation in wild type and mutants. We show that the targeting of *oskar* activity to the posterior pole involves two steps of spatial restriction, cytoskeleton-dependent localization of the mRNA and localization-dependent translation. Furthermore, our experiments demonstrate that two isoforms of Oskar protein are produced by alternative start codon usage. The short isoform, which is translated from the second in-frame AUG of the mRNA, has full *oskar* activity. Finally, we show that when *oskar* RNA is localized, accumulation of Oskar protein requires the functions of *vasa* and *tudor*, as well as *oskar* itself, suggesting a positive feedback mechanism in the induction of pole plasm by *oskar*.

Key words: *oskar*, germ plasm, cytoskeleton, localization, translation, *Drosophila*

INTRODUCTION

The specification of body axes and germline in *Drosophila* depends on the restriction of gene activities to distinct locations in the oocyte (St Johnston and Nüsslein-Volhard, 1992). These localized determinants of cell fate in many cases consist of maternally provided RNAs that are activated at particular locations and stages of oogenesis or embryogenesis. Since inappropriate activation of these determinants can have dramatic consequences on development of the zygote (Driever et al., 1990; Ephrussi and Lehmann, 1992; Gavis and Lehmann, 1992), tight spatial and temporal regulation of their expression is essential.

Different strategies have evolved to restrict the activities encoded by these maternal RNAs. Maternal *hunchback* RNA is initially dispersed uniformly throughout the oocyte and becomes asymmetrically distributed in the embryo through a differential destabilization of the RNA (Wharton and Struhl, 1991). Recent findings indicate that fertilization-mediated activation of translation of the anterior morphogen *bicoid* (*bcd*), is regulated by modulation of the poly(A) status of the localized RNA (Sallés et al., 1994). In contrast, translation of the posterior morphogen *nanos* (*nos*) is not controlled by polyadenylation but requires components of the posterior pole plasm for its localized translation (Gavis and Lehmann, 1994). Finally, *gurken* (*grk*) and *oskar* (*osk*) encode determinants whose localized activities are required in the oocyte itself (Ephrussi et al., 1991; Kim-Ha et al., 1991; Neuman-Silberberg and Schüpbach, 1993). *grk* and *osk* are localized as mRNAs during oogenesis, and it is likely that there is a close relation between RNA localization and translation in order to spatially restrict the activity of these genes.

The function of *osk* during oogenesis is to assemble the posterior pole cytoplasm, or pole plasm, which recruits *nos* RNA and allows its localized translation. This results in the NOS protein gradient necessary for proper patterning of the posterior of the embryo (Ephrussi et al., 1991; Kim-Ha et al., 1991; Barker et al., 1992; Ephrussi and Lehmann, 1992), and is crucial to embryonic development. The pole plasm also contains the determinants of the germline (Illmensee and Mahowald, 1974).

Localization of *osk* to the posterior pole is essential for proper development to occur and several genes required for the formation of pole plasm are involved in this process (for a review, see St Johnston, 1993). In oocytes from females mutant in *cappuccino* (*capu*), *spire* (*spir*), *mago nashi* (*mago*), *TropomyosinII* (*TmII*), *orb* and *staufen* (*stau*), *osk* RNA is stable but is not localized (Ephrussi et al., 1991; Kim-Ha et al., 1991) and mutant embryos develop lacking both abdomen and germline. Conversely, in ovaries from females mutant in *BicaudalD* (*BicD*), *osk* RNA is present at both poles of the oocyte (Ephrussi et al., 1991), resulting in embryos lacking a head and bearing a second abdomen of reverse polarity instead. Finally, when *osk* is deliberately mislocalized to the anterior of...
the oocyte by fusion of its coding region to the 3' untranslated region (3'UTR) of bcd, a functional pole plasm is induced at the anterior pole and bicaudal embryos develop with pole cells at both ends (Ephrussi and Lehmann, 1992). These results demonstrate that tight restriction of osk activity to the posterior pole is essential. In addition, the absence of ectopic abdominal activity in the mutants where osk RNA is not localized indicates that unlocalized osk mRNA is inactive, and raises questions as to the mechanisms underlying the activation of localized osk.

A successful combination of molecular, biochemical and genetic approaches has shed some light on the basis for the spatial restriction of genetic approaches has shed some light on the basis for the anterior pole and bicaudal embryos develop with pole cells at both ends (Ephrussi and Lehmann, 1992). These results lead us to conclude that multiple layers of regulation of pole plasm assembly. From western blot analysis of OSK protein and the mechanisms regulating its translation, we find that during oogenesis and early embryogenesis, at least two isoforms of OSK are produced by alternative start codon usage. The Short OSK isoform has full function, while the Long OSK isoform has little or no pole plasm activity, leading us to propose different roles for the two isoforms in regulation of pole plasm assembly. From western blot analysis of OSK protein in wild type and mutants, we conclude that osk RNA localization is most likely required for OSK translation. In addition, we show that even when osk RNA is localized, accumulation of OSK protein requires the functions of vasa (vas) and tudor (tud), as well as of osk itself. These results lead us to conclude that multiple layers of regulation have evolved to restrict osk activity to the posterior pole. In addition to the localization of osk RNA, osk translation is derepressed upon localization to the posterior pole. Subsequently, translation of osk anchors the mRNA at the posterior pole. As a final positive feedback mechanism, pole plasm components are needed for efficient production of osk activity, the inducer of pole plasm assembly.

MATERIALS AND METHODS

Fly stocks
For the analysis of OSK protein in maternal mutant backgrounds, the following allelic combinations were used: orb<sup>pol</sup>/orb<sup>pol</sup> (Christerson and McKea, 1994); grk<sup>266</sup>/grk<sup>2E12</sup> (González-Reyes et al., 1995); capu<sup>1</sup>/Df(2L)ed-S21 (St Johnstone, unpublished; Reuter and Sztondora, 1983); spir<sup>BP</sup>/Df(2R)TW2 (Manseau and Schüp, 1989); mago/mago<sup>1</sup> (Boswell et al., 1991); TmH<sup>107</sup>/TmH<sup>117</sup> (Erdélyi et al., unpublished data); stauf<sup>BP</sup>/stauf<sup>D1</sup> (Lehmann and Nüsslein-Volhard, 1991); vas<sup>D1</sup>/vas<sup>D2</sup> (Schüp, 1986; Lasko and Ashburner, 1988; Lehmann and Nüsslein-Volhard, 1991); tud<sup>C9</sup>/tud<sup>WES</sup> (Boswell and Mahowald, 1985); nos<sup>Y</sup>/nos<sup>C2</sup> (Lehmann and Nüsslein-Volhard, 1991). The osk alleles and Df(3R)y-pXT103 which uncover osk are described by Lehmann and Nüsslein-Volhard (1986) and in Lindley and Zimm (1992).

Antibodies
The polyclonal rat anti-OSK antibody was generated against the C-terminal half of OSK expressed in bacteria (Ephrussi and Lehmann, 1992). Anti-osk-N was generated against an amino-terminal peptide from osk (amino acids K80 to L95) which was synthesized on an eight branch polylysine core to form a multiple antigenic peptide (Tam, 1988). Anti-osk-C was generated against a carboxy-terminal peptide from osk (amino acids G580 to Q594) which was synthesized as a free peptide and coupled to keyhole limpet hemocyanin as a carrier. Rabbits were injected in the lymph nodes with 200 μg peptide in 50% complete Freund’s adjuvant. The animals were boosted with 100 μg peptide in 50% incomplete Freund’s adjuvant (subcuticular injection) every 30 days. Bleeds were collected 10 days after each boost.

Western blot analysis
Dissected ovaries and devitelinized embryos were kept on ice. Protein extracts and gel samples were prepared and stored as described (Hay et al., 1988), with minor modifications. Tissues were homogenized by sonication in approximately 20 volumes of extraction buffer containing 1 mM EGTA, and protease inhibitors were added at the following concentrations: 0.2 mM benzamidine, 2 mM PMSF, 10 μM leupeptin, 10 μM pepstat A, 4 μM chymostatin, 1 μM aprotinin. The equivalent of approximately one ovary was loaded per lane on SDS-PAGE gels. Proteins were transferred to PVDF membrane (Immobilon P, Millipore) at approximately 7 V/cm for 12 to 18 hours in cold 10% methanol, 10 mM CAPS-NaOH, pH 11. Protein standards (low range, Bio-Rad) were visualized by Ponceau S staining. Antibodies were detected according to the ECL Western blotting protocols (Amersham Life Science) with the antibody indicated. Membranes were stripped as recommended (Amersham) and reprobed with alpha-tubulin monoclonal antibody DM 1A (Sigma).

Whole-mount in situ hybridization and antibody staining
Digoxigenin-labeled DNA probes were used to detect RNA in ovaries as described (Ephrussi et al., 1991). osk DNA probe corresponds to the 2.1 kbp SacI fragment in the osk cDNA. Whole-mount antibody staining was detected using biotinylated secondary antibodies and the Elite Kit (Vector Laboratories). After dissection, ovaries were fixed for 10 minutes in 6% formaldehyde/heptane, permeabilized in methanol:DMO(9:1), rehydrated into phosphate-buffered saline with 0.1% Triton X-100 (PBT), blocked in PBT/0.5% bovine serum albumin and incubated with the appropriate antibodies in blocking solution. Embryos were stained as described (Ephrussi and Lehmann, 1992), with one modification: 0.05% SDS was included in the first blocking solution and in the primary antibody dilution. For unknown reasons, this enhances the signal to background ratio when the peptide antibodies anti-osk-N and anti-osk-C are used.

DNA constructions and germline transformation
Point mutations were made by oligonucleotide-directed mutagenesis using T4 DNA polymerase and single-stranded uracil-DNA (Kunkel, 1987). We mutated a 6.45 kbp genomic Xhol-ApaI osk fragment that fully rescues the osk phenotype. All mutations were verified by DNA sequencing. In the following, the nucleotide positions (nt) refer to the EMBL database osk sequence DMO9785 with accession number M65178. The construct oskMIL carries an A to C mutation at nt 212, which results in the ATG→CTG mutation of the first codon M1. The construct oskMIL9L carries an A to C mutation at nt 626, which results in the ATG→CTG mutation of the codon M139. The construct oskMIL26 carries the same A to C mutation at nt 626, and in addition to the localization of osk RNA, osk translation is derepressed upon localization to the posterior pole. Subsequently, translation of osk anchors the mRNA at the posterior pole. As a final positive feedback mechanism, pole plasm components are needed for efficient production of osk activity, the inducer of pole plasm assembly.
the sequence (nt 584) GAA-ATC has been replaced by the sequence (nt 584) GAG-AAC-AAC-ATG, inserting two codons. This changes E(125)IT of OSK protein to E(125)NNMT so that the I(126) codon of the original osk sequence has been mutated to a start codon with the translation initiation site of M139. The construct Tub-osk140 was made by inserting a PstI restriction site at codon M139 which mutates the M139 but leaves codon T140 (nt 629-631) intact. This site was used to remove all osk sequence upstream of codon T140 from the 6.45 kbp XhoI-Apal fragment. The remaining 3’ osk fragment was inserted in-frame in the PstI-NcoI sites of CaSpeR pTub67c, which contains the alpha4Tub67C promoter (Ferrandon, 1994). The resulting construct expresses the first 10 amino acids of alpha4-Tubulin in fusion with OSK starting at T140 (amino acid sequence MREVVSIGcT140, arte-factual amino acids created by the cloning are in lower case) under the control of the alpha4Tub67C promoter, which is ovary specific. The sequence of each construct is available upon request. Transgenic flies were generated by P element-transformation (Rubin and Spradling, 1982) of a w1118 stock using the CaSpeR vector (Pirrotta, 1988).

**RESULTS**

osk mRNA contains a long open reading-frame which is preceded by an unusually short 5’ untranslated region (Ephrussi et al., 1991; Kim-Ha et al., 1991). The first two in-frame codons for methionine, M1 and M139, both match the consensus sequence for translation initiation in *Drosophila* (Cavener, 1987). Alternative initiation of translation from these two methionines would result in proteins of 606 and 468 amino acids, producing isoforms of predicted Mr = 69,283 (Long OSK) and 53,678 (Short OSK) respectively. By western blot analysis of extracts prepared from wild-type ovaries, we detect three distinct bands of apparent Mr = 71×10^3 (71K), Mr = 57K, and Mr = 55K (Fig. 1). In early embryos, however, the Mr = 57K species is no longer present and an Mr = 59K species appears instead (Fig. 1, emphasized in lower panel). These bands represent OSK since extracts prepared from flies with four copies of *osk* (4x, Ephrussi and Lehmann, 1992) show a specific increase in intensity of these bands. In addition, extracts prepared from three *osk* alleles with nonsense codons in their amino-terminal region (Kim-Ha et al., 1991) show no detectable OSK. These results suggest that the first AUG codon is used as a start codon and raise the possibility that several OSK isoforms

**Fig. 1.** Three distinct OSK bands are detected in western blot analysis of ovary and embryo extracts. Ovary extracts to the left, embryo extracts to the right, as indicated. Wild-type (+) and 4x osk (4x) ovary extracts contain Mr = 55×10^3, 57×10^3 and 71×10^3 OSK bands. 4x osk contains more OSK than wild type. Control lanes with homozygous osk346, osk54 and osk346 (346, 84 and 54, respectively) contain no OSK bands. The blot was probed with anti-OSK, an antibody made against a bacterially expressed OSK fragment overlapping only slightly with the product encoded by osk346, but not with that of osk54 or osk346. The lower panel emphasizes the difference in migration of the lower OSK bands from ovary and embryo extracts, apparent Mr is indicated.

**Fig. 2.** OSK isoforms of 69K and 54K are made by alternative start codon usage. (A) DNA constructs and origin of the antigens used to generate the antibodies for this paper. The constructs are not drawn to proportions. (B) Western blot analysis of ovary extracts from females carrying the construct indicated at the top of each lane. The constructs are in *osk*34/Df-osk background (*Df-osk* is *Df(3R)p-XT103). The left *oskM139L* lane is from a line that overexpresses the construct, presumably due to multiple insertions. Controls: *osk34/Df-osk* encodes a truncated OSK not recognized by anti-OSK, +/Df-osk is from siblings of the *osk*34/Df-osk flies, Oregon R is the wild-type strain used, and 4x is from wild-type flies with two extra copies of *osk*+4. The western blots were probed with anti-OSK and anti-osk-N as indicated. As indicated by arrows, the 71K band of lanes *oskM139L* corresponds to Long OSK, while the 55K and 57K bands of lane *oskM1L* in the left panel correspond to Short OSK. As indicated by the arrowhead, the extended Short OSK encoded by *oskM126* is distinguishable from Short OSK.
are translated from the single detectable osk mRNA species (Ephrussi et al., 1991; Kim-Ha et al., 1991; N. Gunkel and A. E., unpublished), or that full-length OSK protein is processed post-translationally to yield different truncated forms.

**Long and Short OSK isoforms represent translation products that initiate at methionine 1 and 139, respectively**

To determine whether the OSK species detected in western blots are made by alternative initiation from M1 and M139, we examined the consequences of mutating M1 or M139 on the OSK species produced. We generated single base changes (ATG→CTG), thus making conservative substitutions of Leu for M1 or M139. To allow detection of the products after P-element transformation, the constructs were crossed into females hemizygous for osk54, an allele bearing a nonsense codon in the amino-terminal region (Kim-Ha et al., 1991). Ovary extracts from the various lines were prepared for western blot analysis of OSK. The anti-OSK antibody (Fig. 2A,B) revealed that the Short OSK isoform produced from oskM1L comigrates with the 55 and 57K bands, while no band is detected at 71K. Conversely, the Long OSK isoform produced from oskM139L comigrates with the 71K band detected in the wild-type extracts, while no specific band is detected at 55 and 57K. Furthermore, anti-osk-N, an antiserum raised against an OSK amino-terminal peptide (K80-L95) not present in Short OSK (Fig. 2B right panel) does detect the 71K band in extracts from wild type and oskM139L, confirming that this is full-length OSK. Taken together, these results demonstrate that the first AUG of osk is used as a start codon to generate the Long OSK isoform.

Fig. 3. Short OSK rescues the abdomen phenotype of osk. Cuticle preparations of embryos from osk54/Df-osk females carrying the indicated construct (construct maps are in Fig. 2A). oskM1L and oskM126 have full osk activity when compared to osk+, a wild-type osk construct. Tub-osk140 has a lower penetrance and oskM139L typically gives 2–4 abdominal segments (see Table 1 for hatch rates).

The lack of the 55 and 57K bands in extracts from oskM139L suggests that these bands are not the result of proteolytic cleavage. In addition, comigration of Short OSK, expressed from oskM1L, with the 55 and 57K bands raises the possibility that M139 is used as a start codon also in the wild type. To test whether M139 can be used to generate a Short OSK in the normal situation when M1 is not mutated, we modified the oskM139L gene, inserting at position 126 a new M codon embedded in the M139 translation initiation sequence, thus generating oskM126 (Fig. 2A). Translation from M126

blasts are made by alternative initiation from M1 and M139, we examined the consequences of mutating M1 or M139 on the OSK species produced. We generated single base changes (ATG→CTG), thus making conservative substitutions of Leu for M1 or M139. To allow detection of the products after P-element transformation, the constructs were crossed into females hemizygous for osk54, an allele bearing a nonsense codon in the amino-terminal region (Kim-Ha et al., 1991). Ovary extracts from the various lines were prepared for western blot analysis of OSK. The anti-OSK antibody (Fig. 2A,B) revealed that the Short OSK isoform produced from oskM1L comigrates with the 55 and 57K bands, while no band is detected at 71K. Conversely, the Long OSK isoform produced from oskM139L comigrates with the 71K band detected in the wild-type extracts, while no specific band is detected at 55 and 57K. Furthermore, anti-osk-N, an antiserum raised against an OSK amino-terminal peptide (K80-L95) not present in Short OSK (Fig. 2B right panel) does detect the 71K band in extracts from wild type and oskM139L, confirming that this is full-length OSK. Taken together, these results demonstrate that the first AUG of osk is used as a start codon to generate the Long OSK isoform.

The lack of the 55 and 57K bands in extracts from oskM139L suggests that these bands are not the result of proteolytic cleavage. In addition, comigration of Short OSK, expressed from oskM1L, with the 55 and 57K bands raises the possibility that M139 is used as a start codon also in the wild type. To test whether M139 can be used to generate a Short OSK in the normal situation when M1 is not mutated, we modified the oskM139L gene, inserting at position 126 a new M codon embedded in the M139 translation initiation sequence, thus generating oskM126 (Fig. 2A). Translation from M126
produces an OSK isoform that is slightly larger and distinguishable from the Short OSK in wild-type and oskM1L extracts (Fig. 2B, left panel). Furthermore, the anti-osk-N antibody does detect the full-length 71K OSK band in oskM126 extracts (Fig. 2B, right panel), making it unlikely that the 56-58K band detected by anti-OSK is an unstable degradation product of Long OSK. These results show that in the wild type, M139 of osk is used as a start codon to generate a Short OSK isoform, the major OSK species.

Short OSK is necessary and sufficient to induce formation of abdomen and germline

The two isoforms of OSK raise the possibility that they have distinct activities. We therefore analyzed the activity of each isoform in an osk-mutant background, based on two criteria: ability to rescue the lack of abdomen-phenotype in embryos produced by osk females (Fig. 3, panel osk54) and ability to rescue the lack of germline-phenotype. For simplicity, the embryos produced by osk/Df-osk females bearing a particular transgene will be referred to by the name of the transgene (e.g. oskM1L embryos).

oskM1L embryos, which contain only Short OSK, are wild type (Fig. 3). The hatch rates and the fertility of these embryos are similar to those of embryos rescued by the control construct containing wild-type osk (Table 1). By contrast, oskM139L embryos, which contain only Long OSK, do not hatch (Fig. 3), with the exception of one line in which the protein is overexpressed (Fig. 2B left panel, first oskM139L lane) and 11% of the embryos hatch (Fig. 3). The residual osk activity of oskM139L could indicate that Long OSK has a very weak activity or that undetectable amounts of Short OSK are made from the mutated start codon, since translation can initiate from non-AUG condons in a favorable context (Boeck and Kolakofsky, 1994; Grünert and Jackson, 1994). The few adults that emerge from the overexpressing oskM139L line are sterile (Table 1) and our analysis of an oskM139L construct expressed at the anterior (by fusing it to the bcd3 UTR, Ephrussi and

**Fig. 5.** In stage 8/9 egg chambers, OSK protein is detected at the posterior pole at the time when most osk RNA is still distributed throughout the ooplasm. Upper row is osk RNA in stage 3 to 10B egg chambers, middle row is OSK protein in wild type, bottom row is staining control of osk54/osk54 ovaries. OSK protein is first detected in late stage 8 and early stage 9 egg chambers. During stage 9 and 10, OSK accumulates at the posterior pole where it remains into embryogenesis. The osk54 control is overstained to demonstrate the absence of labeling at the posterior pole.

![Image](image-url)

**Fig. 6.** The Long OSK isoform, which lacks pole plasm inducing activity, is localized at the posterior pole. Shown is OSK protein in stage 10 egg chambers from osk54/Df-osk females carrying the constructs indicated (constructs are described in Fig. 2A). OSK was detected using the anti-OSK antibody. The fact that Long OSK (panel oskM139L) is tightly localized in stage 10B egg chambers demonstrates that this isoform does have the RNA localization maintenance activity of osk (Ephrussi et al., 1991; Kim-Ha et al., 1991). In contrast, Short OSK appears less tightly localized at the posterior oocyte cortex (panel oskM1L) than OSK expressed either from the wild-type gene, from oskM126, or even from oskM139L, all of which produce Long isoforms.
Lehmann, 1992), shows that Long OSK is not able to induce pole cells (data not shown). We exclude the possibility that orst at the second start codon in the is inactive due to the conservative mutation M139L, since reinstalling the second start codon in the oskM139L construct [Fig. 2B, lane P(osk⁺)] that has full rescuing activity (Table 1). We also rule out the possibility that oskM139L is inactive due to the conservative mutation M139L, since re-installing the second start codon in the oskM139L construct (oskM126) restores full activity to the gene. oskM126 embryos are wild type (Fig. 3), and hatch rate and fertility is similar to the wild-type control (Table 1). These results show that the amino-terminal 138 amino acids of Long OSK inactivate the pole plasm inducing activity of the protein. They also show that translation of Short OSK is necessary for osk function.

To verify that the Short OSK isoform is sufficient for osk activity, we removed the entire osk promoter and the 139 first codons of osk, replacing them with the promoter and the first ten codons of the alpha4Tub67C gene (Theurkauf et al., 1986). The resulting Tub-osk140 fusion construct (Fig. 2A) expresses less protein than we can detect by western blot analysis (Fig. 2B), yet induces abdomen formation (Fig. 3) and, at a low frequency, germline formation (Table 1). This shows that the Short OSK isoform is sufficient for osk function.

**Long OSK is not detected in the pole cells**

Although the pole plasm inducing activity of Long OSK is strongly inhibited, the protein is readily detected at the posterior pole of oocytes (see below) and pre-pole-cell-stage embryos using the Long OSK-specific anti-osk-N antibody (Fig. 4A). However, when the pole buds begin to form, Long OSK is apparently degraded (or the epitope becomes masked) and is not detectable in the pole cells. By contrast, the anti-osk-C antibody (Fig 2A) stains both the pole plasm and pole cells strongly (Fig. 4D). Hence, Long OSK may be localized or anchored at the posterior pole in a way different from Short OSK and it may be playing a redundant role when pole cells form.

**osk translation appears to require RNA localization to the posterior pole**

The tight localization of OSK protein and mRNA (Ephrussi et al., 1991; Kim-Ha et al., 1991) suggests that the protein is translated at the posterior pole (Fig. 4A,B). During the early stages of oogenesis, osk RNA accumulates in the oocyte, where it remains uniformly distributed throughout the ooplasm until stage 8 when it starts to concentrate at the posterior pole (Fig. 5). At this stage, OSK protein is first detected, but in contrast to the RNA, it is detected exclusively at the posterior pole, initially as a slim crescent close to the oocyte cortex (Fig. 5). Likewise, both the Long and the Short OSK isoforms produced by lines oskM139L and oskM1L, are detected uniquely at the posterior pole in oocytes (Fig. 6). These results indicate that OSK is only translated from mRNA localized at the posterior pole.

To investigate the relation between osk RNA localization and translation, we examined OSK protein in different pole plasm mutants (Fig. 7). orb, capu, spir, mago, stau and Tml are required to localize osk RNA to the posterior pole of the oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991; Christerson and McKearin, 1994; Newmark and Boswell, 1994). Tml is a newly identified mutation that affects osk RNA localization from the anterior margin to the posterior of the oocyte (Erdélyi et al., unpublished data). Mutations in these genes perturb osk RNA localization to the posterior pole, yet the RNA appears stable (data not shown) and remains distributed throughout the oocyte. However, only trace amounts of OSK protein are detected in extracts from orb, capu, spir, mago and Tml (Fig. 7). In grk mutant ovaries where the oocytes have a duplicated anteroposterior axis with an ‘anterior’ margin at each pole, osk RNA is detached from the cortex and mislocalized to the center of the oocytes where it is maintained at least through stage 10 (González-Reyes et al., 1995).
almost no OSK protein is detected in extracts from the greek (grk) ovaries (Fig. 7). Thus, in several mutants in which osk RNA is not localized at the posterior pole, OSK translation is largely inhibited.

In contrast, OSK is translated in pole plasm mutants that do not affect the posterior localization of osk mRNA, i.e. vas, tud, nos (Fig. 7), vls and pum (data not shown). Therefore, unless all mutations that abolish posterior localization of osk RNA also affect trans-acting factors needed for osk translation (as is the case for stau, see below), these results show that, in order to be efficiently translated, osk RNA must be localized to the posterior pole.

In the wild-type oocyte, osk RNA and STAU protein cosegregate during many phases of their localization (Ferrandon et al., 1994), and recent data indicate an involvement of STAU protein in osk translation, independent of its role in osk RNA localization (Kim-Ha et al., 1995). Consistent with this, we do not detect any OSK in stau mutant ovaries (Fig. 7). It is striking that in TmIIp 

### Table 1. Activity of OSK isoforms

<table>
<thead>
<tr>
<th>Construct</th>
<th>No. of lines analyzed</th>
<th>Average no. of eggs scored*</th>
<th>Average hatch rate (%)</th>
<th>No. of females scored per line</th>
<th>% With germ line</th>
<th>% Atrophic</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>59</td>
<td>262</td>
<td>&lt;1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P(osk+)</td>
<td>3</td>
<td>381</td>
<td>82</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P(oskM1L)</td>
<td>3</td>
<td>458</td>
<td>78</td>
<td>3</td>
<td>76</td>
<td>24</td>
</tr>
<tr>
<td>P(oskM139L)</td>
<td>2</td>
<td>318</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P(oskM139L)-1</td>
<td>1†</td>
<td>350</td>
<td>11</td>
<td>14</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>P(oskM126)</td>
<td>3</td>
<td>445</td>
<td>77</td>
<td>35</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>P(Tub-osk140)</td>
<td>3</td>
<td>334</td>
<td>44‡</td>
<td>35</td>
<td>6</td>
<td>94</td>
</tr>
<tr>
<td>+Df(osk)</td>
<td>3</td>
<td>437</td>
<td>89</td>
<td>35</td>
<td>87</td>
<td>13</td>
</tr>
</tbody>
</table>

All the constructs were in osk54/Df(3R)osk-T103. Eggs were collected on apple juice plates from 240 females. The hatch rates were scored by counting hatched and unhatched eggs after aging them for 29 hours at 25°C. The presence of a germline was determined by dissecting offspring females. The atrophic, collapsed, ovaries of females without a germline are easy to distinguish from ovaries with developing egg chambers.

*For all lines ≥262 eggs were scored and the hatch rate determined. The average hatch rate was then calculated.
†This oskM139L line over-expresses the 69K OSK isoform (Fig. 2) and demonstrates that the oskM139L construct alone has a very weak osk activity. Of 13 males dissected (in addition to the 14 females), all had atrophic testes without a germline.
‡The variation of hatch rates between the three Tub-osk140 lines was particularly large: 25%, 37% and 69%.
§The two "lines" here are sibling flies from the crosses of two of the P(osk+) lines.

In the wild-type oocyte, osk RNA and STAU protein cosegregate during many phases of their localization (Ferrandon et al., 1994), and recent data indicate an involvement of STAU protein in osk translation, independent of its role in osk RNA localization (Kim-Ha et al., 1995). Consistent with this, we do not detect any OSK in stau mutant ovaries (Fig. 7). It is striking that in TmIIp 

#### Different functions for the OSK isoforms?

The fact that Short OSK has full activity could mean that this is the genuine OSK and that Long OSK has no function. The striking differential localization of the two isoforms when pole cells form could merely reflect that Long OSK has no function, is not included in pole cells and consequently is degraded.

An alternative interpretation is based on the observation that Short OSK, when expressed alone, is not localized as tightly as wild-type OSK. Since Long OSK, in contrast, is tightly localized and does maintain the localization of osk RNA, it is possible that Long OSK plays a redundant role in anchoring osk RNA to the posterior pole. Consistent with this, Long OSK disappears from the posterior at the same time as osk mRNA (Ephrussi et al., 1991; Kim-Ha et al., 1991). This also coincides with the loss of RNA from polar granules (Mahowald, 1971).
The fact that Short OSK is present as a doublet in extracts from both ovaries and embryos suggests that it is modified. The upshift of the 57K band at fertilization when pole plasm is activated and polar granules associate with polysomes (Mahowald, 1968), and the absence of this modification in osk and vas mutants (which lack pole plasm), indicates a dynamic and active role of OSK in pole plasm assembly and activity.

The mechanism responsible for generating two OSK isoforms is not yet understood. Alternative start codon usage could be achieved in several ways. Differential translation from M1 and M139 could be temporally regulated. Alternatively, the first start codon could be unrecognized by a fraction of the ribosomes engaged by osk, since short 5'UTRs may impair efficient translation initiation (Kozak, 1987), and the osk 5'UTR is only 15 nt long. This leaky scanning (Kozak, 1986) would result in initiation from M139. In yet another scenario, the initiation from M1 is not leaky, making initiation from M139 by ribosomal scanning unlikely. Consequently, translation would have to start from an internal ribosome entry site (Curran and Kolakofsky, 1989). In this respect, Long OSK may have no function per se, but ribosomal read-through of the RNA upstream of M139 may be critical for the translational control of Short OSK.

Localization as a requirement for translation
It has recently been shown that osk RNA is translocated into the oocyte as a translationally repressed mRNA. When the repression is alleviated by mutations in the 3'UTR, the mRNA is translated independently of localization and OSK protein accumulates throughout the oocyte (Kim-Ha et al., 1995). This indicates that degradation of OSK translated from unlocalized RNA cannot account for the localized accumulation of OSK protein at the posterior pole, nor for the absence of OSK in the mutants where osk RNA is uniformly distributed throughout the ooplasm. Rather, we conclude that in these mutants the mRNA most probably remains in the repressed state. In TmlIR mutants, osk mRNA embarks on its transport to the posterior pole but is arrested at the anterior margin (Erdélyi, et al., unpublished data) and, in grk mutants, osk RNA virtually completes transport but is misdirected to the center of the oocyte because the overall polarity of the oocyte is affected (González-Reyes et al., 1995; Roth et al., 1995). The simplest explanation for the fact that osk is not translated in these two mutants is that a trans-acting factor present at the posterior pole is needed for efficient translation of osk. This factor could be activated by the (unknown) signal from the posterior polar follicle cells that is required at stage 7 to induce the anteroposterior axis of the oocyte.
oocyte (González-Reyes et al., 1995; Roth et al., 1995). However, different BicD alleles that cause mislocalization of osk RNA to the anterior (Ephrussi et al., 1991) develop the bicaudal phenotype (Mohler and Wieschaus, 1986), indicating that, in BicD, osk is translated at the anterior. If a posterior trans-acting factor is required for osk translation, the gain-of-function mutation in BicD presumably activates or relocates this factor at the anterior. Alternatively, aspects of osk translational regulation may be bypassed in BicD, since in this mutant STAU is not required to produce osk activity at the anterior (Lehmann and Nüsslein-Volhard, 1991), while it is thought to be essential in the wild type (Kim-Ha et al., 1995). The hypothetical posterior trans-acting factor could be either involved directly in translational control or, indirectly, in aspects of mRNA localization required for translation.

**Positive feedback in the induction of pole plasm**

osk activity is needed for maintenance of osk mRNA localization at the posterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1991). In addition, pole plasm activity is needed for accumulation and modification of Short OSK, the functional isoform, but apparently not for accumulation of Long OSK. It is thus possible that, after osk mRNA translocation to the posterior pole, which is the primary pole plasm localizing step (Ephrussi and Lehmann, 1992), pole plasm nucleation and assembly is subject to several layers of control. These would include derepression of osk translation at the posterior pole and dependence on OSK protein for maintenance of localization, a positive feedback confirming the correct mRNA localization. Subsequently, a regulated shift in start codon usage would lead to production of Short OSK which induces pole plasm. Finally, a second positive feedback by which the accumulation and modification of the Short isoform is dependent on pole plasm components, would assure efficient pole plasm assembly at the posterior pole.

It is unknown whether one of the above mechanisms would suffice for the spatial restriction of a determinant like osk. However, it is tempting to speculate that efficient restriction of such molecules indeed requires the combination of several common cellular mechanisms in order to be evolutionary stable.

We are grateful to Sandra Scanianamico for injecting our P-element constructs. We thank D. St Johnston for the grk fly stocks, for his gift of STAU antibody and for sharing his unpublished results. We thank D. Ferrandon for his transformation vector CaSpeR pTUB67c, the EMBL Protein and Peptide Service for synthesis of peptides, the Animal Facility for expert assistance in antibody generation, and the EMBL DNA Sequencing Service for rapid and accurate sequencing of constructs. We appreciate the suggestions and ideas of S. Eaton and M. Glotzer, and thank S. Cohen for his careful comments on the manuscript. W. Breitwieser is a fellow of Boehringer Ingelheim Fonds.

**REFERENCES**


**Translational control of oskar**
a central role in pattern formation of the *Drosophila* embryo. *Development* 112, 679-691.


(Accepted 11 August 1995)