PKA-R1 spatially restricts Oskar expression for *Drosophila* embryonic patterning

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Summary

Targeting proteins to specific domains within the cell is central to the generation of polarity, which underlies many processes including cell fate specification and pattern formation during development. The anteroposterior and dorsoventral axes of the *Drosophila melanogaster* embryo are determined by the activities of localized maternal gene products. At the posterior pole of the oocyte, Oskar directs the assembly of the pole plasm, and is thus responsible for formation of abdomen and germline in the embryo. Tight restriction of *oskar* activity is achieved by mRNA localization, localization-dependent translation, anchoring of the RNA and protein, and stabilization of Oskar at the posterior pole. Here we report that the type 1 regulatory subunit of *cAMP-dependent protein kinase* (*Pka-R1*) is crucial for the restriction of Oskar protein to the oocyte posterior. Mutations in PKA-R1 cause premature and ectopic accumulation of Oskar protein throughout the oocyte. This phenotype is due to misregulation of PKA catalytic subunit activity and is suppressed by reducing catalytic subunit gene dosage. These data demonstrate that PKA mediates the spatial restriction of Oskar for anteroposterior patterning of the *Drosophila* embryo and that control of PKA activity by PKA-R1 is crucial in this process.

Key words: PKA-R1, *oskar*, Embryonic patterning, *Drosophila*, Oogenesis

Introduction

The asymmetric targeting of proteins within the cell cytoplasm is crucial for many biological processes including cell fate specification and pattern formation during development. In many cases this is an active process relying on a polarized cytoskeleton, and it is often achieved by RNA localization coupled with translational control. *Drosophila* oogenesis has served as an excellent model system for investigating such targeting processes (reviewed by Johnstone and Lasko, 2001). The anteroposterior and dorsoventral axes of the *Drosophila* embryo are determined by the activities of so-called axis determinants, which are localized as RNAs to specific domains of the developing oocyte. The *Drosophila* oocyte develops in a structure called an egg chamber, which consists of the oocyte, the nurse cells and a follicullar epithelium surrounding these cells. Anteroposterior and dorsoventral polarity of the oocyte are established within the egg chamber by cell-cell communication between the oocyte and the follicle cells. RNAs encoding the axis determinants are transported to specific locations along the anteroposterior and dorsoventral axes of the oocyte, where they are then expressed, thereby setting up the body axes of the future embryo (reviewed by Riechmann and Ephrussi, 2001).

At the posterior pole of the oocyte, Oskar plays a central role in abdomen and germline formation. It directs the assembly of the pole plasm, which contains factors responsible for abdomen formation, such as *nanos*, and factors for the formation of the pole cells, the primordial germ cells (Ephrussi et al., 1991; Kim-Ha et al., 1991). In *oskar* loss-of-function mutants, no abdomen and germ cells are formed. Conversely, mis-targeting of Oskar protein to the oocyte anterior (Ephrussi and Lehmann, 1992), or overexpression of Oskar throughout the oocyte (Smith et al., 1992), results in the formation of an ectopic abdomen and germ cells. These observations reveal the importance of restricting *oskar* activity to the posterior pole for correct anteroposterior patterning of the embryo.

Tight localization of Oskar protein is achieved in several steps (reviewed by Riechmann and Ephrussi, 2001). *oskar* RNA is produced in the nurse cells and transported in a translationally quiescent state into the oocyte, then specifically to the oocyte posterior pole. There, localized *oskar* RNA is translationally activated, the RNA and protein complex is anchored (Vanzo and Ephrussi, 2002), and Oskar protein stabilized (Riechmann et al., 2002). It has been proposed that the polarized microtubule network established by cell-cell communication between the oocyte and the posterior follicle cells mediates transport of the RNA (González-Reyes et al., 1995; Roth et al., 1995; Brendza et al., 2000). *oskar* RNA transport depends on cis-acting elements in its 3′UTR (Kim-Ha et al., 1993), on the RNA-binding protein Staufen (St Johnston et al., 1991), on components of the Exon-Junction Complex, such as Y14/Tsunagi (Hachet and Ephrussi, 2001; Mohr et al., 2001) and Mago nashi (Micklem et al., 1997; Newmark et al., 1997), and on Barentsz, a protein enriched

around the nurse cell nuclei and at the posterior pole (van Eeden et al., 2001). Translational repression of the RNA during transport also requires cis-acting elements in the *oskar* 3′ UTR, and proteins such as Bruno and HRP48 bind to these elements and are involved in this process (Kim-Ha et al., 1995; Webster et al., 1997) (T. Yano and A.E., unpublished). Upon localization, translational repression of *oskar* is alleviated. It has been shown that cis-acting elements in the *oskar* 5′UTR (Gunkel et al., 1998) and Staufen protein (Micklem et al., 2000) are involved in the activation of *oskar* translation at the posterior pole. In addition, *oskar* RNA is subject to cytoplasmic polyadenylation, which is mediated by ORB, the *Drosophila* CPEB (cytoplasmic polyadenylation element binding protein) homolog, resulting in an enhancement of *oskar* translation at the posterior pole (Chang et al., 1999; Castagnetti and Ephrussi, 2003).

In this report, we identify the type 1 regulatory subunit of *cAMP-dependent protein kinase (Pka-R1)* as a key factor in the restriction of Oskar protein to the posterior pole. Embryos derived from *Pka-R1* germline clones show a duplication of the posterior structures, the so-called bicaudal phenotype. This phenotype is caused by the premature and ectopic accumulation of Oskar protein in developing *Pka-R1* mutant oocytes, in which anteroposterior polarity and *oskar* RNA localization occur normally. PKA-R1 is a component of the PKA holoenzyme complex, a Serine/Threonine kinase that mediates various developmental processes by controlling the activity of its target proteins. PKA regulatory subunits control PKA activity by inactivating the catalytic subunits in the holoenzyme complex and releasing them in response to the second messenger cAMP (Taylor et al., 1990). We show that PKA catalytic subunit activity is upregulated in the *Pka-R1* mutant, and that the maternal-effect patterning defect is suppressed by reducing catalytic subunit gene dosage, indicating that aberrant accumulation of Oskar results from misregulation of PKA activity. In addition, we show that overexpression of PKA-R1 causes a moderate reduction in PKA activity, resulting in a significant reduction in the amount of Oskar protein produced at the posterior pole of the oocyte. These results reveal that PKA mediates the spatial restriction of Oskar protein during oogenesis and that precise regulation of PKA activity by PKA-R1 is crucial in this process.

Materials and methods

Fly strains

w1118 was used as the wild-type control. 18304 was identified in an FRT-based screen and a stock bearing 18304 and *P{FRT(w^{hs})}2A* was used throughout our analysis (see below). *Df(3L)ri^{-XT106}*, *Df(3L)ri^{-XT1}* and *Df(3L)ME107* fail to complement the 18304 bicaudal phenotype and were used to generate hemizygous mutant flies. To generate 18304, *oskar* double mutant flies, 18304 was recombined with *oskar^{346}*, and *Df(3L)ME107* was recombined with *oskar^{166}*. *DC0^{E95}* and *DC0^{H2}* were used for testing genetic interaction between *Pka-R1* and *DC0*.

For the rescue of 18304 mutants by *Pka-R1*, an EST clone encoding the RA isoform (LD43873) was subcloned into pCaTub67MatPolyA (Micklem et al., 1997) (a gift of D. Ferrandon), or into pUASp2 (Rørth, 1998). The rescue constructs were introduced into the germline of *w1118* embryos, according to standard procedures, and the ability of the transgenes to rescue the 18304 maternal-effect bicaudal phenotype was tested by crossing them into the 18304/ *Df(3L)ME107* and 18304/ E1 mutant backgrounds. In the case of UASp2-RA, expression in the germline was under the control of the pCOGGAL4 driver (Rørth, 1998). For overexpression, expression of the RA isoform of UASp2-RA was achieved using the actinGAL4 or nanosGAL4VP16 drivers (Rørth, 1998) in the *w1118* background. Fertility assays and cuticle preparation were carried out according to Filardo and Ephrussi (Filardo and Ephrussi, 2003).

FRT screen

An X-linked *P{w^{+}mC=lacW}* element was mobilized using *P{ry^{+}t7.2=Delta2-3}* and 1129 lethal P-insertion chromosomes were isolated over a *T(2;3)CyO DTS Ubx SuDCS* compound balancer (M. Erdélyi, A. Guichet, P. Závorszky and A.E., unpublished). Chromosomes bearing lethal mutations (*n*=928) were outcrossed over *Cyo* or *TM3* balancers, respectively, and kept as stocks. The lethal chromosomes were recombined with chromosomes bearing two FRT sites close to the centromere of each chromsome arm: *P{ry^{+}t7.2=neoFRT}40*, *P{w^{+}mW.hs=FRT(w^{hs})}G13* with FRT sites at 40A and 42B for the second chromsome; and *P{FRT(w^{hs})}2A*, *P{neoFRT}82B* with FRT sites at 79DF and 82B for the third chromosome (Chou and Perrimon, 1996). Recombinant chromosomes (*n*=665) were selected based upon resistance to G418 and lethality. Germline clones were generated using X-linked *P{hsFLP}12* and autosomal *P{ovo^{D1}-18}* insertions with the appropriate FRT sites, as described previously (Chou and Perrimon, 1996).

RNA in situ hybridization and immunohistochemistry

Whole-mount antibody staining of ovaries and embryos was performed according to Tomancak et al. (Tomancak et al., 2000), and RNA in situ hybridization of ovaries was carried out according to Filardo and Ephrussi (Filardo and Ephrussi, 2003).

Northern and western blotting

Preparation of ovarian extracts and western blot analysis were perfomed as previously described (Markussen et al., 1995). The following antibodies were used: rabbit anti-Oskar (1:2000) (Vanzo and Ephrussi, 2002); mouse monoclonal anti-α-Tubulin DM1A (Sigma, 1:2000); and rabbit anti-human PKA-R1β (Santa Cruz Biotech, 1:200). The specificity of anti-human PKA-R1β for the *Drosophila* homolog was confirmed by its ability to recognize *Drosophila* RA and RB expressed in bacteria. Extraction of ovarian RNAs and northern blot analysis were carried out as previously described (Castagnetti and Ephrussi, 2003).

EMS mutagenesis

About 200 males of a *w; P{FRT(w^{hs})}2A ru h th st sr e ca* isogenized stock were fed with 1% sucrose containing 40 mM EMS (Sigma) overnight. As the purpose was to isolate new alleles of an existing mutant, we used high concentrations of EMS for the mutagenesis. Flies were allowed to recover by feeding with fresh yeast paste overnight, and were crossed to *w; TM3/TM6b* virgins. In the following generation, single *w; P{FRT(w^{hs})}2A ru h th st sr e ca/TM3* or *TM6b* males were crossed to 4 *w;* 18304/*TM3* virgins. In a first screen, 2,500 lines were tested for semi-lethality in trans to 18304. Females trans-heterozygous for these candidate lines and 18304 were then tested for production of embryos with a bicaudal phenotype, and *e Sb* flies (*P{FRT(w^{hs})}2A ru h th st sr e ca/TM3*) were used for establishing stocks.

Mapping and identification of the 18304 locus

The 18304 bicaudal phenotype was mapped between *st* (73A3) and *P{FRT(w^{hs})}2A* (79D-F) by meiotic recombination. Deficiencies in the region were tested for complementation, and three deficiencies in chromosomal region 77E-78A1, *Df(3L)ri^{-XT106}* *Df(3L)ri^{-XT1}*, and *Df(3L)ME107* failed to complement the 18304 bicaudal phenotype. Although the distal breakpoint of *Df(3L)ri^{-XT106}* was mapped to 77E2-8 and the proximal breakpoint of *Df(3L)ME107* was mapped to 77F3 by analysis of polytene chromosomes (FlyBase), our tests revealed

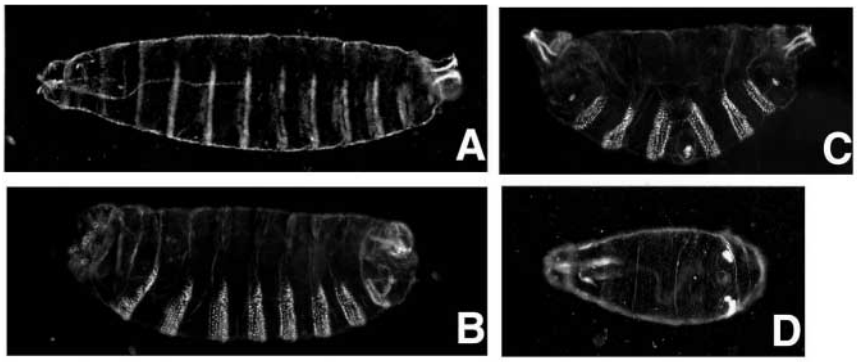

Fig. 1. The 18304 locus is required for the control of *oskar* activity in anteroposterior patterning of the embryo. (A-C) Cuticle preparations of a wild-type embryo (A) and embryos derived from 18304 germline clones (B,C), which show patterning defects ranging from deletion of the head (B), to complete mirror-image duplication of posterior structures (abdominal segments and filzkörper material; C). (D) Embryo derived from 18304, *oskar* double-mutant mother. Such embryos completely lack posterior structures and show the *oskar* single-mutant phenotype.

that they fail to complement the lethality of each other (data not shown). As these two deficiencies failed to complement 18304, we concluded that they have a small overlap in the region 77E2-77F3. To analyze the breakpoints of these deficiencies, first we screened for RFLP markers specific to the 18304 chromosome in the region 77E8-78A1. To identify such RFLPs, a series of genomic fragments in the region were amplified by PCR [about 2 kb of genomic DNA was amplified approximately every 5 kb in the genomic region from DNA of wild-type and 18304/*Df(3L)ri*$^{-XT1}$ (which removes the whole region under analysis) adult flies]. The PCR products were digested with restriction enzymes including *Sau*IIIA1, *Rsa*I and *Hae*III, and fragments showing a different digestion pattern in wild-type and 18304 mutant DNA were identified. To precisely map the breakpoints of *Df(3L)ri*$^{-XT106}$ and *Df(3L)ME107*, genomic fragments containing RFLPs characteristic of 18304 were amplified from 18304/*Df(3L)ri*$^{-XT106}$ and 18304/*Df(3L)ME107* adult flies by PCR, and the products digested with restriction enzymes identifying the polymorphisms. Absence of the genomic region from a deficiency was revealed when only the pattern characteristic of 18304 was obtained, whereas presence of the genomic region in a deficiency was revealed by a mixed pattern of 18304 and wild-type fragments.

Kinase activity assay

Kinase assays were carried out essentially as described by Lane and Kalderon (Lane and Kalderon, 1993). Protein concentration of extracts was determined using a Bio-Rad protein assay kit (Bio-Rad).

Results

The 18304 locus is required for spatial restriction of Oskar protein and anteroposterior patterning of the embryo

To isolate new factors involved in axis formation during *Drosophila* oogenesis, we performed an FRT-based genetic screen for maternal-effect mutants defective in anteroposterior patterning of the embryo. Embryos derived from germline clones of one line, 18304, showed anterior patterning defects ranging from deletion of the head (Fig. 1B) to complete mirror-image duplication of posterior structures (abdominal segments and filzkörper material), the bicaudal phenotype (Fig. 1C, Table 1).

oskar plays a central role in abdomen and germline formation. At the posterior pole, *oskar* assembles the pole plasm, and recruits and activates translation of *nanos* RNA, which encodes the abdominal determinant (Ephrussi et al., 1991; Kim-Ha et al., 1991; Wang and Lehmann, 1991; Gavis and Lehmann, 1994). To address whether 18304 affects *oskar* or downstream factors, flies doubly mutant for 18304 and *oskar* were generated, and the phenotype of their progeny analyzed. Such embryos show a phenotype indistinguishable from that of the *oskar* single mutant (Fig. 1D, Table 1). This indicates that the 18304 locus is required in the germline to control *oskar* activity.

To address which step of *oskar* regulation is affected in the mutant, we first analyzed *oskar* RNA level and localization during oogenesis. Northern blot analysis of ovarian poly(A)$^+$ mRNA revealed no detectable difference in the amount of *oskar* mRNA between wild type and the mutant (Fig. 2I). Examination of the spatial distribution of *oskar* RNA by in situ hybridization showed that *oskar* RNA is localized correctly at the posterior pole, and no appreciable difference between wild type and mutants was observed (Fig. 2A,E). From these results, we conclude that *oskar* RNA level and distribution are not affected in 18304 mutant ovaries. Furthermore, the proper

Table 1. Patterning defects of the 18304 mutant and oskar dependence of the phenotype

Genotype	Wild type	Haed defect	Bicaudal	Posterior group	No cuticle development*
W1118	95	0	0	0	5
18304 germline clone	21	21	29	0	29
18304/*Df(3L)*$^{ri\text{-}XT106}$	12	34	32	0	22
18304/E1	20	25	36	0	19
oskar^{346}/*oskar*^{166}	0	0	0	70	30
18304, *oskar*^{346}/*Df(3L)ME107*, *oskar*^{166}	0	0	0	30	70

The patterning defects of the 18304 mutant in different genetic backgrounds were evaluated by examination of embryonic cuticles and were quantified. More than 200 embryos of each genotype were analyzed. The phenotypes of embryos derived from 18304 in trans to three deficiencies *(Df(3L)ri*$^{-XT106}$, *Df(3L)ri*$^{-XT1}$ and *Df(3L)ME107)* were virtually identical, and the result obtained from *Df(3L)ri*$^{-XT106}$ is shown. The behavior of E1 was indistinguishable from that of the deficiencies, suggesting that E1 is a null allele of the 18304 locus.

*About 30% of *oskar*^{346}/*oskar*^{166} embryos did not develop cuticle, presumably because of second-site mutations accumulated in the stocks. This population was 70% in 18304, *oskar* double mutants. Of the 18304, *oskar* double mutant embryos that developed cuticle, 100% completely lacked an abdomen – the *oskar* phenotype. The suppression of posterior structure formation in the 18304, *oskar* double mutant embryos indicates that the patterning defects in the 18304 mutant are caused by *oskar* activity alone.

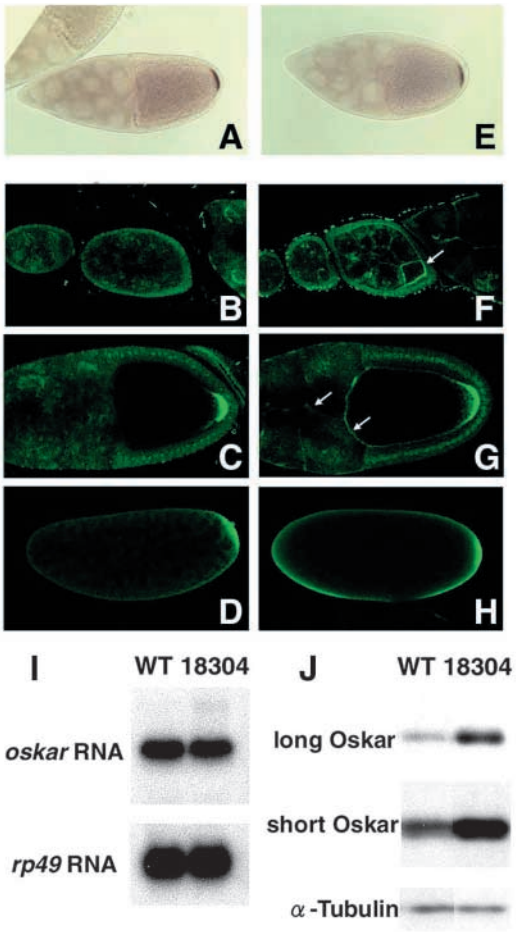

Fig. 2. Oskar protein is ectopically expressed in the 18304 mutant. (A,E) *oskar* RNA localization in stage 10 wild-type and 18304 hemizygote mutant egg chambers revealed by in situ hybridization. *oskar* RNA localizes correctly at the posterior pole in wild-type (A) and 18304 mutant (E) oocytes. (B-D,F-H) Anti-Oskar immunostaining of wild-type and 18304 hemizygous egg chambers and embryos. (B,F) Stage 6 egg chambers of wild-type (B) and 18304 hemizygous (F) females. In the wild type, Oskar protein is not expressed before posterior localization of the RNA and is not detected at this stage (B), whereas it accumulates prematurely (arrow) in 18304 hemizygous oocytes (F). (C,G) Stage 10 egg chambers of wild type (C) and 18304 hemizygotes (G). In the wild type, Oskar protein is detected as a tight crescent at the oocyte posterior pole (C), whereas, in the 18304 mutant, it is observed all around the cortex of nurse cells and the oocyte (arrows), as well as at the posterior pole (G). The signal observed in the nuclei of wild-type and 18304 follicle cells, and in nurse cells is also observed in *oskar* protein-null egg-chambers (data not shown), indicating that it is background. (D,H) 0-2 hour-old embryo of wild type (D) and 18304 hemizygotes (H). In wild-type embryos, Oskar protein is maintained at the posterior pole as a tight crescent (D). In 18304 mutant embryos, Oskar protein is mislocalized all around the cortex, with a higher accumulation at the posterior pole (H). (I) Northern blot analysis of wild-type and 18304 hemizygote ovarian poly(A)$^+$ RNA, probed with an *oskar* cDNA probe (top panel). No difference is detected in *oskar* RNA expression levels between wild type and the 18304 mutant. The blot was re-probed with *rp49* cDNA as a loading control (bottom panel). (J) Western blot analysis of protein extracts of wild-type and 18304 mutant ovaries. Probing with anti-Oskar antiserum reveals that both isoforms of Oskar protein are expressed at higher levels in 18304 than in wild type (top and middle panels). The blot was re-probed with an anti-α-Tubulin antibody as a loading control (bottom panel).

localization of *oskar* RNA also indicates that the intrinsic anteroposterior polarity of the egg chamber is correctly established in the mutants. Dorsoventral polarity also appears normal in the mutant, as no defect in the structure of the eggshells is apparent (Schüpbach, 1987) (data not shown).

We then analyzed Oskar protein localization and abundance in 18304 mutant ovaries. During transport, *oskar* RNA is translationally repressed, and its translation is activated once the RNA is localized at the posterior pole (Kim-Ha et al., 1995; Gunkel et al., 1998). Thus, in wild-type ovaries, Oskar protein is not detected during the early stages of oogenesis (Fig. 2B), until mid-oogenesis, when at stage 9 it accumulates as a tight crescent at the posterior pole (Fig. 2C), where it remains until early embryogenesis (Markussen et al., 1995; Rongo et al., 1995) (Fig. 2D). A clear alteration in Oskar protein distribution is observed in the 18304 mutant, which shows premature accumulation of Oskar protein in stage 6 oocytes, before the onset of *oskar* RNA localization (Fig. 2F). During mid-oogenesis, in addition to its enrichment at the posterior pole, where the RNA is localized, we observe an ectopic accumulation of Oskar protein around the cortex of the oocyte and of the nurse cells (Fig. 2G). In the embryo, a similar ectopic accumulation of the protein all around the cortex, with some enrichment at the posterior pole, is detected (Fig. 2H). To quantitate the amount of Oskar protein in 18304 mutant ovaries, we carried out western blot analysis of ovarian extracts. Both the long and short isoforms of Oskar protein are expressed at significantly higher levels in ovarian extracts of the 18304 mutant than in the wild type (Fig. 2J). These results suggest that premature and ectopic translation of *oskar* is the cause of the posterior-duplication phenotype of 18304 mutant embryos.

18304 encodes *Drosophila Pka-R1*

We mapped the 18304 locus by meiotic recombination to an interval between *st* (73A3) and *P{FRT(w^{hs})}2A* (79D-F) on chromosome 3L. Three deficiencies in chromosomal region 77E-78A1, *Df(3L)ri^{-XT106}*, *Df(3L)ri^{-XT1}* and *Df(3L)ME107* are semi-lethal in trans to 18304, and escaper females produce embryos that display a bicaudal phenotype. We mapped the breakpoint of these deficiencies using RFLP markers specific to the 18304 chromosome (see Materials and methods). A 25 kb candidate domain was determined by the proximal breakpoint of *Df(3L)ri^{-XT106}* and the distal breakpoint of *Df(3L)ME107* (Fig. 3A). In parallel, to characterize the locus, we conducted an EMS screen to isolate new alleles. One line, E1, was identified as an allele of 18304. The behavior of E1 in complementation tests was indistinguishable from deficiencies uncovering the locus, indicating that E1 is a null allele (see Table 1). E1 is lethal over the 3 deficiencies that fail to complement the 18304 maternal-effect phenotype. As 18304 is semi-lethal over these deficiencies, E1 appears to be a stronger allele than 18304 with respect to lethality. About 10% of the eggs derived from E1 germline clones develop into embryos showing patterning defects (head deletion and bicaudal), and many fail to develop a cuticle, suggesting that

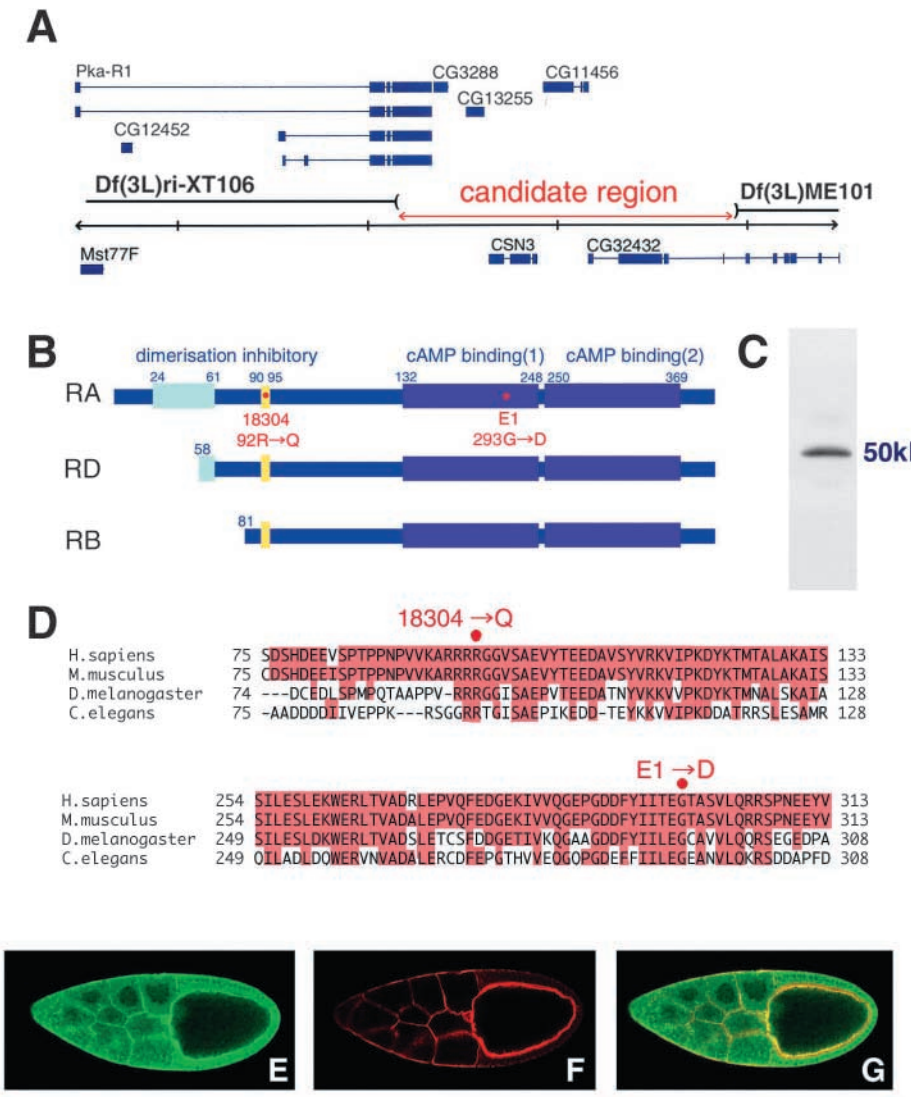

Fig. 3. The 18304 locus encodes *Drosophila Pka-R1*. (A) Diagram of the genomic region defined by the overlap of two deficiencies that fail to complement the 18304 maternal-effect phenotype. The position of the proximal breakpoint of *Df(3L)ri*$^{-XT106}$ and the distal breakpoint of *Df(3L)ME107* (shown in brackets) were identified by RFLPs specific for the 18304 chromosome (see Materials and methods). Six gene candidates for the 18304 locus map to this region: *Pka-R1* (four alternative transcripts are shown), *CG3288*, *CG13255*, *CSN3*, *CG11456* and *CG32432* (Berkeley Drosophila Genome Project). (B) Domain organization of *Drosophila* PKA regulatory subunit type 1. One isoform (RA) comprises a dimerization domain at the N terminus, followed by an inhibitory domain and two cAMP-binding domains. The other two isoforms (RB and RD) lack the dimerization domain. (C) Western blot analysis using human PKA-R1β antibody to reveal the expression of PKA-R1 in the *Drosophila* ovary. A single band of 50 kD is detected in ovarian extracts and corresponds to the size of the RA isoform. (D) Alignment of *D. melanogaster*, *H. sapiens*, *M. musculus* and *C. elegans* PKA-R1. In 18304, a conserved arginine residue in the inhibitory domain is mutated to glutamine and, in E1, a conserved glycine residue is mutated to aspartic acid. (E-G) Subcellular localization of PKA-R1 protein during oogenesis revealed by anti-PKA-R1 immunostaining (E). PKA-R1 is detected in the cytoplasm, with an accumulation at the cell membrane. (F) Rhodamine-conjugated phalloidin reveals the subcortical actin cytoskeleton in the oocyte, nurse cells and follicle cells. (G) Merged image of E and F showing co-localization of PKA-R1 and actin at the cell cortex.

El has an effect on oogenesis in addition to its role in *oskar* regulation. As the bicaudal phenotype was equally penetrant in embryos derived from 18304 germline clones and in 18304 hemizygote embryos, 18304 appears to be a strong, if not a null allele for this phenotype. Sequencing analysis of 18304 and E1 revealed point mutations in *Pka-R1* gene. In both alleles, the mutations are in residues highly conserved among different organisms (Fig. 3D).

The *Pka-R1* locus has been characterized and three protein isoforms annotated by Kalderon and Rubin (Kalderon and Rubin, 1988), and by the Berkeley Drosophila Genome Project. One of these, the RA isoform, has a domain organization similar to its mammalian counterparts. It contains a dimerization domain, which is responsible for homodimer formation and also mediates interaction with the so-called A-kinase anchoring protein. RB and RD have truncations in their N-termini and lack the dimerization domain (Fig. 3B). The maternal-effect bicaudal phenotype of the 18304 and E1 mutants was fully suppressed when a wild-type RA mini-gene was expressed in the germline under the control of the maternal α4-Tubulin promoter (Micklem et al., 1997), or of the Gal4/UAS system driven by pCOG-Gal4 (Rørth, 1998) (data not shown). From these analyses, we conclude that 18304 and E1 are *Pka-R1* mutant alleles, which we will refer to as *Pka-R1*^{18304} and *Pka-R1*^{E1}, respectively.

The Serine/Threonine kinase PKA is a key mediator of the second messenger cAMP in signaling events required for various biological processes. The PKA holoenzyme consists of a cAMP-binding regulatory subunit (R) and a catalytic subunit (C). It is generally described as a hetero-tetrameric complex (R2-C2), which consists of a dimer of two identical regulatory subunits with each subunit bound to a monomeric catalytic subunit. Upon cAMP binding, the regulatory subunits release the catalytic subunits, relieving the active catalytic subunits from inhibition (Taylor et al., 1990). In *Drosophila*, the *DC0* (*Pka-C1* – FlyBase) locus encodes a catalytic subunit that has the highest homology to mammalian PKA, whereas two other genes, *DC1* and *DC2* (*Pka-C2* and *Pka-C3*, respectively – FlyBase), might encode alternative catalytic subunits (Lane and Kalderon, 1993). Two genes encode regulatory subunits

(*Pka-R1* and *Pka-R2* – FlyBase) (Kalderon and Rubin, 1988; Park et al., 2000). Analyses of *DC0* mutants have revealed that this gene is the major source of PKA activity in *Drosophila*, and that PKA is required at various stages for normal growth and development (Lane and Kalderon, 1993), learning (Davis et al., 1995) and behavior (Majercak et al., 1997), and for the control of *hh* signaling (reviewed by Kalderon, 1995). In oogenesis, it has been shown that the *DC0* gene product controls polarization of oocyte microtubules and the integrity of the actin cytoskeleton (Lane and Kalderon, 1993; Lane and Kalderon, 1994). A null mutant of *Pka-R2* has been isolated and characterized. This mutant is viable and fertile, and although adults show defects in behavior, and in formation and maturation of follicle cells in the ovary, no phenotype regarding *oskar* regulation has been observed (Park et al., 2000). In the case of the *Pka-R1* locus, 2 mutant alleles, *7I5* and *11D4*, have been identified. These alleles appear to be hypomorphic, and are viable and fertile, with defects in olfactory learning (Goodwin et al., 1997). These alleles showed no maternal-effect phenotype in trans to $Pka\text{-}R1^{18304}$ or $Pka\text{-}R1^{E1}$, or as hemizygotes (data not shown).

To analyze the expression of PKA-R1 in the *Drosophila* ovary, we performed western blotting using an antibody raised against human PKA-R1β. We detected a single band of 50 kD (Fig. 3C), which is in agreement with what has been proposed to correspond to RA (Goodwin et al., 1997). Consistent with this, bacterially expressed *Drosophila* RA migrates as a 50 kD band on SDS gels, whereas RB and RD migrate as ~40 kD bands (data not shown). Thus it appears that, at the protein level, RA is the only expressed PKA-R1 isoform in the ovary. These observations, and the results of our genetic rescue of *Pka-R1* using RA transgenes, suggest that RA is the functional isoform with regard to the maternal-effect bicaudal phenotype. Subcellular analysis of the distribution of PKA-R1 during oogenesis revealed that PKA-R1 protein is present in the cytoplasm of nurse cells, of the oocyte and of follicle cells, and that the protein accumulates at cell membranes (Fig. 3E-G).

Upregulation of PKA activity in the $Pka\text{-}R1^{18304}$ mutant causes the maternal-effect bicaudal phenotype

To address the effect of $Pka\text{-}R1^{18304}$ on regulation of PKA activity, we carried out kinase activity assays on protein extracts of $Pka\text{-}R1^{18304}$ hemizygotes and wild-type flies. $Pka\text{-}R1^{18304}$ mutant extracts show slightly elevated levels (about 1.2-fold) of PKA activity in the absence of exogenous cAMP, indicating an upregulation of PKA activity in the mutant. The mutant extracts show an increase in PKA activity in response to an addition of exogenous cAMP, and showed a 1.5- to 2-fold elevation in activity compared with wild-type extracts (Fig. 4), revealing an excess of total PKA catalytic subunit activity in the $Pka\text{-}R1^{18304}$ mutant.

To test whether the defect in *oskar* regulation in $Pka\text{-}R1^{18304}$ is indeed due to upregulation of the *DC0* catalytic subunit, we reduced *DC0* dosage by removing one wild-type copy of the gene in the $Pka\text{-}R1^{18304}$ background. Both the maternal-effect bicaudal phenotype and the semi-lethality of $Pka\text{-}R1^{18304}$ were completely suppressed by using two independent loss-of-function *DC0* alleles, *E95* and *H2* (Fig. 5 and data not shown). Thus, the aberrant accumulation of Oskar in $Pka\text{-}R1^{18304}$ mutant oocytes is caused by upregulation of PKA catalytic activity.

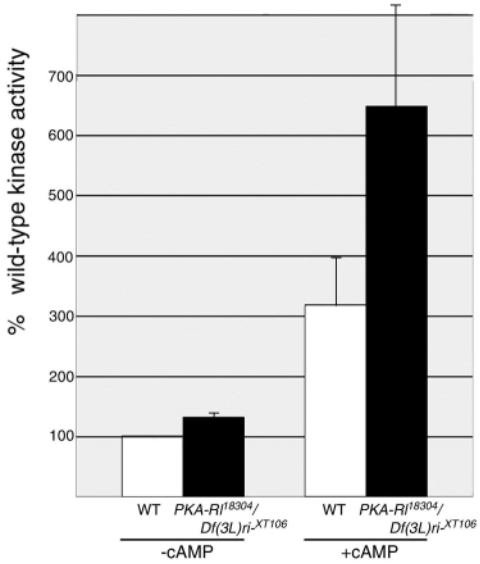

Fig. 4. PKA catalytic activity is increased in $Pka\text{-}R1^{18304}$. PKA activity in extracts of $Pka\text{-}R1^{18304}/Df(3L)ri^{-XT106}$ adult flies was measured in the absence or in the presence of 5 μM cAMP. In the absence of exogenous cAMP, $Pka\text{-}R1^{18304}$ mutant extracts show an approximately 1.2-fold increase in PKA activity over wild type (left panel). In the presence of saturating concentrations of cAMP, mutant extracts show a 1.5- to 2-fold elevation in PKA activity over the wild type, revealing an excess of PKA catalytic subunit activity in the $Pka\text{-}R1^{18304}$ mutant (right panel). This is a summary of assays performed independently three times; bars indicate s.e.m. The differences in PKA activities between wild type and mutant in the presence or absence of exogenous cAMP are statistically significant as determined by a two-sample *t*-test ($P<0.001$).

Overexpression of RA a causes reduction in PKA activity and in Oskar protein accumulation at the posterior pole

The fact that PKA activity above the normal level results in Oskar protein expression outside of the posterior domain indicates that PKA mediates the spatial restriction of Oskar. This suggests that, in wild-type egg chambers, PKA may play a role in *oskar* translational activation at the posterior pole. To address this directly, we wished to analyze Oskar expression in egg chambers in which PKA activity is reduced or abolished. However, direct analysis of Oskar protein regulation in PKA loss-of-function mutants is difficult, because loss of PKA activity results in defects in microtubule polarity in the oocyte and a failure in *oskar* mRNA localization to the posterior pole (Lane and Kalderon, 1994). As *oskar* mRNA translation is repressed before posterior localization of the transcript, this effect of PKA on *oskar* mRNA localization has precluded analysis of the role of PKA on *oskar* translation at the posterior pole. Therefore we sought to reduce PKA activity to a level that does not impair oocyte polarization and *oskar* mRNA localization. We reasoned that this might be achieved by overexpressing PKA-R1, which should promote formation of the inactive holoenzyme complex. When the RA isoform was overexpressed ubiquitously in the fly using an actinGAL4

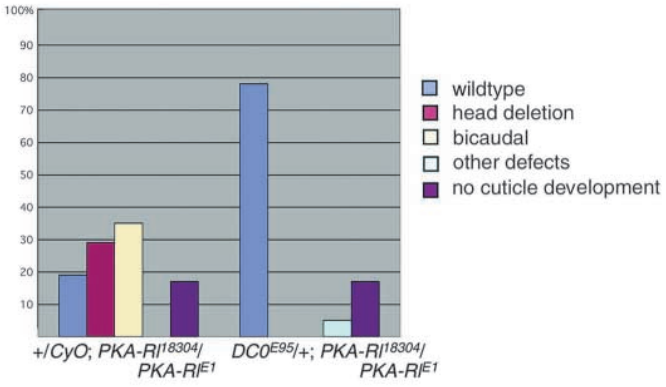

Fig. 5. Mutations in the *Drosophila* PKA catalytic subunit *DC0* suppress the *Pka-R1*^{18304} maternal-effect bicaudal phenotype. The patterning defects of embryos derived from *CyO*/+; *Pka-R1*^{18304}/*Pka-R1*^{E1} (*Pka-R1* single mutant) and *DC0*^{E95}/+; *Pka-R1*^{18304}/*Pka-R1*^{E1} (*Pka-R1*^{18304} mutant lacking one wild-type copy of *DC0*) mothers were evaluated by cuticle analysis. In the *Pka-R1* single mutant, only 18% of the embryos appear wild type, and 65% of the embryos display a phenotype reflecting ectopic posterior patterning activity (29% head deletion, 36% bicaudal). Removal of one wild-type copy of *DC0* completely suppresses this phenotype, and 88% percent of the embryos display a wild-type cuticle pattern. A similar suppression was observed when the *DCO*^{H2} allele was tested.

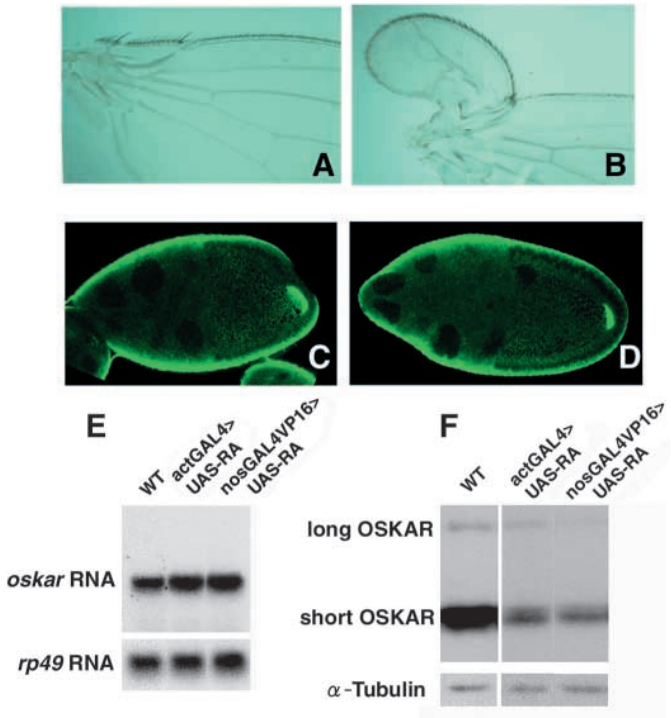

Fig. 6. Overexpression of the RA isoform of PKA-R1 causes a reduction in PKA activity and Oskar protein accumulation. (A) Wild-type adult wing. (B) An ectopic wing outgrowth, which is observed when the RA isoform is overexpressed ubiquitously using an actinGAL4 driver. The penetrance of this phenotype is about 30%. (C,D) *oskar* RNA localization in stage 10 wild-type egg-chambers and in egg chambers in which RA was overexpressed using the actinGAL4 driver, revealed by in situ hybridization. *oskar* RNA localizes correctly at the posterior pole in wild type (C) and in RA-overexpressing oocytes (D). (E) Northern blot analysis of wild-type and 18304 hemizygote ovarian poly(A)$^+$ RNA probed with an *oskar* cDNA probe (top panel). The blot was re-probed with *rp49* cDNA as a loading control (bottom panel), revealing that there was no difference in *oskar* RNA expression levels between wild-type and RA-overexpressing ovaries. (F) Western blot analysis of protein extracts of wild-type and RA-overexpressing ovaries (actinGAL4 and nanosGAL4VP16), probed with anti-Oskar antiserum. Both isoforms of Oskar protein are expressed at significantly lower levels in the extracts of ovaries in which RA is overexpressed (top). The blot was re-probed with anti-α-Tubulin antibody as a loading control (bottom).

driver, a wing outgrowth was observed in about 30% of the animals (Fig. 6B). This phenotype is very similar to that observed in a dominant-negative *DC0* mutant in wings (Pan and Rubin, 1995). This suggests that overexpression of the PKA-R1 RA isoform indeed results in a reduction in PKA catalytic subunit activity in the cell.

We then analyzed the effect of RA overexpression in the germline on oskar regulation, using either the actinGAL4 or nanosGAL4VP16 driver. We first carried out in situ hybridization and observed that overexpression of RA does not cause detectable defects in oocyte polarity or oskar mRNA localization at the posterior pole (Fig. 6C,D). In addition, northern blot analysis revealed no difference in the amount of oskar mRNA in wild-type and R1-overexpressing ovaries (Fig. 6E). We then quantitated by western blot analysis the amount of Oskar protein in RA-overexpressing ovaries. A significant reduction in Oskar protein levels, both the long and short isoforms, is observed in RA-overexpressing ovaries compared with wild type (Fig. 6F). However, abdomen formation is not affected in these embryos and the adults are fertile, indicating that the reduced amount of Oskar in R1-overexpressing oocytes is sufficient for abdomen and germline formation in the embryo. This is consistent with previous observations that very small amounts of Oskar are enough to support abdomen (Erdélyi et al., 1995) and germline (Van Eeden et al., 2001) formation. This result demonstrates that PKA activity is indeed required for accumulation of Oskar in wild-type egg chambers.

Discussion

PKA-R1 is essential during *Drosophila* oogenesis for restriction of Oskar to the posterior pole

Our results reveal that *Pka-R1* is an essential gene in *D. melanogaster* and that it is specifically required during oogenesis for post-transcriptional regulation of *oskar*. PKA-R1 forms a complex with the PKA catalytic subunit and controls its activity in response to cAMP. In *Drosophila*, the *DC0* locus was identified as the catalytic subunit gene with the highest homology to its mammalian counterparts, and it serves as the major source of PKA catalytic activity (Lane and Kalderon, 1993). *DC0* mutant germline clones show defective actin structures in the nurse cells and oocyte, indicating a role of PKA in organization of the actin cytoskeleton. Analysis of *DC0* loss-of-function alleles in the germline has also revealed a requirement for PKA catalytic activity in the establishment of microtubule polarity along the anteroposterior axis of the oocyte (Lane and Kalderon, 1993; Lane and Kalderon, 1994), a prerequisite for *oskar* RNA localization. The effect of loss of *DCO* activity on microtubule polarity has prevented analysis

of the role of PKA in regulation of Oskar protein expression, because *oskar* RNA is translationally repressed before its localization, and translation is not activated if the RNA fails to localize to the posterior pole.

Our analysis demonstrates a requirement for precise modulation of PKA activity in the *Drosophila* germline for correct spatial distribution of Oskar protein. We have shown that, in the *Pka-R1^{18304}* mutant, where PKA activity is upregulated, Oskar protein is overexpressed and accumulates ectopically thoughout the oocyte, although *oskar* RNA localization and levels are normal. Dorsoventral patterning is correctly established. Anterior patterning also appears normal in the mutant, as revealed by the fact that *Pka-R1^{18304}*, *oskar* double mutants develop a normal head and thorax. In addition, we have shown that overexpression of the regulatory subunit causes a modest reduction in PKA activity, without affecting *oskar* RNA localization in the ovary. In such egg chambers, Oskar is underexpressed, suggesting that PKA activity is indeed required for Oskar expression. Taken together, our results suggest that, in addition to its role in the establishment of microtubule polarity and actin cytoskeleton integrity, PKA has a positive role in the regulation of Oskar protein expression at the posterior pole.

Regulation of PKA by PKA-R1 is required during *Drosophila* development

We observe that, in addition to defects in oogenesis, both of our *Pka-R1* alleles have reduced viability, indicating that the control of PKA activity by PKA-R1 is required in several developmental processes. It has been shown that *Pka-R1* is expressed throughout the cell-body layer of the central brain and optic lobes, and strongly accumulates in mushroom bodies (Goodwin et al., 1997). Analysis of hypomorphic alleles has revealed that *Pka-R1* is involved in olfactory learning (Goodwin et al., 1997) and courtship conditioning (O'Dell et al., 1999). The novel *Pka-R1* mutants we have described appear to be stronger alleles, and their analysis should prove useful for investigating the role of *Pka-R1* in brain development and function. In addition, the role of PKA in different process of development has been investigated by making use of mutants in *DC0*, *PKA-R2*, and factors that modulate cAMP levels such as *dunce* and *rutabaga*. For example, it has been demonstrated that PKA antagonizes *hh* signaling by phosphorylating and inactivating the downstream transcription factor Cubitus interruputus (Wang et al., 1999; Price and Kalderon, 1999). In addition, PKA-mediated signaling was shown to be involved in learning and behavior, and drug responses. Regulation of PKA by PKA-R1 is likely to be crucial in these processes as well.

The *Pka-R1^{18304}* mutant phenotype is caused by a defect in PKA repression

The increase in PKA activity in *Pka-R1^{18304}* mutant extracts and the suppression of both the semi-lethality and the maternal-effect bicaudal phenotype by reduction of a functional copy of the *DC0* catalytic subunit reveals that the phenotype of *Pka-R1^{18304}* is due to its failure to repress PKA activity in the mutant. Release of active PKA catalytic subunits from the inactive PKA holoenzyme is controlled by cAMP levels. It is also known that free catalytic subunits are more susceptible to proteolytic degradation than are catalytic subunits in the holoenzyme complex (Park et al., 2000). We observed an excess of PKA catalytic activity both in the absence and the presence of exogenous cAMP in the mutant extract, suggesting that upregulation of PKA catalytic subunit activity in *Pka-R1^{18304}* is due to a defect of mutant PKA-R1 in inhibiting catalytic subunit activity. In addition, the mutant extract still shows an increase in PKA activity in response to cAMP, which suggests the existence of a holoenzyme complex in the mutant. This is likely to be the case, as the point mutation in *Pka-R1^{18304}* is in a conserved arginine in the 'inhibitory domain' that acts as a catalytic unit pseudosubstrate (Gibson et al., 1997). However, it is also possible that an autoregulatory feedback loop controlling expression or stability of catalytic subunits contributes to the increase in total PKA activity.

A PKA-dependent mechanism regulates Oskar expression and anteroposterior patterning

The premature and ectopic accumulation of Oskar in *Pka-R1^{18304}* suggests a role for PKA-R1 in *oskar* localization-dependent translation. An alternative explanation is that PKA-R1 is involved in the control of Oskar protein stability. In the case of *C. elegans*, some germ plasm components are excluded from the somatic cells by cullin-dependent degradation (DeRenzo et al., 2003). A similar process might operate to restrict Oskar to the posterior pole. This assumes the existence of a mechanism whereby precociously and ectopically translated Oskar is degraded, and that this process requires PKA-R1 and its inhibition of PKA activity. However, there is no evidence to date of translation of *oskar* RNA prior to its posterior localization (Markussen et al., 1995; Rongo et al., 1995; Gunkel et al., 1998), or of active degradation of mislocalized Oskar (Ephrussi et al., 1991; Smith et al., 1992; Kim-Ha et al., 1995; Riechmann et al., 2002).

We have previously shown that Oskar degradation is inhibited by phosphorylation (Riechmann et al., 2002). We also showed that both the protection and the degradation machineries operate throughout the oocyte and not just at the posterior pole. As nucleotide substitutions in the 3′UTR of *oskar* lead to its ectopic translation and detectable accumulation (Kim-Ha et al., 1995), under normal circumstances, *oskar* translational regulation seems fairly tight, and is responsible for the specific accumulation of the protein at the posterior. Therefore we speculate that the ectopic accumulation of Oskar in PKA-R1 mutants reflects a role of PKA in activation of *oskar* translation at the posterior pole, and that phosphorylation of translation regulatory proteins by PKA might cause the release of *oskar* mRNA from translational repression outside of the posterior domain.

Lane and Kalderon demonstrated that PKA is involved in the reception of the signal from the posterior follicle cells for the establishment of oocyte polarity at mid-oogenesis, specifically for the destabilization of the posterior microtubule organizing center (Lane and Kalderon, 1994). The signal from the posterior follicle cells might activate PKA at the posterior pole of the oocyte, and this local activation might in turn be responsible for the localized activation of *oskar* expression. It has been shown that the PKA holoenzyme can be targeted to specific subcellular domains by association with A-kinase anchoring proteins (AKAPs) (Huang et al., 1997) (reviewed by Colledge and Scott, 1999), a mechanism that has been proposed to regulate the spatial distribution of PKA activity. It

is tempting to speculate that, in wild-type egg chambers, PKA holoenzyme complexes are targeted to specific AKAPs through PKA-R1, which blocks phosphorylation of specific targets, thus preventing ectopic expression of Oskar outside of the posterior domain. However, an alternative explanation is possible, whereby it is not PKA, but rather a PKA target involved in *oskar* activation that is asymmetrically distributed, with an enrichment at the posterior pole – as is the case for *oskar* mRNA. Uniformly moderate levels of PKA activity throughout the oocyte would result in phosphorylation/activation of the target exclusively at the posterior pole; over-activation of PKA throughout the oocyte would result in activation of the target protein throughout the oocyte, which would be sufficient for ectopic activation of Oskar expression outside of the posterior pole. To address these possibilities, and to address directly the mechanism by which PKA controls the spatial restriction of *oskar*, it will be important to visualize the localization of the kinase activity, and to identify and determine the subcellular localization of the targets of PKA in this process.

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