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Oocyte polarity depends on regulation of *gurken* by Vasa

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SUMMARY

Vasa, a DEAD box mRNA helicase similar to eIF4A, is involved in pole plasm assembly in the *Drosophila* oocyte and appears to regulate translation of *oskar* and *nanos* mRNAs. However, several *vasa* alleles exhibit a wide range of early oogenesis phenotypes. Here we report a detailed analysis of Vasa function during early oogenesis using novel as well as previously identified hypomorphic *vasa* alleles. We find that *vasa* is required for the establishment of both anterior-posterior and dorsal-ventral polarity of the oocyte. The polarity defects of *vasa* mutants appear to be caused by a reduction in the amount of Gurken protein at stages of oogenesis critical for the establishment of polarity. Vasa is required for translation of *gurken* mRNA during early oogenesis and for achieving wild-type levels of *gurken* mRNA expression later in oogenesis. A variety of early oogenesis phenotypes observed in *vasa* ovaries, which cannot be attributed to the defect in *gurken* expression, suggest that *vasa* also affects expression of other mRNAs.

Key words: *vasa, gurken,* Polarity, Translation, Oogenesis

INTRODUCTION

The freshly laid *Drosophila* egg and the developing embryo are polarised along both the anterior-posterior (A/P) and dorsal-ventral (D/V) axes. Distinct structures such as dorsal appendages and anterior micropyle are positioned asymmetrically, reflecting the pattern and polarity within the somatic follicle cell layer that secretes the egg shell. Maternal determinants such as *bicoid (bcd)* at the anterior and *nanos (nos)* at posterior define the A/P body plan of the embryo (reviewed in St Johnston and Nüsslein-Volhard, 1992). The patterning of the follicular epithelium and the polarisation of the oocyte axis by differential localisation of maternal mRNAs is achieved during oogenesis and involves multiple reciprocal communication events between the germline and somatic components of the egg chamber (reviewed by Ray and Schüpbach, 1996). The initial asymmetry is established by the posterior positioning of the oocyte within the egg chamber (Gonzáles-Reyes and St Johnston, 1994). Next, the *gurken/torpedo* (*grk*/*top*) signalling pathway, homologous to the vertebrate TGFα/EGF receptor signalling cascade (Price et al., 1989; Neuman-Silberberg and Schüpbach, 1993), imposes polarity on the follicular epithelium by specifying as posterior the fate of the follicle cells surrounding the asymmetrically positioned oocyte (Gonzáles-Reyes et al., 1995; Roth et al., 1995). The posterior follicle cells are thought to generate a second signal, of unknown nature, directed to the oocyte to polarise its A/P axis (Ruohola et al., 1991; Gonzáles-Reyes and St Johnston, 1994; Gonzáles-Reyes et al., 1995; Roth et al., 1995). Posterior follicle cell signal-dependent disruption of the microtubule organising center (MTOC) at the posterior of the oocyte, and the nucleation of microtubules at the anterior (Theurkauf et al., 1992), result in the reversal of microtubule polarity within the oocyte and differential localisation of maternal mRNAs along its A/P axis. Rearrangement of the microtubules is thought to lead to the translocation of the oocyte nucleus (Gutzeit and Koppa, 1982; Koch and Spitzer, 1983) and the associated *grk* mRNA to the anterior cortex of the oocyte. This movement is asymmetric with respect to the perpendicular D/V axis and defines the polarity of this axis by a second round of *grk/top*-mediated signalling to specify dorsal follicle cell fate (Gonzáles-Reyes et al., 1995; Roth et al., 1995). Later, during embryogenesis, the D/V patterning of the follicular epithelium is translated into D/V polarity of the developing embryo (reviewed in St Johnston and Nüsslein-Volhard, 1992).

Two major features of the establishment of polarity in *Drosophila*, the dependence of the D/V axis on the previous establishment of the A/P axis and the shared *grk/top* signalling pathway necessary for polarisation of both axes explain the finding that many mutations affect both A/P and D/V polarity. The superimposition of A/P and D/V defects often leads to severe abnormalities in oogenesis and the predominance of D/V phenotypes. Therefore, to uncover possible defects in the establishment of A/P polarity, it is important to examine the A/P axis in apparent D/V mutants. One way of achieving this is to analyse the localisation of maternal mRNAs such as *oskar* (*osk*), *grk* and *bcd,* whose distributions have been shown to be significantly altered in A/P mutants (Gonzáles-Reyes et al., 1995; Roth et al., 1995). Taking this approach, we isolated a novel allele of *vasa* (*vas*), which exhibits strong polarity phenotypes.

The *vas* locus codes for a protein belonging to the family of DEAD box RNA helicases that are able to bind and unwind RNA in an ATP-dependent manner (Lasko and Ashburner, 1988; Hay et al., 1988b; Liang et al., 1994). Absence of Vas during embryogenesis leads to abdominal defects and the failure to form pole cells (Schüpbach and Wieschaus, 1986). *vas* has been placed downstream of *osk* in the hierarchy of posterior group genes that are required for pole plasm formation (Hay et al., 1990; Lasko and Ashburner, 1990; Breitwieser et al., 1996). However, there are indications that *vas* is also required upstream of *osk,* for efficient translation of *osk* mRNA (Markussen et al., 1995; Rongo et al., 1995). Recently, it has been demonstrated that translation of the posterior determinant *nos* also requires *vas* activity (Gavis et al., 1996).

Vas protein is detected throughout the development of the germline. Maternal Vas protein is incorporated into the germline precursors, the pole cells, is found in the embryonic gonads and is expressed in the germline during oogenesis. During mid-oogenesis, Vas protein starts to accumulate at the posterior pole of the oocyte, where it participates in pole plasm formation (Hay et al., 1988a; Hay et al., 1990; Lasko and Ashburner, 1990; Liang et al., 1994). This posterior accumulation is mediated by interaction of Vas with Oskar protein (Breitwieser et al., 1996).

Although many alleles of *vas* exhibit oogenesis defects ranging from early arrest of oogenesis to weak ventralisation of the egg shell (Lasko and Ashburner, 1990), the function of *vas* during early oogenesis has not been studied. Here we report a detailed analysis of *vas* oogenesis phenotypes. *vas* is required for a variety of processes during early oogenesis and participates in the establishment of A/P and D/V polarity. We find that the polarity defects in *vas* mutants are due to a reduction in Gurken (Grk) protein levels. Our results suggest that *vas* is required for translation of *grk* mRNA during early oogenesis and for accumulation of *grk* mRNA to wild-type levels later in oogenesis. We propose that *grk* regulation required for the establishment of A/P polarity of the egg chamber is distinct from the regulation of *grk* involved in the establishment of D/V polarity.

MATERIALS AND METHODS

Drosophila strains

The following fly strains were used in this study: OregonR (wild type), *b* vas^{Q7} *pr*/CyO, vas^{PD} *cn bw*/CyO, *b* vas^{D1} *cn*/CyO, *Df*(2L)A267 *b cn bw*/CyO, *b* vas^{Q6} *pr*/ CyO (Tearle and Nüsslein-Volhard, 1987). vas^{15}/SM6 was generated in a P-element mutagenesis screen performed in our laboratory (Erdélyi et al., 1995). Flies were grown at 22°C or 25°C on corn/agar medium supplemented with dry yeast. The wild-type flies used for western analysis were grown on a medium without dry yeast, to ensure that the size and stage distribution of egg chambers in the ovaries would be comparable to those in the underdeveloped ovaries of *vas* mutants.

P-element mapping

5 primers (a-e) spanning the *vas* locus were designed based on the sequence of a genomic contig from the BDGP database. The positions of the primers were chosen such that the maximal distance between the primer pairs on opposite strands is not more than 7 kb, and that the primers, including a primer pointing into the putative *vas* promoter region, cover the whole *vas* locus. PCR reactions were conducted using a P-element-specific primer (pz), together with each of the *vas*-specific primers. The P-element-specific primer is complementary to the P-element-inverted repeats and can be extended only outside of the P element in both directions. The distribution of *vas*-specific primers ensures that the PCR product generated using any P-element-specific primer pair would not be longer than 3.5 kb, which is readily amplifiable in a standard PCR reaction (1 minute 95°C, 1 minute 55°C, 2 minutes 30 seconds 72°C, 35 cycles). We were able to obtain an amplification product only in the case of two primer pairs, pz-a and pz-b. The sum of these two PCR products corresponds to the size of the PCR fragment expected to be generated using the a-b primer pair, suggesting that the P-element inserted between these two primers. The pz-a and pz-b PCR fragments were cloned and sequenced by the EMBL sequencing service.

Primer sequences:
(a) GTTAAAAATGCCACCACCATC;
(b) AAGCTCTCTAATTGCTACAG;
(pz) CGATCGGGACCACCTTATGTTATTTCATCAT.

Western blot analysis

Western blot analysis was performed as described by Markussen et al. (1995). The anti-Vas polyclonal antiserum was raised by injection of full-length recombinant Vas protein, expressed as inclusion bodies in *E. coli*, into rats. The rat anti-Grk polyclonal antibody was a gift of Trudi Schüpbach (Neuman-Silberberg and Schüpbach, 1996). The anti-rat HRP-conjugated secondary antibody (Amersham) was diluted 1:2000. Equal loading of protein on the gel was verified using a mouse anti-tubulin antibody (Sigma).

Competitive PCR amplification

A single ovary containing only non-vitellinogenic stages of oogenesis was dissected in PBT (0.1% Tween), transferred into 10 µl of lysis and binding buffer from Dynabeads mRNA direct extraction kit, and incubated on ice 20 minutes. The sample was then spun for 2 minutes in a table-top centrifuge at full speed and incubated with 10 µl of preconditioned Dynabeads for 5 minutes. The beads were then washed once with wash buffer containing LiDS and twice with wash buffer without LiDS (100 µl each wash). The sample was split into two and each half was incubated for 2 hours at 37°C with 20 µl of RT reaction mix made of 1× first-strand buffer (GibcoBRL), 0.1 mM dNTPs, 10 mM DTT, 1 unit of RNAase inhibitor and 1 µl of reverse transcriptase (GibcoBRL) or 1 µl of water for the RT^- control. The RT reaction was primed from an oligo(dT) anchor immobilised on the beads. For quantification of actin RNA, a 5-fold dilution series of each sample was made and amplified by PCR within the linear range (30 cycles) in the presence of ^{32}P CTP nucleotide. The products were run on an agarose gel, dried, exposed using a phosphorimager screen and quantified by ImageScan software.

For competitive PCR quantitation (Gilliland et al., 1990), a master mix was prepared containing per reaction 1× PCR buffer (Perkin-Elmer), 0.1 mM dNTPs, 2×0.5 µl of 0.1 µg/µl RTgrkU and RTgrkL primers (see below), 5 units of Taq polymerase and 1 µl of RT template. 3-fold dilutions of genomic competitor were added to each reaction and, after PCR amplification (40 cycles, 94°C 45 seconds, 63°C 1 minute, 72°C 1 minute), analysed by agarose gel electrophoresis.

Primer sequences:
(RTgrkU) TGTTACCCACACCGGAGAACCGCCACCTGA;
(RTgrkL) GCGATAATCCGGCACATTGCGGAGCATGAA.

RNA in situ hybridisation

In situ hybridisation was performed as described (Glotzer et al., 1997). RNA probes corresponding to the full-length RNAs were transcribed from plasmids containing *osk* (Ephrussi et al., 1991), *bcd* (Berleth et al., 1988) and *grk* (Neuman-Silberberg and Schüpbach, 1993) cDNAs. Fluorescent *osk* probe was prepared as described in Glotzer et al.

(1997). *bcd* and *grk* antisense probes were labelled using the digoxigenin RNA labelling and detection kit from Boehringer Mannheim.

Double in situ hybridisation for *osk* mRNA and *grk* mRNAs was performed with a digoxigenin-labelled antisense RNA for *grk* and with a biotin-labelled antisense RNA for *osk*.

Whole-mount antibody staining

The polyclonal rabbit anti-Grk antibody was a gift from Siegfried Roth. Secondary antibody was goat anti-rabbit AP-conjugate (Amersham), and was used at 1:1000 to stain the early stages and 1:2500 to stain the late stages of oogenesis.

Analysis of egg chamber morphology

Ovaries from 4-day-old females were dissected in PBT (0.1% Triton), fixed 20 minutes in 4% paraformaldehyde in PBS, washed 10 minutes in PBT, incubated 2.5 hours in PBT (1% Triton) and washed again in PBT (0.1% Triton) for 40 minutes. Ovaries were then incubated with FITC-phalloidin (1:250) in PBT (0.1% Triton) in the dark for 1 hour and 30 minutes. After washing 20 minutes in PBT, RNA was digested by incubation with 0.4 mg/ml RNase in PBT. Ovaries were stained with propidium iodide (50 µg/ml in PBT), washed twice for 10 minutes in PBT and equilibrated overnight in N-propylgallate before mounting.

Egg shell preparation

For egg shell preparation, eggs were collected in a staining basket, rinsed with tap water, fixed in a 1:1 glycerol:acetic acid mixture at 60°C for 2 hours, mounted in Hoyer's medium and incubated at 60°C overnight.

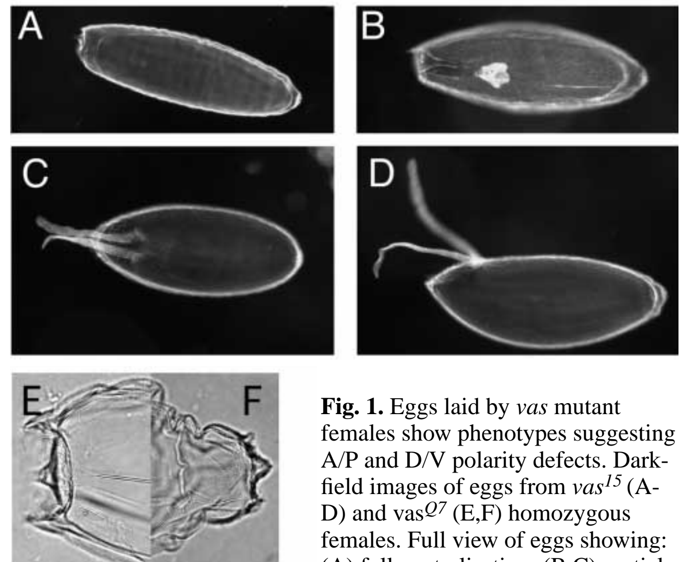

Fig. 1. Eggs laid by *vas* mutant females show phenotypes suggesting A/P and D/V polarity defects. Dark-field images of eggs from vas^{15} (A-D) and vas^{Q7} (E,F) homozygous females. Full view of eggs showing: (A) full ventralisation; (B,C) partial loss of appendage material; (D) wild-type dorsal appendages. Magnified view of an anterior micropyle (E) and of a posterior micropyle (F).

RESULTS

vas is required for proper establishment of A/P and D/V polarity

To identify new components of signalling pathways involved in the establishment of polarity of the *Drosophila* oocyte, we searched a group of P-element-induced maternal effect female-sterile mutations for A/P and D/V polarity defects. D/V defects were assessed by examining the dorsal appendages of eggs laid by homozygous mutant females. A/P polarity defects were revealed by in situ hybridisation to ovaries using *osk* and *grk* mRNAs as probes. The site of P-element insertion in interesting mutants was mapped by in situ hybridisation of a P-element-specific probe to polytene chromosomes.

We identified a mutant that had a P-element insertion at the cytological locus 35C and failed to complement an overlapping deficiency, *Df*(2L)A267. Complementation analysis with previously identified mutants in the 35C region revealed that the mutation is most likely a novel allele of *vas*. We refer to this mutant as vas^{15}.

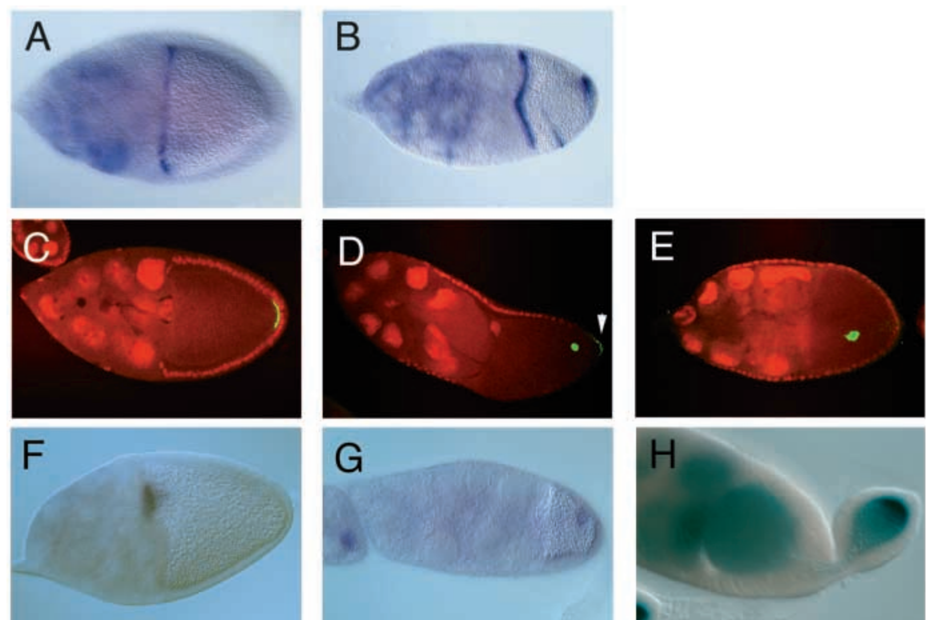

Fig. 2. Aberrant localisation of maternal mRNAs in *vas* mutant oocytes indicates a defect in A/P axis specification. Distribution of *bcd* RNA in wt (A) and vas^{Q7} mutant (B) egg chambers, revealed by RNA in situ hybridisation of digoxigenin-labelled probe and Nomarski optics. (C-E) Confocal images of *osk* mRNA distribution in wt (C) and vas^{15} mutant (D,E), revealed by in situ hybridisation of fluorescently labelled mRNA probe (green). Ovaries were simultaneously stained with DAPI (red) to reveal the overall morphology of the egg chamber. (D) Oocyte showing partial mislocalisation of *osk* mRNA (arrowhead highlights residual *osk* RNA at the posterior pole). (E) Oocyte in which most *osk* mRNA is detected in the middle of the egg chamber. (F,G) Distribution of *grk* mRNA in wild-type (F) and vas^{Q7} mutant (G) oocytes, revealed by in situ hybridisation of digoxigenin-labelled probe and Nomarski optics. In the mutant, *grk* mRNA remains at the posterior of the egg chamber, in an area that coincides with the yolk-free region, and is most likely the oocyte nucleus. (H) vas^{15} mutant egg chambers frequently exhibit a constriction in the middle of the oocyte. The staining at the posterior of the egg chamber represents beta-galactosidase activity produced from a *lacZ* reporter construct that was crossed into the vas^{15} mutant background to reveal the posterior pole of the oocyte.

Females homozygous for *vas*^{15} lay ventralised eggs. Ventralisation of the egg shell is variable and ranges from a complete (Fig. 1A) or partial (Fig. 1B,C) loss of appendage material, to appendages of wild-type appearance (Fig. 1D). Within the ovary, we occasionally observe immature eggs with a micropyle at both the anterior and posterior pole, suggesting a defect in A/P polarity (data not shown). Such eggs are not laid. One of the previously identified alleles of *vas*, *vas*^{Q7} (Tearle and Nüsslein-Volhard, 1987), also exhibits the two-micropyle phenotype, but at a higher frequency (Fig. 1E,F) (Lehmann and Nüsslein-Volhard, 1991).

The formation of a micropyle at the posterior pole of eggs produced by *vas*^{Q7} and *vas*^{15} females indicates that posterior follicle cell fate was not properly established during oogenesis and that the follicle cells at the posterior pole of the egg chamber adopted the default anterior fates. A failure in proper commitment of posterior follicle cells has been shown to cause an abnormal reorganisation of the oocyte cytoskeleton, resulting in a bipolar array of microtubules. Under such circumstances, A/P asymmetry within the oocyte is not established, leading to abnormal localisation of maternal mRNAs (Gonzáles-Reyes et al., 1995; Roth et al., 1995). To determine whether the A/P polarity defects of the follicular epithelium observed in *vas*^{15} and *vas*^{Q7} mutants are accompanied by A/P polarity defects in the oocyte, we performed in situ hybridisations to ovaries using *bcd*, *osk* and *grk* mRNA probes.

bcd mRNA is localised to the anterior cortex in wild-type oocytes at stage 9 (Fig. 2A) (Berleth et al., 1988; St Johnston et al., 1989). In 30% of stage 9 egg chambers of *vas*^{Q7} homozygous mutant ovaries, *bcd* mRNA is found at both the anterior and posterior of the oocyte (Fig. 2B), suggesting a duplication of anterior structures at the posterior pole (Gonzáles-Reyes et al., 1995; Roth et al., 1995). *osk* mRNA, normally localised tightly to the posterior pole of the oocyte (Fig. 2C) (Ephrussi et al., 1991; Kim-Ha et al., 1991), is fully or partially mislocalised to the middle of the developing oocyte in 30% of *vas*^{15} mutant egg chambers, indicating a symmetrical arrangement of the oocyte cytoskeleton (Fig. 2D,E) (Ruohola et al., 1991; Gonzáles-Reyes et al., 1995; Roth et al., 1995). *grk* mRNA is localised together with the oocyte nucleus to the anterior-dorsal corner of stage 7-10 wild-type egg chambers (Fig. 2F) (Neuman-Silberberg and Schüpbach, 1993). In *vas*^{Q7} ovaries, *grk* mRNA and the associated oocyte nucleus occasionally fail to translocate to the anterior dorsal corner of the oocyte and remain at the posterior pole (Fig. 2G). The frequency of *grk* mislocalisation is significantly lower than the frequency of mislocalisation of *osk* and *bcd* mRNAs.

In *vas*^{15} mutant ovaries, we frequently observe stage 10 egg chambers with an abnormal morphology characterised by a constriction close to the posterior pole of the oocyte (Fig. 2H). Similar defects have been described previously in several mutants affecting the establishment of A/P polarity of the egg chamber and are thought to be due to the migration of anteriorised follicle cells (Gonzáles-Reyes et al., 1995; Roth et al., 1995).

Taken together, the A/P and D/V polarity defects of the egg shell, the abnormal localisation pattern of *osk*, *bcd* and *grk* mRNAs, the mispositioning of the oocyte nucleus and the morphological egg chamber defects observed in *vas*^{Q7} and *vas*^{15} mutants indicate that *vas* is required for the establishment of polarity along both body axes.

Establishment of polarity requires wild-type expression of Vas protein early in oogenesis

Since in the case of *vas*^{15} the mutation seemed to be associated with a P-element insertion, we mapped the insertion at the molecular level. An initial BLAST search of the Berkeley Drosophila Genome Project (BDGP) EST database revealed three nearly identical but independent cDNAs homologous to *vas* (accession numbers AA246989, AA392889, AA536466), all of which include a sequence at the 5′ end not present in the published *vas* cDNAs (accession numbers g433675, g158795). By computer analysis of a genomic contig spanning the *vas* locus (P1 clone DS00929), this additional sequence was mapped to the genomic DNA 6.6 kb upstream of the published N-terminal exon of *vas*. (Lasko and Ashburner, 1988; Hay et al., 1988b). The 78 bp long 5′ sequence is joined with the published terminal exon, but the junction is situated 10 bp upstream of the longest published cDNA identified (Lasko and Ashburner, 1988). The splice site matches the consensus on both sides and the extra sequence does not contribute a new start codon, hence the identity of the *vas* ORF is not altered. This indicates that the 5′ sequence identified in the BDGP represents a previously missed non-coding exon of *vas*. By PCR-based primer scanning (Fig. 3A), we found that the P element inserted at the beginning of this 5′ initial exon of *vas* (position 69187 in DS00929). The insertion of a P element at this location should severely affect the expression of *vas*.

Consistent with this assumption, western blot analysis of ovary extracts from females homozygous for *vas*^{15} reveals that the level of Vas protein is strongly reduced (Fig. 3B). We did not observe any novel bands that would represent a truncated version of Vas protein. Conversely, western blot analysis of ovary extract from *vas*^{Q7} failed to reveal any residual 72 kDa Vas protein (Fig. 3B). Instead, a novel band of about 35 kDa is recognised by the anti-Vas antibody. Since sequencing of the *vas*^{Q7} allele failed to reveal any mutation in the *vas* coding sequence (Liang et al., 1994), the truncated Vas protein could be the product of an aberrantly spliced transcript.

We examined the behaviour of *vas*^{15} and *vas*^{Q7} in combination with previously identified *vas* alleles (Table 1). *vas*^{D1} represents a group of *vas* alleles that fail to complete oogenesis and lays no eggs (Lasko and Ashburner, 1990). Combination with *vas*^{D1} (or deficiency *Df*(2L)A267 which uncovers the *vas* locus) causes an increase in severity of the A/P and D/V polarity phenotypes observed in *vas*^{15}, but has no significant effect on the *vas*^{Q7} phenotype. In *vas*^{D1} no Vas protein is detected in the ovaries by whole-mount antibody staining (Lasko and Ashburner, 1990). Hence *vas*^{D1} as well as *Df*(2L)A267 may represent protein nulls. Taken together with the western analysis, these results indicate that in *vas*^{15} the severity of the polarity phenotype correlates with the extent of reduction in the level of Vas protein. They also indicate that the truncated Vas protein present in *vas*^{Q7} lacks the activity required for the establishment of polarity.

vas^{Q6} belongs to a group of eight *vas* alleles in which the lesion has been mapped on a molecular level to the 425 aa region that is highly homologous among DEAD box proteins (Liang et al., 1994). Suprisingly, 46% of the eggs laid by *vas*^{Q6}/*vas*^{Q7} flies have defects in A/P polarity, as indicated by presence of micropyle at the posterior end of the egg, but exhibit only partial or no defects in D/V axis specification, as deduced from the presence of dorsal appendages. This novel

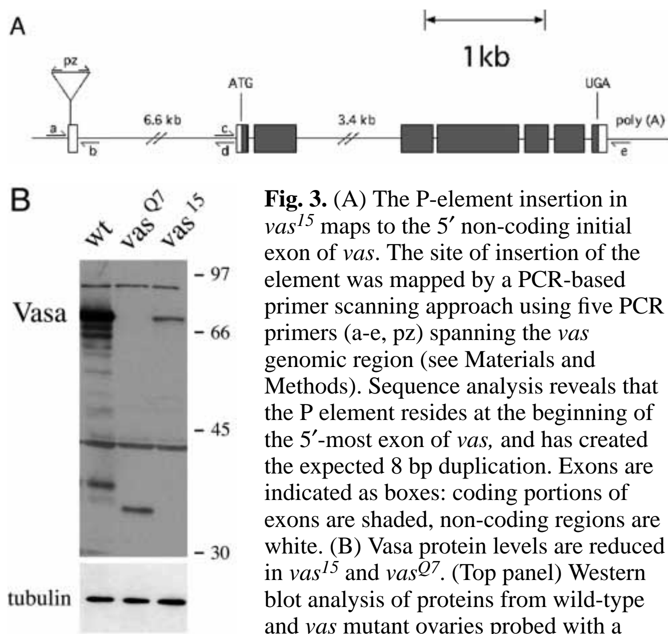

Fig. 3. (A) The P-element insertion in vas^{15} maps to the 5′ non-coding initial exon of *vas*. The site of insertion of the element was mapped by a PCR-based primer scanning approach using five PCR primers (a-e, pz) spanning the *vas* genomic region (see Materials and Methods). Sequence analysis reveals that the P element resides at the beginning of the 5′-most exon of *vas,* and has created the expected 8 bp duplication. Exons are indicated as boxes: coding portions of exons are shaded, non-coding regions are white. (B) Vasa protein levels are reduced in vas^{15} and vas^{Q7}. (Top panel) Western blot analysis of proteins from wild-type and *vas* mutant ovaries probed with a polyclonal anti-Vasa antiserum. The amount of Vasa protein is reduced in vas^{15} mutant ovaries compared to wild type. In extracts from vas^{Q7} ovaries, a novel band of about 35 kDa is detected by the Vas antibody. All exons and small introns of vas^{Q7} have been sequenced and revealed no mutation, ruling out that the 35 kDa protein is a truncated form of Vas resulting from a novel termination codon in *vas*. We cannot exclude the possibility that a truncated version of Vas protein is produced from an aberrantly spliced mRNA caused by insertion of the P element. (Bottom panel) Loading control: same blot stripped and reprobed with a monoclonal anti-tubulin antibody.

phenotype indicates that vas^{Q6} possesses enough Vas activity for the establishment of D/V polarity, but lacks the activity necessary to polarise the A/P axis early during oogenesis. This result also suggests that the D/V axis can be established even when A/P polarity is not specified.

vas^{PD} represents a group of *vas* alleles that do not exhibit any oogenesis defects. When combined with vas^{15}, vas^{Q7} and vas^{Q6}, the oogenesis defects observed in those alleles are rescued by the Vas activity provided by one copy of the vas^{PD} allele. In vas^{PD}, Vas is expressed at wild-type levels in the germarium and during the early stages of oogenesis, but is absent later in oogenesis (Hay et al., 1990; Lasko and Ashburner, 1990), indicating that the critical period when the reduction of Vas activity in vas^{15}, vas^{Q7} and vas^{Q6} causes the observed polarity defects occurs in early oogenesis.

Vas is required for two different aspects of *grk* expression during early and mid-oogenesis

The fact that some *vas* mutations affect both A/P and D/V polarity suggests that Vas protein must be required either for the initial steps in the establishment of A/P polarity, and/or that Vas affects shared components of the pathways establishing A/P and D/V polarity. Since Vas protein is expressed exclusively in the germline (Hay et al., 1988a, 1990; Lasko and Ashburner, 1990), it most likely regulates germline components of the polarity establishment pathways. Since the phenotypes observed in *vas* mutants are reminiscent of the phenotypes described for *grk*, a key germline signalling molecule required for proper establishment of both A/P and D/V polarity (Gonzáles-Reyes et al., 1995; Roth et al., 1995), we examined the expression of Grk protein in ovaries from females homozygous for different *vas* mutations by western blotting.

Grk expression is normal in combinations of *vas* alleles that do not exhibit oogenesis defects (vas^{PD}/vas^{D1}) (Fig. 4A). In *vas* alleles that affect oogenesis (vas^{Q7}, vas^{15}), the amount of Grk protein is strongly reduced (Fig. 4A). The extent of the reduction in Grk protein expression correlates with the reduction of Vas activity in the mutants and with the severity of the A/P and D/V phenotype. It is therefore possible that the A/P and D/V polarity defects observed in *vas* mutants are due to the reduction in Grk protein levels.

In order to investigate the correlation between the polarity phenotypes and the distribution of Grk protein throughout oogenesis, we performed antibody staining on ovaries from different *vas* mutant combinations, using anti-Gurken antibody. In vas^{15}, which exhibits weak A/P and intermediate D/V polarity defects, Grk protein expression is weak during early oogenesis and variable in mid-oogenesis (data not shown). In vas^{Q7}, where the A/P and D/V defects are strong

Table 1. Complementation behaviour of *vas* alleles

	Egg shell phenotype (%)						
Genotype	Wild type	Posterior aeropyle and strong ventralisation*	Posterior aeropyle and weak ventralisation†	Posterior micropyle and strong ventralisation*	Posterior micropyle and weak ventralisation†	Posterior micropyle and no ventralisation defects	*n*
15/15	5	30	65	0	0	0	408
15/Df	5	52	42	2	0	0	505
15/D1	6	44	50	0	0	0	494
15/PD	100	0	0	0	0	0	450
Q7/Q7	2	67	11	20	0	0	320
Q7/Df	0	68	9	23	0	0	364
Q7/D1	0	67	26	7	0	0	357
Q7/PD	100	0	0	0	0	0	350
Q6/Q7	10	3	0	40	23	23	133
Q6/PD	100	0	0	0	0	0	150

Eggs were collected on apple juice plates from more than 40 females and aged for 24 hours at 25°C. Phenotypes were assessed by examination of the egg shells with a dissecting microscope and of egg shell preparations with a Zeiss Axiophot.

*Eggs without any visible dorsal appendage material.

†Eggs with residual dorsal appendage material, single dorsal appendage.

We were not able to recover any vas^{Q6} homozygous females, although, when originally isolated, this allele was homozygous viable.

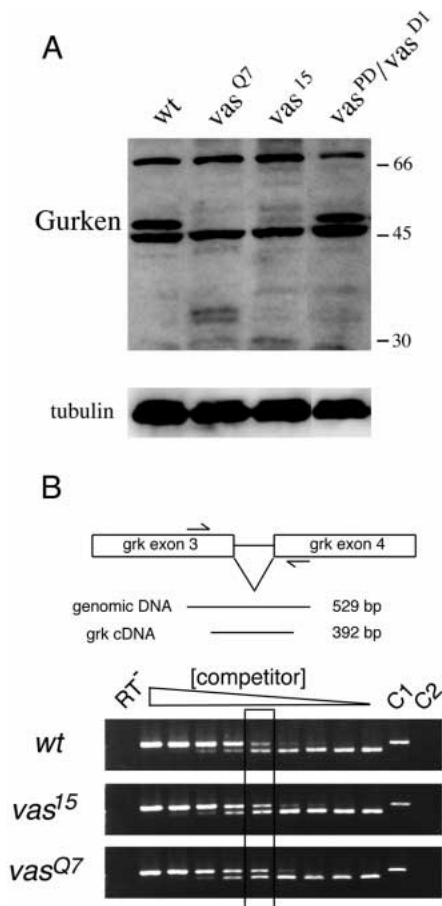

Fig. 4. (A) Gurken protein levels are reduced in *vas* mutants that show A/P and D/V polarity defects. (Top panel) Western blot analysis of proteins from wild-type and *vas* mutant ovaries probed with a polyclonal anti-Gurken antiserum. We observe a reproducible subtle difference between the amount of Gurken protein present in *vas*^{15} and *vas*^{Q7} mutant ovaries. In the *vas*^{PD}/*vas*^{D1} trans-heterozygous combination of alleles, Grk protein level appears wild type. The strong band present just below the Grk-specific band is most likely due to crossreactivity of the antibody with an abundant yolk protein. (Bottom panel) Loading control: same blot stripped and reprobed with a monoclonal anti-tubulin antibody. (B) Competitive RT-PCR analysis shows that levels of *grk* mRNA are not affected during the early stages of oogenesis. Poly(A) mRNA for each sample was isolated from a single young nonvitellinogenic ovary, reverse transcribed into a first strand of cDNA, as described in the Materials and Methods section. The mRNA levels were normalised by linear range hot PCR, using actin primers (data not shown). The competitive PCR assay was performed using a pair of primers which amplify a 392 bp fragment from the *grk* cDNA, but a 529 bp fragment from the *grk* genomic clone (top part of the figure). The products were analysed by agarose gel electrophoresis. The RT^{-} lane shows the amplification products from an aliquot of poly(A) mRNA that was subjected to the RT reaction, but in the absence of reverse transcriptase. Ten different concentrations of competitor genomic DNA are shown; the difference between each lane is 3-fold. Lane C1 shows amplification of genomic competitor alone; C2 is a PCR control with no template added. Box indicates dilutions where the concentration of competitor and *grk* transcript amplification are approximately the same, as judged by the intensity of the ethidium bromide staining of the two bands, and comparison with the adjacent bands. The minor differences in this lane between the wild-type and mutant samples indicates that the difference in mRNA levels is less than three-fold, which is consistent with the actin amplification results (data not shown).

(Fig. 5B), Grk protein is absent throughout oogenesis (Fig. 5L,M). In *vas*^{Q6}/*vas*^{Q7} egg chambers, which exhibit strong A/P but weak D/V polarity defects (Fig. 5C), Grk is never detected during early oogenesis (Fig. 5N) but Grk expression is normal in 51% of the egg chambers at mid-oogenesis (Fig. 5O). Taken together, these results indicate that the various polarity defects in *vas* mutants parallel the defects in Grk protein expression during relevant stages of oogenesis. We conclude that *vas* participates in the establishment of oocyte polarity by regulating expression of Grk.

To understand the way in which *vas* affects *grk* expression, we examined the distribution of *grk* mRNA in *vas* mutant ovaries. In *vas*^{Q7}, *grk* mRNA appears wild type during the early stages of oogenesis but disappears at stage 9/10 (Fig. 5F,G). Since Grk protein is not detected in this mutant (Fig. 5L,M), it seems that *vas* affects *grk* expression on two distinct levels. During early oogenesis the presence of *grk* RNA, but absence of the protein, indicates a defect in translation of *grk* mRNA. At mid-oogenesis, *vas* appears to be required for the accumulation of *grk* mRNA to wild-type levels.

To rule out the possibility that *grk* mRNA levels are also affected during early oogenesis, we quantified the amount of *grk* mRNA from young non-vitellinogenic *vas*^{15} and *vas*^{Q7} mutant ovaries, by competitive PCR (Fig. 4B). We observe no significant difference between the *grk* RNA levels in wild-type and in *vas* mutant ovaries. Since the level of Grk protein is severely reduced in the mutants, we propose that *vas* is required for efficient translation of *grk* mRNA during early oogenesis.

In *vas*^{Q6}/*vas*^{Q7} egg chambers, *grk* mRNA expression during early oogenesis is indistinguishable from the wild type (Fig. 5H); however, Grk protein is not detected (Fig. 5N). During mid-oogenesis, *grk* mRNA is distributed along the anterior cortex in 47% (Fig. 5I) and correctly localised to an anterodorsal location in 51% (data not shown) of *vas*^{Q6}/*vas*^{Q7} egg chambers. During these later stages, Grk protein is detected (Fig. 5O). Taken together with the expression of *grk* in *vas*^{Q7} homozygous egg chambers, these results indicate that *vas*^{Q6} lacks the activity required for translation of *grk* mRNA during early oogenesis, but possesses the activity necessary for *grk* expression during mid-oogenesis.

Vas is required for a variety of processes during early oogenesis

vas^{Q7} and *vas*^{15} mutant ovaries are significantly smaller than wild-type ovaries and exhibit various structural defects. To characterise those defects more precisely, we examined the morphology of mutant egg chambers.

Previous analysis of *vas* mutants reported the apparent degeneration of some ovarioles during mid-oogenesis (Lasko and Ashburner, 1990; Schüpbach and Wieschaus, 1991). We observe similar defects in *vas*^{15}, in which some egg chambers of stage 10 and older lose most of the normal morphological characteristics and appear as amorphic clusters (data not shown).

In wild-type ovaries, the oocyte is positioned posterior to the nurse cells (Spradling, 1993). In 5% of *vas*^{15} egg chambers, the oocyte is in the middle or on the side, surrounded by nurse cells (Fig. 6A). This suggests that Vas is required for the positioning of the oocyte at the posterior of the egg chamber.

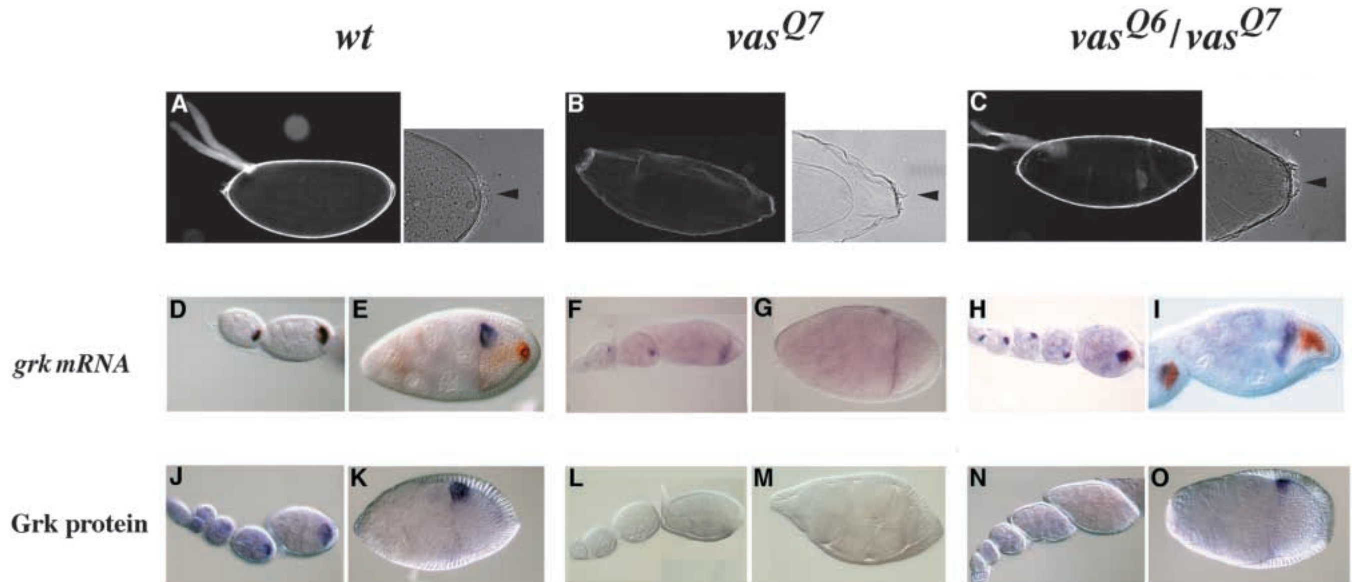

Fig. 5. Polarity defects in *vas* mutants correlate with defects in *grk* expression. (A) Lateral view of a wild-type egg, showing the two dorsal appendages and a magnification of the posterior aeropyle (arrowhead). (B) Lateral view of a completely ventralised egg laid by a vas^{Q7} mutant female, showing the absence of dorsal appendages and a magnification of an ectopic posterior micropyle (arrowhead). (C) Lateral view of an egg laid by a vas^{Q6}/vas^{Q7}mutant female, showing dorsal appendages and an ectopic posterior micropyle (arrowhead). (D,E) *grk* mRNA (blue) and *osk* mRNA (brown) in wild-type egg chambers. (D) *grk* and *osk* mRNAs accumulate in the growing oocyte at stages 5 and 6. (E) *grk* mRNA localised to an anterior-dorsal corner and *osk* mRNA localised to the posterior pole of a stage 9 oocyte. (F,G) *grk* mRNA in vas^{Q7} egg chambers. (F) *grk* accumulates in the growing oocyte at stages 5 and 6. (G) *grk* mRNA is not detected in the stage 9 oocyte. (H,I) *grk* mRNA (blue) and *osk* mRNA (brown) in vas^{Q6}/vas^{Q7} egg chambers. (H) *grk* and *osk* mRNAs accumulate in the growing oocyte at stages 5 and 6. (I) *grk* mRNA is mislocalised along the anterior cortex in 20% of stage 9 oocytes. *grk* mRNA is localised to an anterior-dorsal corner in 51% of oocytes (data not shown) and *osk* mRNA is mislocalised to the middle of the oocyte. (J,K) Grk protein distribution in wild-type egg chambers. (J) Grk accumulates in the growing oocyte at stages 5 and 6. (K) Grk localised to an anterior-dorsal corner in stage 9 oocytes. (L,M) Grk is not detected in vas^{Q7} egg chambers. (N,O) Grk in vas^{Q6}/vas^{Q7} egg chambers. (N) Grk is not detected in oocytes at stages 5 and 6. (O) Grk is localised to an anterior-dorsal corner in stage 9 oocytes in 51% of the egg chambers. In 9% of egg chambers, Grk is found at the posterior, associated with the oocyte nucleus (data not shown).

grk mRNA has been shown to accumulate in the oocyte and thus serves as an oocyte marker (Neuman-Silberberg and Schüpbach, 1993). In vas^{Q7}, at a relatively low frequency, in situ hybridisation with *grk* mRNA probe reveals the presence of two oocytes (Fig. 6B). Frequently egg chambers containing two oocytes are immediately adjacent to egg chambers that lack an oocyte and contain a reduced number of nurse cells. A similar phenotype has been observed in *egghead* and *brainiac* mutants (Goode et al., 1996), and can be explained by the

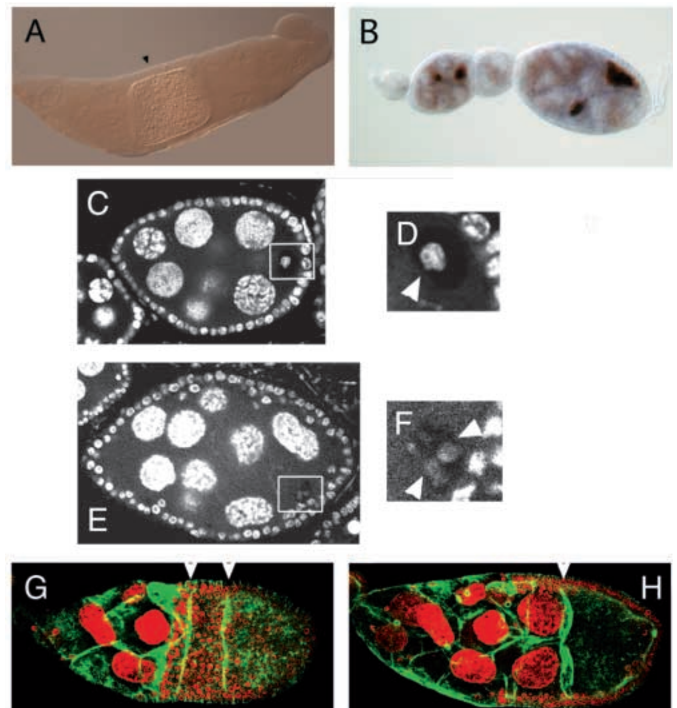

Fig. 6. Early oogenesis defects displayed by *vas* mutants. (A,B) Nomarski images of (A) a fixed unstained vas^{15} egg chamber exhibiting mispositioning of the oocyte (arrowhead) and (B) a vas^{Q7}/vas^{Q6} ovariole containing alternate egg chambers containing either two oocytes or no oocyte. (C-F) Confocal images of propidium iodide-stained wild-type (C,D) and vas^{Q7} (E,F) ovaries. In the wild-type oocyte nucleus, DNA appears as a single compact dot (C,D, arrowhead). In the vas^{Q7} oocyte nucleus, DNA is fragmented (E,F, arrowheads). (G,H) Confocal images of vas^{Q7} egg chambers double-labelled with rhodamine-conjugated phalloidin and propidium iodide. (G) Projection of 20 optical sections to emphasise the position of the columnar follicle cells (red dots) with respect to the two anterior cortical actin rings (arrowheads). (H) A single focal plane from the same confocal series as G, emphasising the border between the nurse cells and the oocyte. The border coincides with the position of the anterior actin cortical ring (arrowhead).

abnormal migration of follicle cells between encapsulated cysts in the germarium. Interestingly, such a two-oocyte phenotype has been observed at a similarly low frequency in *grk* mutant egg chambers (Neuman-Silberberg and Schüpbach, 1993).

vas mutant ovaries exhibit two additional phenotypes. In wild-type oocytes, the DNA of the oocyte nucleus condenses as a karyosome which appears as a compact, single, white dot within the dark area delimiting the oocyte nucleus (Fig. 6C,D). We have noted that, in *vas* mutants, the karyosome is often fragmented and appears as two or three fuzzy spots within the oocyte nucleus (Fig. 6E,F). A similar phenotype has been observed in *spindle* (*spn*) mutants (González-Reyes et al., 1997).

At stage 10 of oogenesis, wild-type egg chambers adopt a characteristic appearance where the oocyte is as large as the nurse cell cluster (Cummings and King, 1970). The border between the oocyte and the nurse cells coincides with the border between the columnar and the squamous follicle cells and appears to overlap with a cortical ring of actin at the anterior margin of the oocyte. In *vas* mutant egg chambers of the same stage, the oocyte appears smaller than the nurse cells (Fig. 6H) and the columnar follicle cell epithelium covers roughly half of the egg chamber, surrounding not only the oocyte, but also a portion of the adjacent nurse cells (Fig. 6G,H). Optical sectioning of such an egg chamber reveals two anterior cortical actin rings (Fig. 6G, arrows). The first ring coincides with the border between the columnar and squamous follicle cells and is positioned at approximately half of the length of the egg chamber, as in the wild type. The second ring is positioned more posteriorly and coincides with the apparent border between the nurse cells and the oocyte, at approximately two thirds of the length of the egg chamber. These observations suggest that the size of the oocyte and its association with the columnar follicle cells are normal, and that the observed topological defect in the *vas* mutants is due to the pushing of the nurse cells towards the oocyte. Such a defect is also observed in *BicD* mutant ovaries (Swan and Suter, 1996).

DISCUSSION

Vas participates in the establishment of A/P and D/V polarity by regulating *grk* expression

The analysis of a newly identified mutation in the *vas* locus, together with the detailed characterisation of previously identified *vas* alleles, has revealed a novel function of *vas*. Ventralisation of the egg shell, formation of a posterior micropyle and the aberrant localisation pattern of *bcd*, *osk* and *grk* mRNAs observed in *vas* mutants indicates that *vas* is required for the establishment of both A/P and D/V polarity.

The strong correlation between the polarity phenotypes observed in *vas* mutants and the defects in Grk protein levels at relevant stages of oogenesis suggest that *vas* is required for regulation of Grk expression. The defects imposed by reduction of *vas* activity upon *grk* expression can be divided into two categories. During early stages of oogenesis, when *grk*-mediated signalling is responsible for the establishment of posterior follicle cell fate and subsequent polarisation of the A/P axis (Gonzáles-Reyes et al., 1995; Roth et al., 1995), *vas* appears to affect translation of *grk* mRNA. However, later during oogenesis, when localised *grk* activity is required to specify dorsal follicle cell fate and thus polarise the D/V axis (Schüpbach, 1987; Neuman-Silberberg and Schüpbach, 1993), absence of *vas* causes a reduction in *grk* mRNA levels.

The role of *vas* during the later phase of *grk* expression is not clear. Vas could directly affect some aspect of *grk* mRNA processing, such as splicing or mRNA stability. However, the fact that the vas^{PD} mutation, which lacks detectable *vas* activity during mid-oogenesis (Hay et al., 1990; Lasko and Ashburner, 1990), is able to rescue the mid-oogenesis D/V polarity defects caused by stronger *vas* mutations, suggests that the effect of *vas* on *grk* mRNA expression at this stage is an indirect consequence of a disruption of earlier oogenesis processes. One possibility would be that fragmentation of the oocyte nucleus results in an inability to express or maintain wild-type *grk* RNA expression or localisation during mid-oogenesis. Alternatively, Vas could be required during early oogenesis for the expression of factors regulating *grk* mRNA transcription or stability during mid-oogenesis. It is also possible that the instability of *grk* mRNA is a consequence of the failure to translate the mRNA. However, this seems unlikely since, in vas^{Q6}/vas^{Q7} ovaries, Grk translation is affected only during the early stages, suggesting that Vas is not required for translation during mid-oogenesis.

Recently a group of six *spn* mutants has been implicated in translational regulation of *grk* mRNA (González-Reyes et al., 1997). *spn* mutants share some features with *vas* mutants described in this study. Both *vas* and *spn* affect the process of oocyte positioning, exhibit fragmentation of the oocyte nucleus and affect expression of Grk protein and distribution of *grk* mRNA (González-Reyes et al., 1997). However there are also several differences. *spnA,B,C,D,* unlike *vas,* only weakly affect the polarisation of the A/P axis when the oocyte is correctly positioned at the posterior of the egg chamber (González-Reyes et al., 1997). Furthermore, in those *spn* mutants, *grk* mRNA is present during mid-oogenesis but is not translated (González-Reyes et al., 1997), whereas in *vas, grk* mRNA is either absent *(vas^{Q7})* or expressed and translated *(vas^{Q6}/vas^{Q7})*.

Taken together, these data suggest that, during early oogenesis, *vas* is required, whereas *spn* activity is partially dispensable, for *grk* translation. During mid-oogenesis, *grk* mRNA localisation around the oocyte nucleus requires both *vas* and *spn*; however, only *spn* activity is required for translation of *grk* mRNA. Therefore *grk* expression appears to be differentially regulated during the establishment of A/P and D/V polarity. This hypothesis is supported by the fact that several mutations such as *K10* and *squid* (*sqd*) (Wieschaus et al., 1978; Kelley, 1993) affect only *grk*-dependent D/V patterning processes, whereas vas^{Q6}/vas^{Q7} predominantly affects the establishment A/P polarity.

Interestingly, *spnE* (*homeless*) encodes a protein that belongs to the same protein family of DEAD box RNA helicases as *vas* (Gillespie and Berg, 1995). *spnE,* unlike other *spn* mutants, is critical for the establishment of the A/P axis once the oocyte is posteriorly localised within the egg chamber. As in *vas* mutants, *grk* transcript is frequently absent or mislocalised in stage 10 *spnE* egg chambers (Gillespie and Berg, 1995). Overall, *spnE* appears to be more similar to *vas* than to *spn* mutants. The *spn* mutations may therefore be divided into two groups, one including *spnA,B,C,D,* which mainly affects the late phase of *grk* expression, and the other comprising helicases *spnE* and *vas,* which are required for both the early and late phases of *grk* expression.

The egg shell phenotypes and the pattern of *grk* expression in vas^{Q6}/vas^{Q7} ovaries, as well as the low frequency of mislocalisation of the oocyte nucleus in vas^{Q7} and vas^{15}, indicates that D/V polarity can be established even when proper A/P axis specification has failed to occur. The partial mislocalisation of *osk* mRNA to the middle of vas^{15} oocytes indicates that the organisation of the oocyte cytoskeleton is only partially aberrant in *vas* mutants. It is therefore possible that, in this mutant, the organisation of the microtubules allows wild-type localisation of the oocyte nucleus, but interferes with localisation of maternal mRNAs. This suggests that the process of nuclear migration is less sensitive to reductions in Grk signalling than is mRNA localisation. This conclusion is supported by the observation of similar discrepancies between the frequency of mislocalisation of mRNAs and of the nucleus in hypomorphic *top* mutants (Gonzáles-Reyes et al., 1995; Roth et al., 1995).

Vas as a translational regulator of *grk*

In this study, we propose that during early oogenesis *vas* may be required for *grk* mRNA translation. Our conclusion is based on the fact that, in early stage egg chambers, *grk* mRNA levels are normal, but levels of Grk protein are reduced below the threshold of detectability by antibody staining in situ and by western blotting.

During early and mid-oogenesis, *grk* mRNA is tightly localised to a specific subcompartment of the oocyte (Neuman-Silberberg and Schüpbach, 1993), ensuring the proper spatial and temporal specificity of Grk-mediated signalling processes. It has been demonstrated that mislocalisation of *grk* mRNA and the resulting misplacement of Grk patterning activity causes severe defects in follicular cell fate specification and ultimately leads to embryonic lethality (Neuman-Silberberg and Schüpbach, 1993; Christerson and McKearin, 1994; Roth and Schüpbach, 1994). Therefore, tight regulation of the expression of Grk protein within the egg chamber is critical. Although the temporal and spatial regulation of *grk* activity appears to be largely achieved by *grk* mRNA localisation, a mechanism must exist that ensures that *grk* mRNA is translated only in the oocyte and at the proper location within this cell. In the case of *osk* mRNA, it has been demonstrated that translational repression prior to localisation is mediated by the RNA-binding protein Bruno (Bru) (Kim-Ha et al., 1995). Bru recognises distinct RNA elements called Bruno Response Elements (BRE) in the 3′UTR of the *osk* transcript (Kim-Ha et al., 1995). Although we cannot rule out that the effect of Vas on Grk expression is indirect, the fact that the 3′UTR of *grk* mRNA contains at least one BRE that can bind Bru protein (Kim-Ha et al., 1995) and that Bru interacts with Vas (Webster et al., 1997) raises the possibility that Vas and Bru cooperate in translational regulation of *grk* mRNA. It has also been shown that, in *aubergine* mutants, which have A/P and D/V defects (Schüpbach and Wieschaus, 1991), translation of both *osk* and *grk* mRNAs is severely reduced. This further supports the notion that some components of the machinery for translational regulation of *osk* and *grk* are shared (Wilson et al., 1996).

Vas is a DEAD box RNA helicase similar to eukaryotic eIF4A (Lasko and Ashburner, 1988; Hay et al., 1988b) and has been shown to be required for efficient translation of *osk* and *nos* mRNAs (Markussen et al., 1995, Rongo et al., 1995, Gavis et al., 1996). Our data suggest that *grk* is a third maternal mRNA that requires Vas for its translation. Since even a combination of deficiencies that removes the *vas* locus, and thus represents a true null mutation, allows oogenesis to proceed until stage 6 (Lasko and Ashburner, 1990), it is clear that *vas* is not absolutely required for translation of all mRNAs in the oocyte. Nonetheless, the pleiotropy of *vas* phenotypes suggests that many Vas target mRNAs are yet to be identified. So far Vas has not been shown to possess RNA-binding specificity in vitro (Liang et al., 1994), but it does specifically interact with proteins such as Osk and Bru (Breitwieser et al., 1996; Webster et al., 1997). It is therefore possible that Vas recognises its target mRNAs by interaction with the proteins that associate with those mRNAs. Given the ubiquitous expression of *vas* in the germline, it is unlikely that Vas acts as the factor responsible for differential translation of many different mRNAs. Rather Vas may represent a germline-specific translational activator that provides a link between the general translational machinery and the specific regulatory proteins associated with different mRNAs, resulting in temporally and spatially regulated translation.

vas is required for many aspects of germline development

Our analysis of egg chamber morphology in *vas* mutant ovaries reveals that *vas* is required not only for the establishment of polarity, but also for proper encapsulation of the oocyte by the follicular epithelium, for positioning of the oocyte within the egg chamber, for the integrity of the oocyte nucleus and for the completion of oogenesis. Vas is required for the formation of the pole cells and is expressed in the germline throughout embryonic, larval and pupal development of both the male and the female germline (Hay et al., 1990; Lasko and Ashburner, 1990). Hence, *vas* seems to be required for most aspects of germline development. RNA helicases similar to *vas* are expressed specifically in the germline of other species such as *Caenorhabditis elegans* (Roussell and Bennett, 1993), *Xenopus laevis* (Komiya et al., 1994), *Danio rerio* (Olsen et al., 1997; Yoon et al., 1997) and *Mus musculus* (Fujiwara et al., 1994), suggesting that the requirement for *vas* in germline development is conserved throughout the animal kingdom. Since *vas* acts at least in part through translational regulation of different mRNAs in the germline, one possibility is that *vas* may represent a factor that imposes germline specificity on the translation machinery.

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