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

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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 *osk* activity. Localization of *osk* RNA to the posterior pole of the oocyte is mediated through the *osk* 3′UTR (Kim-Ha et al., 1993), is dependent on the cytoskeleton (Clark et al., 1994; Pokrywka and Stephenson, 1995; Erdelyi et al., 1995), and requires *osk* function (Ephrussi et al., 1991; Kim-Ha et al., 1991). Mutations in RNA elements (called BREs) in the *osk* 3′UTR result in translation of unlocalized *osk* RNA (Kim-Ha et al., 1995). This demonstrates that in the normal situation unlocalized *osk* RNA is translationally repressed. These studies have also shown that, in addition to its function in *osk* RNA localization, *stau* plays an important role in *osk* translation. Finally, the fact that *bcd* RNA is translationally repressed until fertilization (Driever and Nüsslein-Volhard, 1988) and yet *osk-bcd* RNA, which lacks the *osk* 3′UTR, induces anteriorly localized pole plasm in oogenesis (Ephrussi and Lehmann, 1992), indicates that RNA elements upstream of the *osk* 3′UTR regulate temporal expression of *osk* RNA.

To gain a deeper understanding of the nature of *osk* activity and how it is restricted to the posterior pole, we have carried out an 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^{mel}/orb^{mel} (Christerson and McKearin, 1994); grk^{2B6}/grk^{2E12} (González-Reyes et al., 1995); $capu^{7L}/Df(2L)ed\text{-}SZ1$ (St Johnston, unpublished; Reuter and Szidonya, 1983); $spir^{RP}/Df(2R)TW2$ (Manseau and Schüpbach, 1989); $mago^{1}/mago^{1}$ (Boswell et al., 1991); $TmII^{gs1}/TmII^{gs1}$ (Erdélyi et al., unpublished data); $stau^{D3}/stau^{D3}$ (Lehmann and Nüsslein-Volhard, 1991); vas^{D1}/vas^{PD} (Schüpbach and Wieschaus, 1986; Lasko and Ashburner, 1988; Lehmann and Nüsslein-Volhard, 1991); tud^{WC8}/tud^{WC8} (Boswell and Mahowald, 1985); nos^{L7}/nos^{L7} (Lehmann and Nüsslein-Volhard, 1991). The *osk* alleles and *Df(3R)p-XT103* which uncover *osk* are described by Lehmann and Nüsslein-Volhard (1986) and in Lindsley 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 (subscapular 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 pepstatin 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. Antigen was 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 *Sac*I 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 buffered 6% formaldehyde/heptane, permeabilized in methanol:DMSO (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 *Xho*I-*Apa*I *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 DMOSKAR with accession number M65178. The construct *oskM1L* carries an A to C mutation at nt 212, which results in the ATG→CTG mutation of the first codon M1. The construct *oskM139L* carries an A to C mutation at nt 626, which results in the ATG→CTG mutation of the codon M139. The construct *oskM126* carries the same A to C mutation at nt 626, and in addition

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 *Pst*I 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 *Xho*I-*Apa*I fragment. The remaining 3′ *osk* fragment was inserted in-frame in the *Pst*I-*Not*I 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 MREVVSIQIGtcsT140, artefactual 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 w^{1118} 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 M_r=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 M_r=71×10^3 (71K), M_r=57K, and M_r=55K (Fig. 1). In early embryos, however, the M_r=57K species is no longer present and an M_r=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

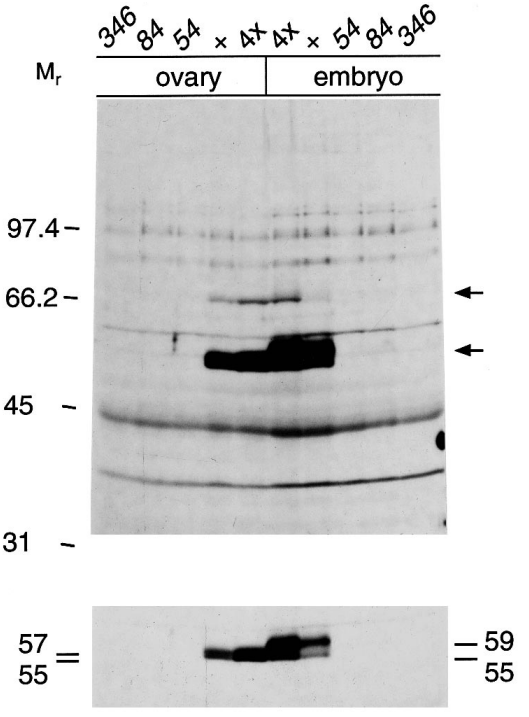

Fig. 1. Three distinct OSK bands are detected in western blot analysis of ovary and embryo extracts. Ovary extracts to the left, embryo extracts to the right, as indicated. Wild-type (+) and 4x *osk* (4x) ovary extracts contain M_r=55×10^3, 57×10^3 and 71×10^3 OSK bands. 4x *osk* contains more OSK than wild type. Control lanes with homozygous osk^{346}, osk^{84} and osk^{54} (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 osk^{346}, but not with that of osk^{84} or osk^{54}. The lower panel emphasizes the difference in migration of the lower OSK bands from ovary and embryo extracts, apparent M_r is indicated.

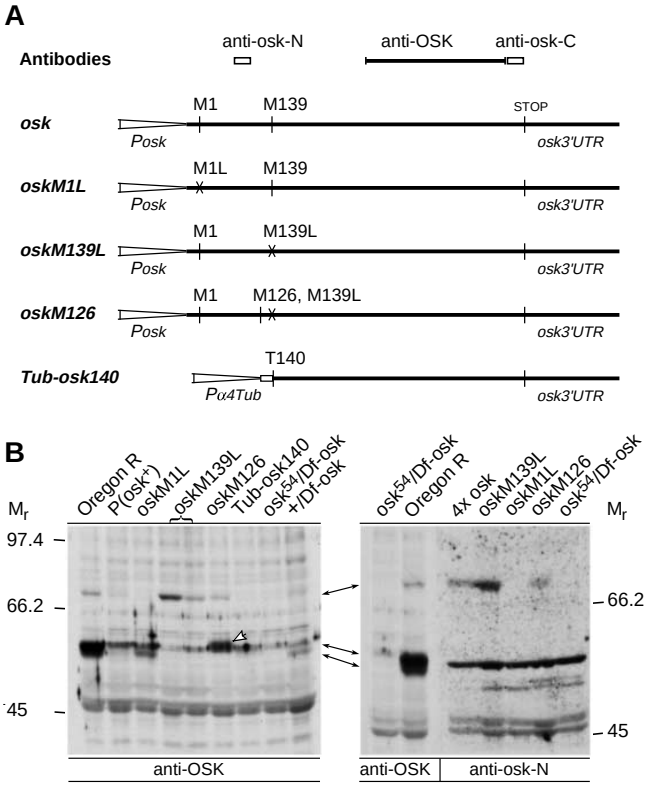

Fig. 2. OSK isoforms of 69K and 54K are made by alternative start codon usage. (A) DNA constructs and origin of the antigens used to generate the antibodies for this paper. The constructs are not drawn to proportions. (B) Western blot analysis of ovary extracts from females carrying the construct indicated at the top of each lane. The constructs are in osk^{54}/*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: *osk54/Df-osk* encodes a truncated OSK not recognized by anti-OSK, *+/Df-osk* is from siblings of the osk^{54}/*Df-osk* flies, Oregon R is the wild-type strain used, and 4x is from wild-type flies with two extra copies of osk^+. 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.

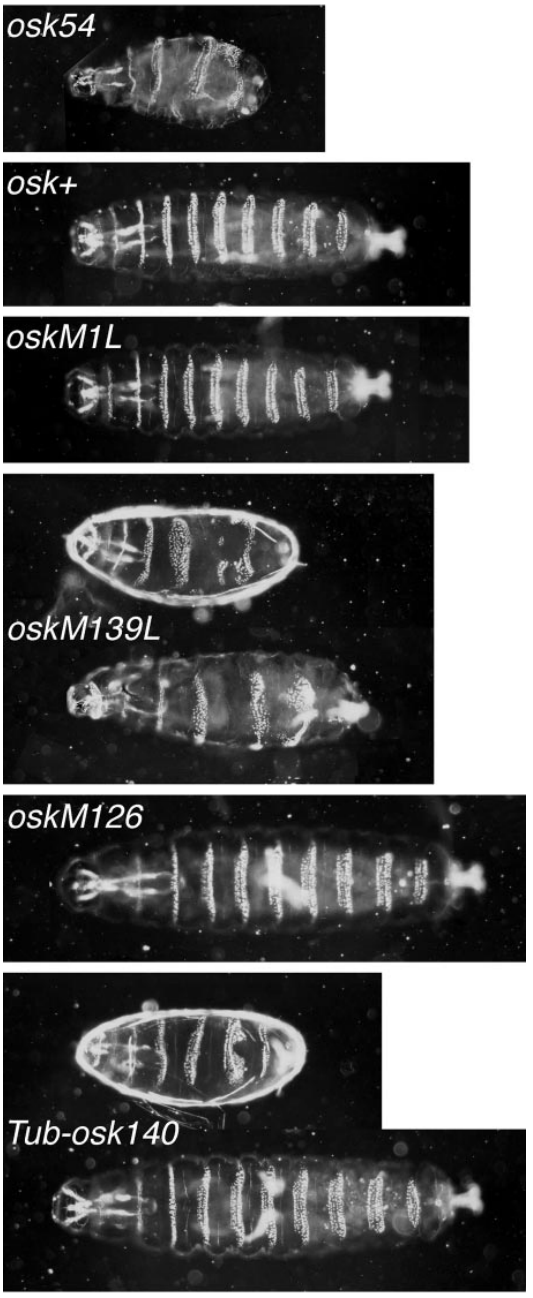

Fig. 3. Short OSK rescues the abdomen phenotype of *osk*. Cuticle preparations of embryos from *osk54/Df-osk* females carrying the indicated construct (construct maps are in Fig. 2A). *oskM1L* and *oskM126* have full *osk* activity when compared to *osk+*, a wild-type *osk* construct. *Tub-osk140* has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

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

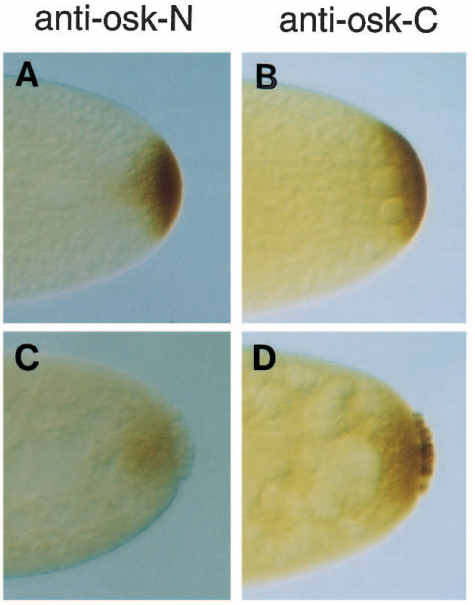

Fig. 4. Long OSK is not detected in pole cells. (A,B) Embryos of stage 1-2, i.e. nuclear division cycle 1-8. (C,D) Preblastoderm embryos of pole cell stage. The Long OSK-specific antibody anti-osk-N (A,C) does not label pole cells (C), indicating that Long OSK does not enter pole cells and may be degraded. By contrast, anti-osk-C, which recognizes the C terminus, does stain pole cells, suggesting that it is Short OSK that is included in pole cells.

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 osk^{54}, 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

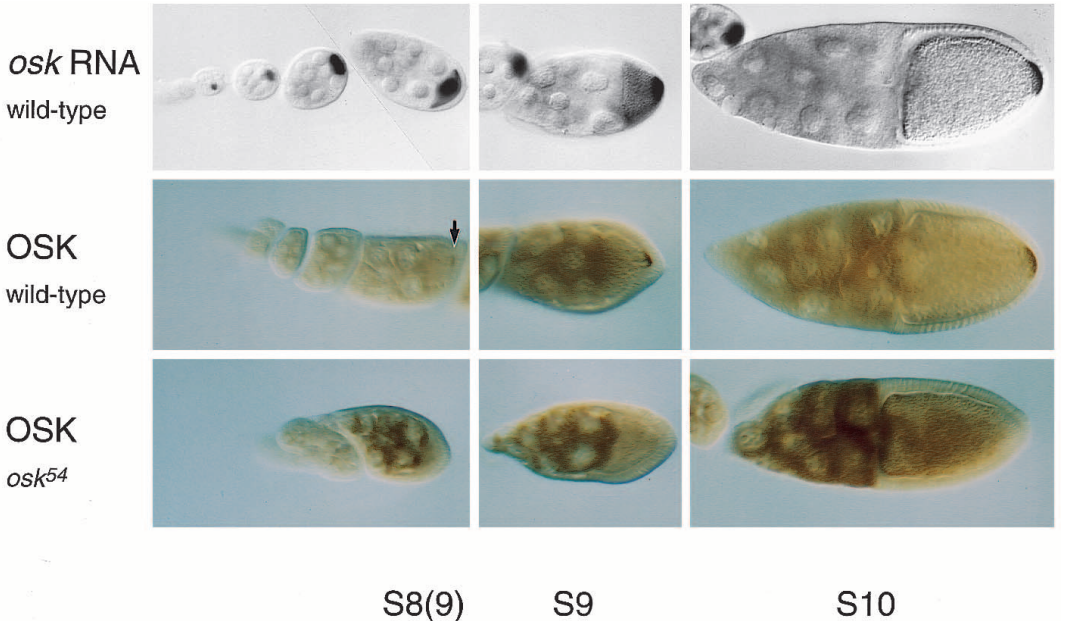

Fig. 5. In stage 8/9 egg chambers, OSK protein is detected at the posterior pole at the time when most *osk* RNA is still distributed throughout the ooplasm. Upper row is *osk* RNA in stage 3 to 10B egg chambers, middle row is OSK protein in wild type, bottom row is staining control of osk^{54}/osk^{54} 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 osk^{54} control is overstained to demonstrate the absence of labeling at the posterior pole.

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 (Table 1). *oskM139L* embryos typically develop 2 to 4 abdominal segments (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

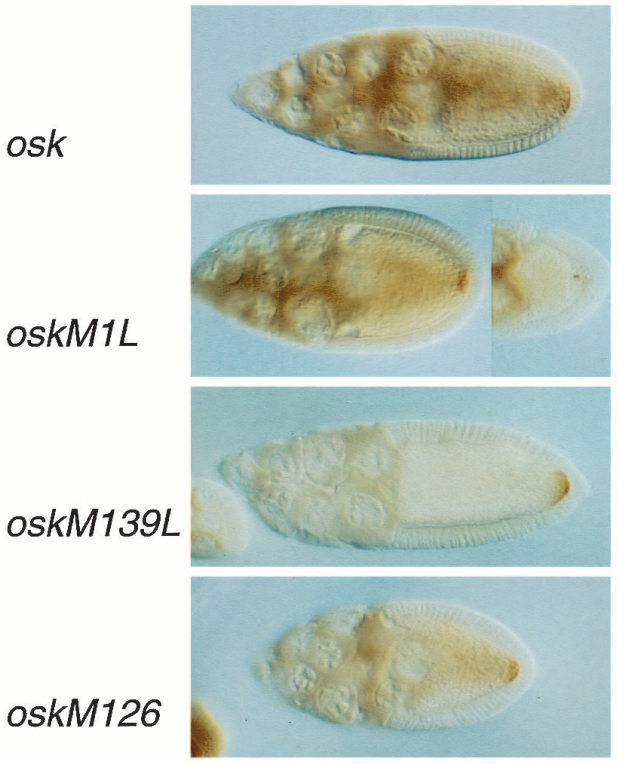

Fig. 6. The Long OSK isoform, which lacks pole plasm inducing activity, is localized at the posterior pole. Shown is OSK protein in stage 10 egg chambers from *osk54/Df-osk* females carrying the constructs indicated (constructs are described in Fig. 2A). OSK was detected using the anti-OSK antibody. 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 *oskM139L* is inactive as a result of inefficient production of Long OSK, since the amount produced by *oskM139L* is greater than the total amount of OSK protein produced by a wild-type *osk* 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 reinstalling 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 *TmII* 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). *TmII^{gs}* 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 *TmII^{gs}* (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 mislocalised to the center of the oocytes where it is maintained at least through stage 10 (González-Reyes et

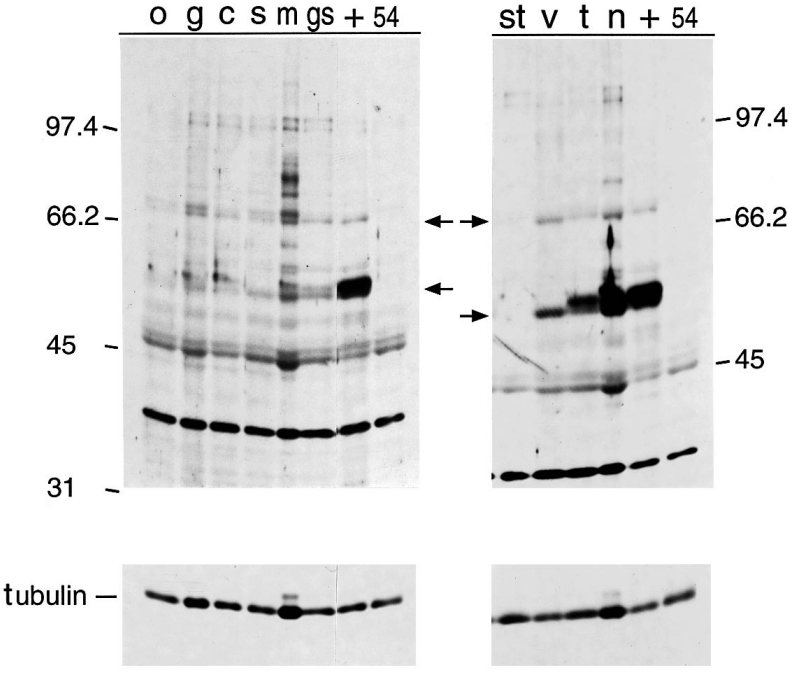

Fig. 7. OSK translation is inhibited in mutants where *osk* mRNA is not localized at the posterior pole. Lanes: (o) *orb*; (g) *grk*; (c) *capu*; (s) *spir*; (m) *mago*; (gs) *TmII^{gs}*; (st) *stau*; (v) *vas*; (t) *tud*; (n) *nos*; (+) and (54) are controls as in Fig. 1. Top panels show western blot analyses of ovary extracts probed with the anti-OSK antibody (Fig. 2A). Bottom panels show the same western blots probed with an anti-alpha-tubulin antibody as a loading control. In *orb*, *grk*, *capu* and *spir* very little, if any, OSK is detected. In *mago* and *TmII^{gs}*, a small amount of OSK is present. Although localized *osk* RNA has not been detected at the posterior pole by in situ hybridization in *orb*, *capu*, *spir*, *mago* or *TmII^{gs}*, the alleles used are not fully penetrant, and a certain percentage of embryos develop abdominal segments and have normal polarity. We infer that in these embryos a small amount of *osk* RNA is localized to the posterior pole and that the small amount of protein detected is translated from the localized RNA. The same may be the case for *grk* since, although all the egg chambers show a duplication of anterior follicle cells at the posterior pole, *osk* mRNA is only mislocalized to the center in 87% of the oocytes in the allelic combination used (González-Reyes et al., 1995). In *stau* ovaries no OSK protein is detected. In *vas*, *tud* and *nos*, where *osk* mRNA is localized to the posterior pole, OSK is translated. Hence, efficient translation of *osk* requires localization at the posterior pole.

Table 1. Activity of OSK isoforms

Construct	No. of lines analyzed	Hatch rate: Average no. of eggs scored*	Hatch rate: Average hatch rate (%)	Germ line: No. of females scored per line	Germ line: % With germ line	Germ line: % Atrophic
None	–	262	<1	–	–	–
P(*osk+*)	3	381	82	35	76	24
P(*oskM1L*)	3	458	78	35	99	1
P(*oskM139L*)	2	318	2	–	–	–
P(*oskM139L*)*-1*	1†	350	11	14	0	100
P(*oskM126*)	3	445	77	35	97	3
P(*Tub-osk140*)	3	334	44‡	35	6	94
+/Df-osk	2§	437	89	35	87	13

All the constructs were in *osk54/Df(3R)p-XT103*. Eggs were collected on apple juice plates from ≥40 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.

al., 1995; Roth et al., 1995). Almost no OSK protein is detected in extracts from the *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 *TmII*^{gs}, although *osk* RNA and STAU protein colocalise at the anterior of the oocyte until early stage 10 (Fig. 8A,B), no OSK protein is detected at the anterior margin (Fig. 8C). The failure to translate even small amounts of OSK at the anterior in *TmII*^{gs}, at the time when *osk* is translated at the posterior in the wild type (Fig. 5), again suggests that *osk* RNA cannot be translated prior to its localization at the posterior pole.

Pole plasm activity is needed for the accumulation of OSK protein

Although OSK is translated in pole plasm mutants that do not affect *osk* RNA localization, the relative amount of Short OSK is severely reduced in *vas* and *tud*, compared with wild type (Fig. 7). This raises the possibility that pole plasm integrity affects translation or stability of Short OSK. To examine this, we analyzed OSK in *osk*^{150} mutant ovaries. In this allele, *osk* RNA is stably localized at the posterior pole (Ephrussi et al., 1991), but none of the known downstream pole plasm components become localized. In *osk*^{150}, *vas* and *tud*, Long OSK is present at levels similar to wild type, but the amount of Short OSK is substantially reduced (Fig. 9). In *osk* and *vas*, only the 55K but not the 57K band of Short OSK is present, suggesting that a pole plasm-induced modification of Short OSK has failed. *tud*^{WC8} is not a null allele but has a weak pole plasm activity (Boswell and Mahowald, 1985), which may account for the intermediate phenotype where both the 55K and the 57K bands are present at reduced levels (Fig. 7,9). This result shows that other pole plasm components are needed for the stable accumulation of OSK.

DISCUSSION

oskar mRNA and protein are tightly localized at the posterior pole of oocytes where OSK nucleates the pole plasm. Our data support three conclusions about the nature of *osk* activity and the mechanism that restricts it to the posterior pole. First, alternative start codon usage generates two distinct OSK isoforms, and Short OSK is required and sufficient for *osk* activity. Second, the OSK isoforms are only translated after the mRNA is localized close to the oocyte cortex, at the posterior pole. This restricts *osk*-induced nucleation of pole plasm to the posterior of the oocyte. Third, pole plasm components are required for accumulation and modification of the Short OSK isoform. This provides yet another level of control, presumably restricting efficient pole plasm assembly to its site of nucleation, at the posterior pole.

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).

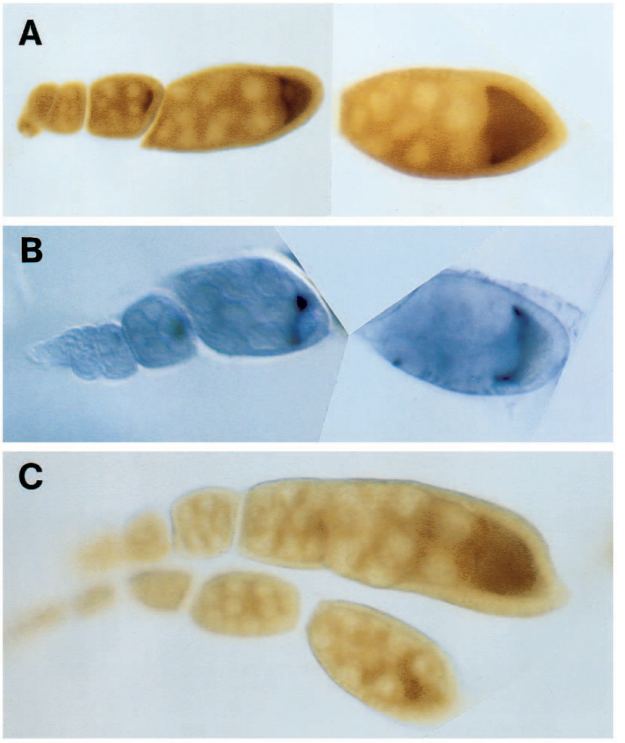

Fig. 8. *osk* RNA colocalized with STAU protein at the anterior margin of $TmII^{gs}$ oocytes is not translated. (A-C) $TmII^{gs1}$ oocytes stained for (A) STAU protein, (B) *osk* mRNA, and (C) OSK protein. The anti-OSK antibody (Fig. 2A) was used to stain OSK. There is a large concentration of *osk* mRNA and STAU protein at the anterior margin at the time when *osk* is translated in the wild type, yet no OSK protein can be detected.

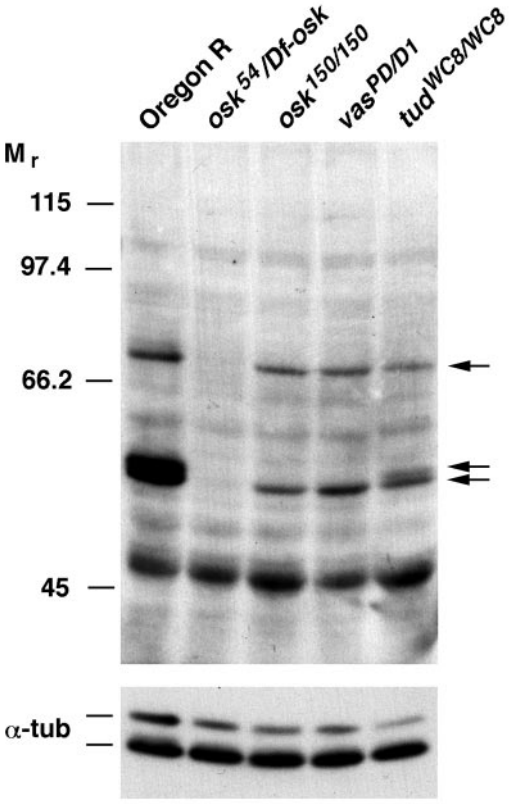

Fig. 9. Pole plasm activity is required for accumulation and modification of Short OSK. The top panel shows western blot analysis of ovary extracts probed with the anti-OSK antibody (Fig. 2A). The bottom panel shows the same western blot probed with an anti-alpha-tubulin antibody as a loading control. Genotypes are indicated at the top of each lane. In *osk*, *vas* and *tud* Long OSK is present at levels similar to wild type, while the amount of Short OSK is substantially reduced. In *osk* and in *vas*, the 57K OSK-specific band is missing, indicating that the modification of Short OSK is altered. In *tud*, the modification of Short OSK appears unaltered (also see Fig. 7, lane t).

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 $TmII^{gs}$ and *grk*, the situation is different. In $TmII^{gs}$ 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 (González-Reyes et al., 1995; Roth et al., 1995). However, dominant *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.

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