vasa is required for GURKEN accumulation in the oocyte, and is involved in oocyte differentiation and germline cyst development

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SUMMARY

The *Drosophila* gene *vasa* is required for pole plasm assembly and function, and also for completion of oogenesis. To investigate the role of *vasa* in oocyte development, we generated a new null mutation of *vasa*, which deletes the entire coding region. Analysis of *vasa*-null ovaries revealed that the gene is involved in the growth of germline cysts. In *vasa*-null ovaries, germaria are atrophied, and contain far fewer developing cysts than do wild-type germaria; a phenotype similar to, but less severe than, that of a null *nanos* allele. The null mutant also revealed roles for *vasa* in oocyte differentiation, anterior-posterior egg chamber patterning, and dorsal-ventral follicle patterning, in addition to its better-characterized functions in posterior embryonic patterning and pole cell specification. The anterior-posterior and dorsal-ventral patterning phenotypes resemble those observed in *gurken* mutants. *vasa*-null oocytes fail to efficiently accumulate many localized RNAs, such as *Bicaudal-D*, *orb*, *oskar*, and *nanos*, but still accumulate *gurken* RNA. However, GRK accumulation in the oocyte is severely reduced in the absence of *vasa* function, suggesting a function for VASA in activating *gurken* translation in wild-type ovaries.

Key words: *Drosophila*, RNA localization, axis patterning, *vasa* (*vas*), oogenesis

INTRODUCTION

Segregation of the germline from the soma is a central feature of animal development. In *Drosophila*, the germline is determined through the activities of maternally expressed RNAs and proteins which colocalize in the pole plasm at the posterior pole of the egg (reviewed by Rongo and Lehmann, 1996). Pole cells, the progenitors of the germline, form very early in embryogenesis, then, beginning at gastrulation, they migrate into the interior of the embryo and ultimately associate with the gonadal mesoderm to form the embryonic gonads (reviewed by Williamson and Lehmann, 1996). Beginning in larval development, germ cells proliferate and differentiate in order to carry out spermatogenesis and oogenesis; among the structures assembled during oogenesis is new pole plasm, which specifies the germline for the subsequent generation of individuals.

Genetic and molecular studies have identified numerous genes which are required for pole plasm assembly and subsequent posterior segment specification and germ cell formation; many of these genes are expressed during oogenesis and produce mRNAs and/or proteins which localize in pole plasm or in polar granules, specialized organelles contained within the pole plasm (reviewed by Rongo and Lehmann, 1996). Analysis of the expression of these genes supports an early hypothesis (Mahowald, 1968) that translational control is a major mechanism regulating *Drosophila* germline

development. The product of the *vasa* (*vas*) gene, a DEAD-box-family protein which is localized in polar granules and which shares the enzymatic functions of the translation initiation factor eIF4A (Hay et al., 1988; Lasko and Ashburner, 1988; Liang et al., 1994), is a candidate germline-specific translational regulator. For instance, levels of the short isoform of OSKAR protein (OSK), a molecule central to pole plasm assembly (Ephrussi et al., 1991; Kim-Ha et al., 1991; Ephrussi and Lehmann, 1992), are greatly reduced in *vas* mutant ovaries (Markussen et al., 1995; Rongo et al., 1995). Another pole plasm mRNA whose translation may be activated by VAS is *nanos* (*nos*), as *nos* RNA carrying an intact translational regulation element in its 3′UTR is completely inactive in embryos derived from *vas* mutant ovaries (Gavis et al., 1996; Dahanukar and Wharton, 1996).

While the activities of pole plasm components such as VAS have been most thoroughly studied with respect to their function in pole cell formation and specification of the posterior soma, clearly some genes involved in pole plasm assembly also function in other stages of germline development. For instance, females homozygous for either of two strong *nos* alleles exhibit defects in germ cell proliferation (Lehmann and Nüsslein-Volhard, 1991; Wang et al., 1994). Furthermore, pole cells lacking maternal *nos* function fail to complete migration and do not associate with the embryonic gonadal mesoderm (Kobayashi et al., 1996), indicating a role for *nos* in the transition from pole cell to functional germ cell.

Similarly, various *vas* alleles have defects in oogenesis and lay few or no eggs (Lasko and Ashburner, 1988, 1990; Lehmann and Nüsslein-Volhard, 1991; Schüpbach and Wieschaus, 1991). Females *trans*-heterozygous for *Df(2L)A267* and *Df(2L)TE116-GW18*, two large deletion mutations which both include *vas*, were reported to be blocked in early vitellogenic stages of oogenesis (Lasko and Ashburner, 1988). Analysis of whether this phenotype was caused solely by loss of *vas* function has been confounded by the fact that these *trans*-heterozygous deficiency lines are haploid for a large number of genes, but that, aside from large deficiencies, a clearly null allele of *vas* did not exist. Four EMS-induced alleles of *vas*, *vasD1*, *vasQ6*, *vasQ7* and *vasD5*, also lead to greatly reduced fertility, with many egg chambers blocked as for the *trans*-heterozygous deficiency females (Lehmann and Nüsslein-Volhard, 1991). The few eggs produced by females homozygous for these alleles often lack dorsal appendages and have the micropyle, a specialized vitelline membrane structure normally found only at the anterior of the egg, duplicated at the posterior (Lehmann and Nüsslein-Volhard, 1991). Again, whether these phenotypes represent the results of a complete loss of *vas* function is unknown. *vasQ6* and *vasD5* are missense mutations which alter single amino acids of VAS and both alleles produce substantial amounts of mutant protein (Liang et al., 1994), so neither of these mutations is likely to be null. For *vasD1* and *vasQ7* the molecular nature of the mutation is unknown, but the *vas* coding region is unaffected in these mutant alleles.

In this paper, we have used a new *vas* null allele, *vasPH165*, a small deletion which we generated by imprecise P-element excision, to investigate in detail the role of *vas* in events of oogenesis prior to pole plasm assembly. We found that abrogation of *vas* function results in defects in many aspects of oogenesis including control of cystocyte divisions, oocyte differentiation, and specification of posterior and dorsal follicle cell-derived structures. Furthermore, *vasPH165* oocytes only weakly concentrate many oocyte-localized RNAs, although some oocyte-specific molecules, including *gurken* (*grk*; Schüpbach, 1987; Neuman-Silberberg and Schüpbach, 1993) RNA, remain concentrated in the oocyte in *vas* mutant ovaries. However, in the case of *grk*, translation is severely reduced in the absence of *vas* function. This provides evidence that VAS is involved in translational control mechanisms operating in early stages of oogenesis.

MATERIALS AND METHODS

Fly stocks

To create a null allele of *vas*, excision lines were generated through the introduction of the *Δ2-3* transposase source into *vasP(ry[+])LYG2* (Rittenhouse and Berg, 1995). To do this, *+/Y*; *vasP(ry[+])LYG2 cn*; *ry506* males were crossed to *w*; *Bic-DPA66 Su(Bic-DPA66) cn/CyO*; *Δ2-3Sb/TM3 Ser* virgin females. *w/Y*; *vasP(ry[+])LYG2 cn/CyO*; *Δ2-3Sb/ry506* males were then crossed to *+/+*; *l(2)05084P(ry[+]l(2)05084)/CyO*; *ry506* virgin females. Individual *ry* F[1] males representing excisions of *vasP(ry[+])LYG2* were then individually crossed to *l(2)05084P(ry[+]l(2)05084)/CyO*; *ry506* virgin females. Balanced *ry* stocks were generated and females homozygous for an excision chromosome were crossed to Oregon R males to test for fertility. Excision lines were screened for deletions through Southern analysis using a 1.9-kb *Eco*RI genomic fragment from the *vas* first intron, and which includes

the *vasP(ry[+])LYG2* insertion site, as a probe. VAS protein levels from excision lines were determined by western analysis.

vasP(ry[+])LYG2 was provided by Celeste Berg (University of Washington, Seattle); *l(2)05084P(ry[+]l(2)05084)/CyO* was provided by the Berkeley *Drosophila* Genome Project; *nosRC*, which disrupts a splice donor site and is null for RNA and protein expression (Wang et al., 1994; Curtis et al., 1997) was received from Takahiro Akiyama (Azabu University, Kanagawa, Japan); and *grkHK36*, a strong *grk* allele (Schüpbach, 1987) was obtained from Trudi Schüpbach (Princeton University). The wild-type strain employed was Oregon R.

In situ hybridization and antibody staining

In situ hybridizations with DIG-labeled RNA probes and antibody stainings were carried out on ovaries and embryos as described by Kobayashi et al. (1998), except that DMSO was omitted from the fixation solution used for ovaries. Primary antibodies were used at the following dilutions: α-ORB, 1:20; α-BIC-D, 1:10; α-NOS, 1:150; α-GRK, 1:3000; α-ADD-87, 1:20. α-NOS was pre-adsorbed with embryos from *nosBN* females, and α-GRK was preadsorbed with *grkHK36* ovaries. For bright-field microscopy, antibody stainings were detected with DAB, enhanced using the Vectastain ABC or ABC Elite kits (Vector Laboratories) and biotinylated secondary antibodies. For confocal microscopy, antibody stainings were detected using Texas Red-conjugated secondary antibodies (Molecular Probes). β-gal staining of *khc:lacZ* ovaries was carried out as described by Clark et al. (1994).

OliGreen/Texas Red-phalloidin staining

Ovaries were dissected in PBS and fixed for 20 minutes in a mixture of 600 μl heptane, 200 μl 4% paraformaldehyde in PBS + 0.2% Tween-20, and 20 μl DMSO. Following fixation, samples were rinsed 3 times with PBT (PBS + 0.1% Tween-20), incubated for 1 hour in RNase A (100 mg/ml), and rinsed again 3 times with PBT. Ovaries were incubated for 1 hour in OliGreen (Molecular Probes, 1:1000 dilution) and Texas Red-phalloidin (Molecular Probes), washed with PBT, and mounted in 70% glycerol in PBS.

RESULTS

vasPH165 is a *vas* null allele

Identification of additional *vas* cDNA clones has indicated the presence of a 127-bp exon upstream of the previously reported 5′ end of *vas*, which extends the 5′ UTR of the gene. This exon is separated by a large intron of 6603 bp from the remainder of the gene (Fig. 1A,B; Berkeley *Drosophila* Genome Project, unpublished results, submitted to GenBank under accession numbers L81347, L81348, L81449, AC000466, and AC000469). *vasLYG2* is a P-element-induced *vas* allele (Rittenhouse and Berg, 1995), which we mapped to the large first intron of *vas* (Fig. 1A), and which produces about 2% of the wild-type level of VAS (Fig. 1C), a level essentially undetectable in tissue staining experiments. Despite the very low level of VAS in *vasLYG2* ovaries its phenotype is hypomorphic; oogenesis in *vasLYG2* females is less severely compromised than in *Df(2L)A267/Df(2L)TE116-GW18* flies, and *vasLYG2* females lay numerous eggs. This suggested to us either that trace amounts of VAS are sufficient for oogenesis to often proceed to completion, or that the more severe phenotype observed in the double-deficiency lines results from the effects of a reduction in dosage of a second gene which enhances the *vas* phenotype.

To distinguish between these possibilities, it was necessary to obtain a molecularly characterizable null *vas* allele. For this

Fig. 1. (A) Organization of the *vas* gene. Exons (boxes) are numbered with Roman numerals (I-VIII), the translational start codon is in exon II; E, *Eco*RI site. The site of the *P{ry+, vasLYG2}* insertion is indicated, and the insertion site is immediately after position 75581 in the BDGP P1 clone DS00929 (GenBank accession number AC002502). *vasPH165* results from an imprecise excision of *P{ry+, vasLYG2}*, in which 7343 bp of genomic DNA, including the entire *vas* coding region, are deleted, and replaced with 16 bp from the P element (sequence highlighted). (B) The 5′ UTR of *vas*. The first 127 nucleotides make up exon I, the 6603-bp first intron follows after nucleotide 127 (solid triangle) and nucleotide 128 corresponds to nucleotide 76 as reported in Lasko and Ashburner (1988). Nucleotides 151-153 are the initiator ATG (underlined). (C; top panel) Western blot probed with α-VAS antiserum to compare the levels of VAS in Oregon R, *vasLYG2* and *vasPH165* ovarian extracts. Extracts were loaded in various amounts as indicated at the top of the figure. (Bottom panel) The same blot probed with α-eIF4A antiserum (Lavoie, 1995) as a loading control.
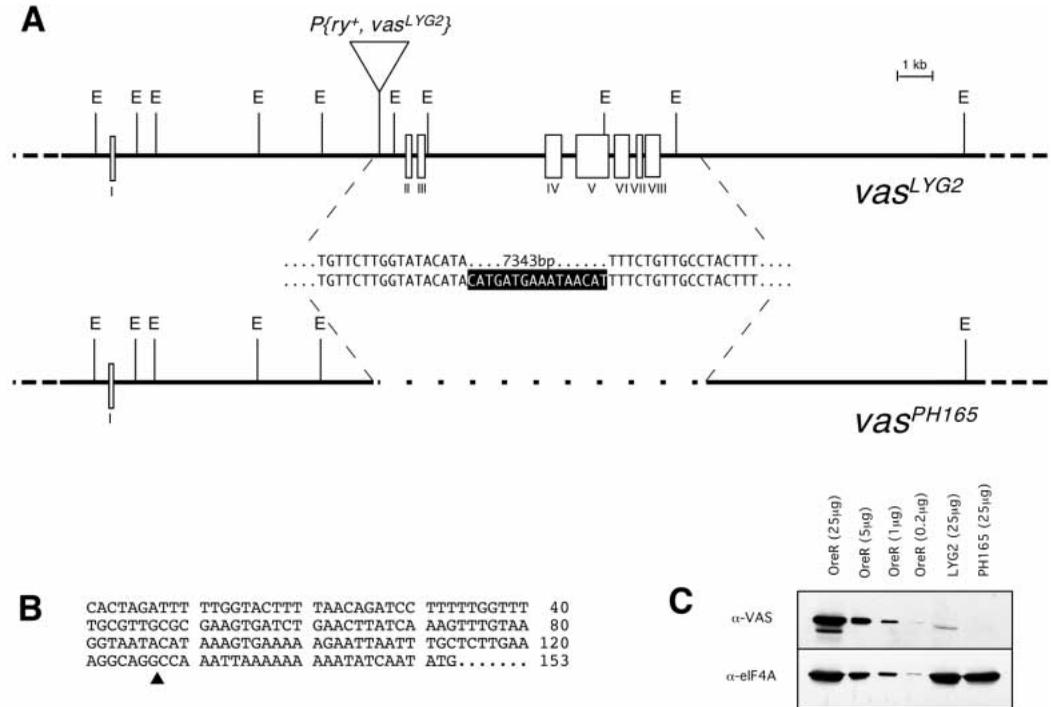
purpose, we generated a series of derivative lines by mobilizing the P element in *vasLYG2*. Out of 201 excision events, stable lines were generated from 129. Of these, 119 caused reversion to a wild-type phenotype, indicating that the *vasLYG2* phenotype results solely from the P-element insertion, and that the *vasLYG2* chromosome is free of other female-sterile mutations. Three other derivatives were recessive lethal, and seven, although having excised the *ry+* marker on the P element of *vasLYG2*, remained defective in oogenesis. These were checked by PCR and Southern blots to determine whether they carried a deletion confined to the *vas* gene, and by western blots using anti-VAS antiserum to determine whether they expressed VAS. From these analyses, one line, *vasPH165*, was identified, which produced no detectable VAS protein and carried a 7343-bp deletion which removes the entire coding region of *vas* (Fig. 1A,C). We confirmed the breakpoints of *vasPH165* by nucleotide sequencing, comparing the mutant sequence to wild-type sequence provided by the Berkeley *Drosophila* Genome Project. From the nature of the *vasPH165* mutation and the fact that no VAS protein is detectable on western blots even on long overexposures (Fig. 1C), we conclude that *vasPH165* is null for VAS. The *vasPH165* deletion is mostly limited to *vas*, as one of its breakpoints lies within *vas* and the other is 1270 bp downstream of its 3′ end. A nested gene may be located within the 3.5-kb third intron of *vas*, which is deleted in *vasPH165*, as a 3-kb transcript present throughout all developmental stages is detected on northern blots using genomic probes including this intron but not with *vas* cDNA probes (Lasko and Ashburner, 1988). However, any gene other than *vas* which may be disrupted in *vasPH165* is almost certainly irrelevant to the discussion below, as a *vas*-GFP transgene, constructed from a *vas* cDNA fused to the *vas* promoter, and therefore lacking any nested or 3′-flanking genes,

rescues *vasPH165* homozygotes, and *Df(2L)A267/ Df(2L)TE116-GW18* flies to fertility. The phenotypes of *vasPH165/vasPH165*, *vasPH165/ Df(2L)A267*, and *Df(2L)A267/ Df(2L)TE116-GW18* ovaries are essentially identical.

VAS is involved in the maintenance of germline cysts

Upon cursory examination of *vasPH165* ovaries under the light microscope, we noticed an obvious atrophy of the germaria as compared with the wild-type, suggesting that fewer germ cells were present. To investigate this more closely, we used an antibody recognizing ADD-87 protein (Lin et al., 1994; Zaccai and Lipshitz, 1996) which stains spectrosomes and fusomes, specific structures present in stem cells, cystoblasts and dividing cystocyte clusters. In *vasPH165* ovaries, a reduction in the number of ADD-87-staining structures, and therefore a reduction in the number of developing cysts, is readily apparent as compared with wild-type (Fig. 2A-D). This phenotype has high expressivity, and increases in severity with the age of the female (Fig. 2C-D). In germaria from 7-day-old *vasPH165* females, region 1 frequently consists of only a few stem cells and developing cysts. Posterior to these there is often what appears to be an extended interfollicular stalk (compare Fig. 2D with Fig. 2C), likely formed by follicle cells in the absence of cystocyte clusters. This suggests that the cystocyte clusters remaining in the germarium have ceased to develop further and have degenerated, while the follicle cells continue to divide.

Two other genes that function in the same pathway as *vas* in posterior patterning, *nos* and *pum*, have also been implicated in germ cell proliferation (Lehmann and Nüsslein-Volhard, 1991; Wang et al., 1994; Lin and Spradling, 1997), raising the possibility that they also may be interacting with *vas* in the
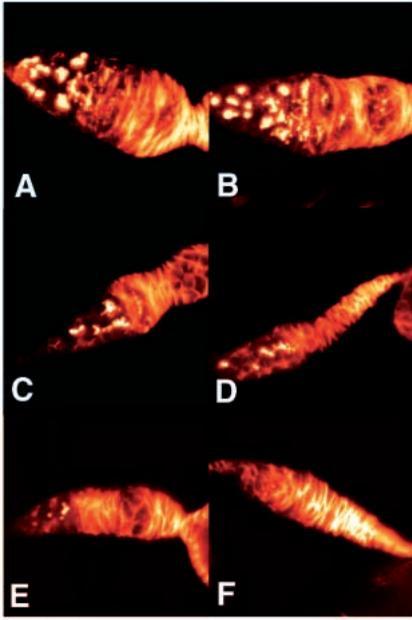
Fig. 2. Confocal micrographs of germaria from (A) wild-type, (B) grk^{HK36}/grk^{HK36}, (C) 4-day-old vas^{PH165}/vas^{PH165}, (D) 7-day-old vas^{PH165}/vas^{PH165}, and (E,F) 1- to 2-day old nos^{RC}/nos^{RC} ovaries stained for the ADD-87 protein, which marks spectrosome and fusome structures and which is diagnostic for stem cells and developing germline cysts. Substantially fewer foci of ADD-87 are observed in all vas^{PH165} or nos^{RC} germaria as compared with the wild-type or with grk^{HK36}. Similar phenotypes were observed in $vas^{PH165}/Df(2L)A267$ ovaries.

germarium. *pum* mutants produce ovarioles which contain only two or three clusters of undifferentiated germ cells which lack spectrosome/fusome structures or ring canals (Lin and Spradling, 1997), a phenotype we never observed in *vas* mutants. Upon examination of nos^{RC} mutant ovaries with the α-ADD-87 antibody, we observed a phenotype which appears similar, but somewhat more severe, than that of vas^{PH165}. Many nos^{RC} ovarioles consist of a germarium with one to three cysts (Fig. 2E), followed by an extended stalk and one to three normal-looking egg chambers. In more extreme cases, only remnants of spectrosome/fusome material can be detected in the anterior of the germarium (Fig. 2F), consistent with the conclusion that these germline cells have arrested development.

VAS is involved in oocyte differentiation

At a low frequency (aprox. 1% for each), we observed defects in germline differentiation and oocyte determination in vas^{PH165} ovaries, including tumorous egg chambers (Fig. 3A), egg chambers with 16 nurse cells and no oocyte, others with two oocytes, and again others with a mislocalized oocyte (Fig. 3B-D). Far more frequently the normal 15 nurse cells and one oocyte are present; however, by at least two criteria the oocytes produced in vas^{PH165} egg chambers are not fully differentiated. In wild-type development, the nurse cell nuclei endoreplicate during pre-vitellogenic oogenesis and become highly polyploid, whereas the oocyte nucleus remains diploid and condenses into a tight karyosome (Mahowald and Kambysellis, 1980). However, in vas^{PH165} the oocyte nucleus appears more diffuse than does the wild-type oocyte nucleus (Fig. 3E,F).
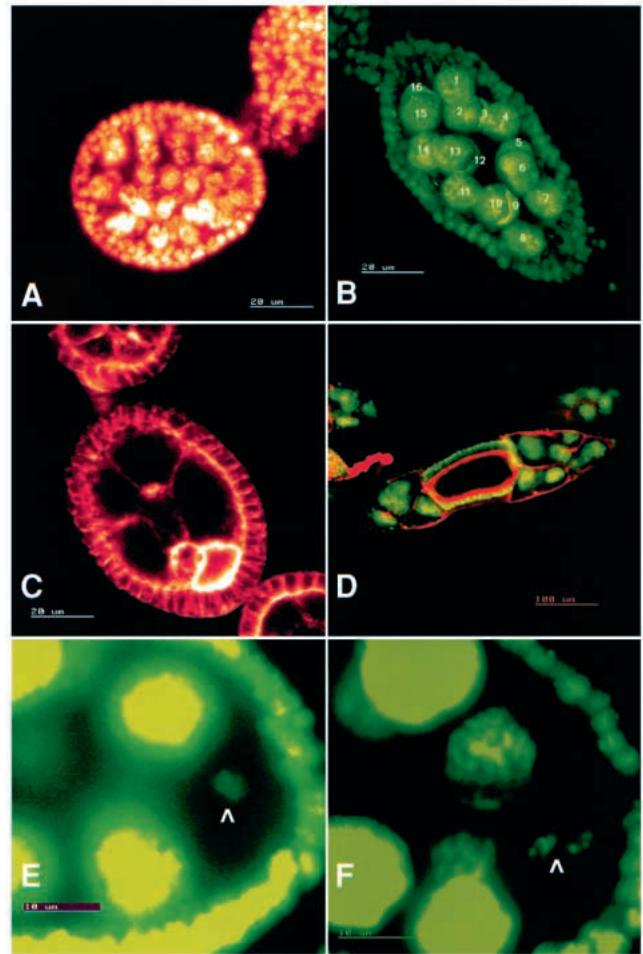

Fig. 3. Phenotypes of *vas*-null egg chambers affecting oocyte determination. Confocal micrographs of (A-B) vas^{PH165}/vas^{PH165} ovaries stained with the nuclear dye Oli-Green (Molecular Probes), illustrating the following phenotypes: (A) tumorous germline cyst, (B) 16 nurse cells and no oocyte. The 16 polyploid nuclei are numbered in this panel. (C) vas^{PH165}/vas^{PH165} egg chamber with two oocytes, stained for F-actin with Texas Red-phalloidin (Molecular Probes). (D) $vas^{PH165}/Df(2L)A267$ egg chamber doubly stained with Oli-Green and with Texas Red-phalloidin, illustrating a bipolar egg chamber with its oocyte in the center. We also observed bipolar egg chambers in vas^{PH165}/vas^{PH165} ovaries. (E,F) Higher-magnification view of oocyte nuclei stained with Oli-Green; (E) wild-type, (F) vas^{PH165}/vas^{PH165}. In each of these panels, an arrowhead points to the oocyte nucleus; note the more diffuse staining in F as compared with E, apparent in almost all vas^{PH165} egg chambers. Similar phenotypes were observed in $vas^{PH165}/Df(2L)A267$ ovaries. Scale bars 20 μm (A-C), 100 μm (D), 10 μm (E,F).

This would be consistent either with a failure to form the karyosome structure, perhaps involving a premature meiotic arrest in diplotene rather than in metaphase, or an increase in ploidy in vas^{PH165} oocytes. A very similar nuclear morphology has been observed in *spindle* (*spn*) mutant oocytes, which has been interpreted as resulting from a delay in oocyte determination (González-Reyes et al., 1997).

Secondly, vas^{PH165} oocytes do not efficiently accumulate at least four oocyte-localized RNAs. In wild-type ovaries, the *Bicaudal-D*, *orb*, *osk* and *nos* RNAs all accumulate efficiently in the oocyte within the germarium and remain concentrated

Fig. 4. Localization of various RNAs and proteins in wild-type and *vas^PH165* ovaries. Micrographs of wild-type ovaries are arranged on the left of the vertical bar, and micrographs of mutant ovaries are arranged on the right. (A-C) In situ hybridizations illustrating the distribution of *Bic-D* RNA in (A,B) wild-type and (C) *vas^PH165/vas^PH165* ovaries. Localized signal is virtually absent in the *vas* mutant. (D-F) In situ hybridizations illustrating the distribution of *orb* RNA in (D,E) wild-type and (F) *vas^PH165/vas^PH165* ovaries. Localized signal is much fainter in the *vas* mutant. (G,H) Ovaries from (G) wild-type and (H) *vas^PH165/vas^PH165* females stained with an antiserum recognizing ORB protein. Despite the weak localization of *orb* RNA to the oocyte in the *vas* mutant, ORB protein is abundant and oocyte-specific from about stage 5. In earlier stages of oogenesis, ORB staining is much more diffuse in the *vas* mutant than in the wild-type. (I-L) In situ hybridizations illustrating the distribution of *osk* RNA in (I,J) wild-type and (K,L) *vas^PH165/vas^PH165* ovaries. Accumulation of *osk* RNA in the oocyte is greatly reduced in the *vas* mutant, and diffuse signal is often apparent in the center of egg chambers (K). Weak localization to the oocyte is also frequently observed (L). (M-O) In situ hybridizations illustrating the distribution of *nos* RNA in (M) wild-type and (N,O) *vas^PH165/vas^PH165* ovaries. The distribution of *nos* RNA in *vas* mutant egg chambers is far more diffuse than in the wild-type, but a higher concentration of *nos* RNA in the oocyte than in the nurse cells is usually apparent. (P,Q) Ovaries from (P) wild-type and (Q) *vas^PH165/vas^PH165* females stained with an antiserum recognizing NOS protein. The peak of NOS expression in developing cysts at the posterior of germarium region 1 (Wang et al., 1994) is apparent in both the wild-type and mutant ovaries (arrowheads). For each probe and antiserum presented, the control and *vas^PH165/vas^PH165* stainings were processed in parallel, and color reactions proceeded for identical lengths of time.
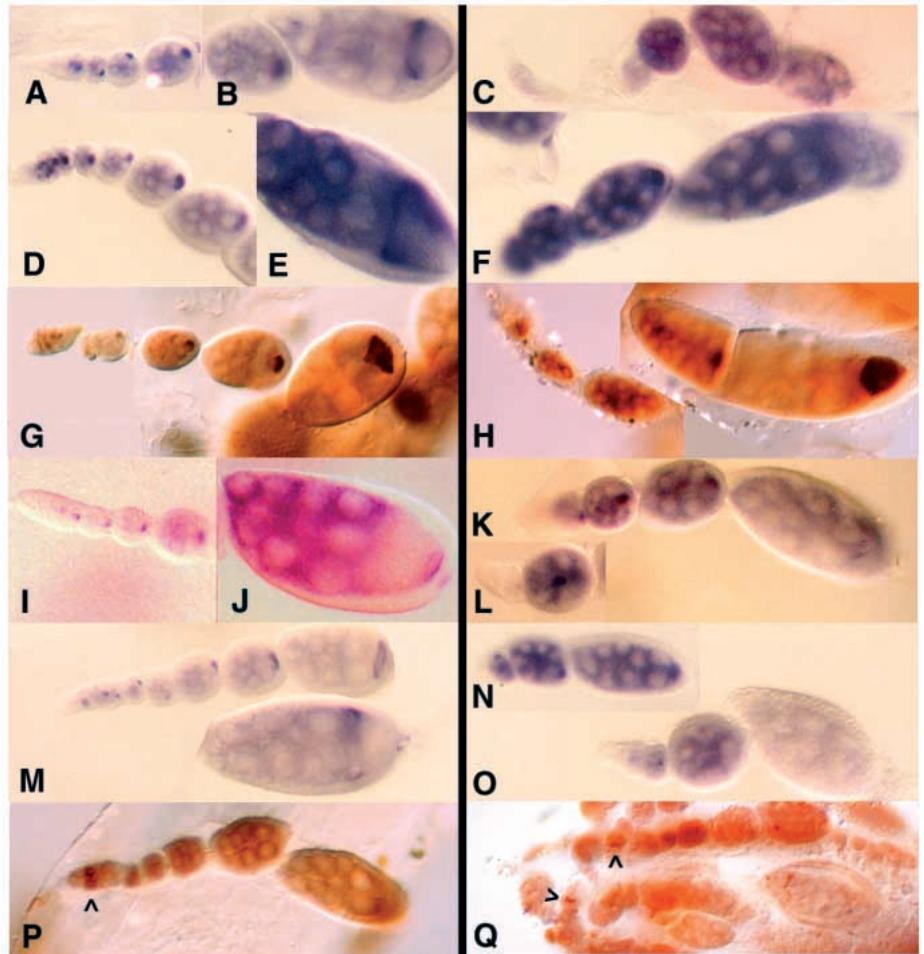
therein throughout the early stages of oogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991; Suter and Steward, 1991; Lantz et al., 1992; Wang et al., 1994; Fig. 4A,B,D,E,I,J,M). Oocyte accumulation of these RNAs is much less pronounced in *vas^PH165* egg chambers in which *Bic-D* RNA localization is essentially undetectable (Fig. 4C), and the concentration of *orb* RNA in the oocyte is only slightly above the levels in the nurse cells (Fig. 4F). In *vas^PH165* egg chambers, *osk* RNA is also not observed to accumulate in the oocyte in the germarium or first vitellarial stages of development. Rather, *osk* RNA tends to concentrate in a somewhat diffuse manner in the center of the egg chamber (Fig. 4K,L). Finally, loss of *vas* function has the least pronounced effect on the accumulation of *nos* RNA into the oocyte, but even in this case oocyte localization is incomplete and poorly maintained (Fig. 4N,O).

Many oocyte-specific RNAs are still translated, and accumulate in *vas*-null oocytes despite defects in RNA localization

Despite the poor localization of *orb* RNA, ORB protein accumulates efficiently in *vas^PH165* oocytes, although somewhat later in oogenesis than in the wild-type (compare

Fig. 4H with Fig. 4G). NOS protein expression is also broadly similar to wild-type in *vas^PH165* ovaries. The reduced number of developing cysts in *vas^PH165* germaria complicates the analysis, but the peak of NOS protein expression in 4- to 8-cell cysts at the posterior of region 1 (Wang et al., 1994) remains apparent in *vas^PH165* (Fig. 4P,Q). OSK protein is not detectably translated in early oogenesis in wild-type nor in *vas^PH165* egg chambers (Kim-Ha et al., 1995; Rongo et al., 1995; data not shown).

In wild-type oocytes, BIC-D and EGALITARIAN (EGL) proteins colocalize in the oocyte cytoplasm, in a posterior crescent from stages 2-7 (Suter and Steward, 1991; Mach and Lehmann, 1997; Fig. 5A,C,D). In *vas^PH165*, BIC-D and EGL are efficiently translated and are localized to the oocyte. However, the two proteins are tightly concentrated in a focus which, in higher magnification in both bright-field and confocal microscopy, appears to be adjacent to the oocyte nucleus (Fig. 5B,E, and data not shown).

vas^PH165 egg chambers often degenerate, usually at stage 6 but occasionally later

Most *vas^PH165* egg chambers develop into germline cysts

containing 15 nurse cells and a posteriorly localized oocyte. These appear normal until about stage 6, whereupon all the nuclei undergo pycnosis and further development ceases (Fig. 6A). Approximately 70% of all *vas^PH165* egg chambers examined terminate development in this manner. A minority (approx. 20%) of *vas^PH165* oocytes continue developing beyond stage 6; most of these are blocked at stage 9-10, after which the oocyte often loses its integrity at the anterior end, and nurse cell nuclei invade the region of the egg chamber normally occupied only by the oocyte (Fig. 6B). Finally, we found that a small number of oocytes produced by *vas^PH165* females complete oogenesis, but that these frequently have duplicated micropyles at both the anterior and posterior ends (Fig. 6C). These eggs also often have dorsal appendage defects consistent with ventralization of the chorion: of 73 eggs analyzed, 14 (19%) lacked dorsal appendages; 48 (66%) had a single fused dorsal appendage; and only 11 (15%) had two dorsal appendages.

vas activity is required for efficient accumulation of GRK protein

The phenotypes of late-stage *vas^PH165* oocytes suggest that the GRK signalling pathway (González-Reyes et al., 1995; Roth et al., 1995) may be inactive in these ovaries, and led us to investigate the expression and distribution of *grk* RNA and protein in *vas^PH165*. Unlike *Bic-D*, *orb*, *osk* and *nos* RNA, localization of *grk* RNA to the oocyte remains highly efficient in *vas^PH165* egg chambers (Fig. 7). However, while in wild-type oocytes from about stage 2-3 the distribution of *grk* RNA forms an obvious posterior crescent (Fig. 7A), *grk* RNA remains tightly concentrated in an extremely small area in *vas^PH165* oocytes (Fig. 7B), similar to the BIC-D/EGL localization pattern in these oocytes (Fig. 5). Later in oogenesis, *grk* RNA becomes anteriorly localized in both wild-type and *vas^PH165* oocytes, although in the mutant its distribution may extend further ventrally (Fig. 7C and D).

Despite the relatively normal accumulation of *grk* RNA in *vas^PH165* oocytes, and unlike the other proteins discussed above, the effects of a loss of *vas* activity are striking with regard to GRK protein accumulation; essentially no localized GRK is observed in *vas^PH165* oocytes (Fig. 7E and F). Furthermore, as measured on western blots, the level of GRK protein is greatly reduced in *vas^PH165* ovaries as compared with wild-type (Fig. 7G). Upon overexposure of such western blots a small amount of GRK can be detected in both *grk^HK36* and *vas^PH165* extracts (Neuman-Silberberg and Schüpbach, 1996; data not shown). As *grk* is required for specification of dorsal and posterior follicle structures, the duplicated micropyles and dorsal appendage defects found in *vas^PH165* eggs (and in eggs produced by other *vas* alleles; Lehmann and Nüsslein-Volhard, 1991; Schüpbach and Wieschaus, 1991) are likely to be caused by the reduced level of GRK in *vas* mutants. These results also suggest that VAS may activate GRK translation in wild-type oocytes.

We examined the organization of the microtubule cytoskeleton in *vas^PH165* oocytes using a *khc:lacZ* reporter gene construct (Clark et al., 1994). In the minority of *vas^PH165* egg chambers that develop normally through stage 10 and can thus be assessed in this way, the distribution of KHC-β-gal in oocytes is similar to that in the wild-type (Fig. 7H-M), with the exception that the intensity of signal is much lower in

mutant egg chambers. The reduced signal may indicate that only a subset of microtubules are correctly organized in *vas^PH165* oocytes. An alternative explanation, that VAS may be required for efficient translation of KHC-β-gal protein in oocytes, is formally possible.

DISCUSSION

Partially redundant roles for VAS in early oogenesis

We have isolated a null allele of *vas*, *vas^PH165*, and its analysis reveals that VAS is involved in many stages of oogenesis, including cystocyte differentiation, oocyte differentiation, and specification of anterior-posterior polarity in the developing cysts. However, the phenotype of *vas^PH165* is inconsistent with a total failure of any of these processes, indicating that VAS has a partially redundant role in bringing them about. Some *vas*-null egg chambers, namely those with two oocytes or with mislocalized oocytes (Fig. 3C,D) are similar to those produced in *spn-A*, *spn-B*, *spn-C*, *spn-D* and *homeless* (*spn-E*) mutants (González-Reyes and St Johnston, 1994; Gillespie and Berg, 1995; González-Reyes et al., 1997). Furthermore, the *vas*-null oocyte nucleus appears to be very similar to those observed in *spn* mutants (Fig. 3F, González-Reyes et al., 1997), and defects in anterodorsal accumulation of *grk* mRNA in stage 9 have also been reported for *spn* mutants (Gillespie and Berg, 1995; González-Reyes et al., 1997). It is likely that *vas* and the *spn* genes have overlapping functions in oocyte determination, and it is noteworthy in this context that *homeless*, which, like *vas,* is required in the germline, encodes a DE-H-box protein somewhat related to VAS. A role for the *spn* gene products in translational activation of *grk* has been suggested (González-Reyes et al., 1997). Construction of multiple mutants with *vas^PH165* and *spn* mutations will be important in understanding the degree to which their functions overlap.

vas and *nos* are both required for germline cyst development

We have observed a decrease in the number of developing germline cysts in *vas^PH165* ovaries, and a similar, but more severe, phenotype occurs in ovaries from females null for *nos* function (Fig. 2; Wang et al., 1994; Curtis et al., 1997). Because of the similarities between this aspect of the *vas* and *nos* mutant phenotypes, and because VAS has been implicated in translational activation of *nos* in the pole plasm (Gavis et al., 1996; Dahanukar and Wharton, 1996), it is tempting to speculate that VAS and NOS function in the same pathway to promote germline cyst development, and that VAS may activate (but not be absolutely essential for) translation of NOS in this stage of germline development as well. Our immunostaining for NOS neither supports nor refutes this possibility. While we see no obvious lack of NOS in *vas^PH165* germaria (Fig. 4Q), it is presently impossible to detect differences in NOS levels in stem cells or cystoblasts, as even in wild-type ovaries these cells are not detectably stained with NOS antibody (Wang et al., 1994; Fig. 4Q). The higher level of NOS in 4-8 cell cysts, which appears to be unaffected by *vas^PH165*, would not be responsible for cyst development, as it occurs at a later developmental stage than that at which the cysts are blocked in *nos^RC* ovaries.

The reduction in the number of developing germline cysts we observe in *vas* and *nos* null mutants could result from a failure of the pole cells to migrate to the gonad during embryogenesis, a failure of the germ cells to regulate the cell cycle or to respond to cell division signals, or from inappropriate differentiation of stem cells into cystoblasts, depleting the germarium of proliferative stem cells. Of these three possibilities, we do not think the defect involves pole cell migration, as we never observe completely agametic ovaries in *vas* or *nos* mutants. Agametic ovaries are observed even in cases where pole cell migration is incompletely blocked, such as in the progeny of *AS-Pgc* females (Nakamura et al., 1996). Direct assessment of the role of zygotic VAS in pole cell migration is confounded by the fact that maternal VAS perdures until embryonic stage 15-16, long after pole cell migration is complete.

Different threshold levels of VAS are required for its various activities

In wild-type flies, *vas* is abundantly expressed in the female germline, and, while VAS is concentrated at the posterior pole of the oocyte from stage 10 of oogenesis, easily detectable levels of VAS are present uniformly throughout the early embryo (Lasko and Ashburner, 1990). These uniform levels are sufficient for ectopic posterior somatic structures to develop in circumstances where OSK is present outside of the pole plasm, for instance in females overexpressing *osk* or carrying the P[*oskBRE*−] transgene (Smith et al., 1992; Kim-Ha et al., 1995). For embryos produced by females carrying a *Bic-D* dominant allele, ectopic posterior segments form dependent on *vas* activity (Mohler and Wieschaus, 1986), but ectopic pole cells do not form, suggesting that the threshold level of VAS necessary for determination of the posterior soma is less than that necessary to induce formation of pole cells (Wharton and Struhl, 1989). A much lower threshold level of VAS is required in early oogenesis than for either later function, since vas^{LYG2} homozygotes, which produce very low levels of unaltered VAS (Fig. 1C), lay a far larger number of eggs than do vas^{PH165} homozygotes, and show defects in oogenesis at lower penetrance and of less severity than for the null (S. S. and P. L., unpublished observations).

RNA localization is dispensable for localizing proteins to the early oocyte

A comparison of the distribution of RNAs and proteins in *vas*-null egg chambers suggests that localization of RNAs, such as *Bic-D* and *orb*, is largely redundant for targeting their proteins to the oocyte. Furthermore, as most *vas*-null egg chambers proceed through development at least to stage 6, despite the very weak localization of such RNAs, it can also be argued that RNA localization is relatively unimportant for the functions of these genes. Therefore translational control, or protein targeting by means independent of RNA localization, must have a critical role in establishing the correct distribution of these molecules. This is perhaps similar to the interplay between translational control and RNA localization apparent in later oogenesis and in embryos; targeting of OSK and NOS to the posterior pole is accomplished through a combination of RNA localization and translational repression of the unlocalized RNA (Kim-Ha et al. 1995; Markussen et al., 1995; Rongo et al., 1995; Gavis et al., 1996; Smibert et al., 1996),

and, at least for *nos*, it has been argued that RNA localization comes about as a consequence of translational regulation (Dahanukar and Wharton, 1996).

Two separable stages of oocyte localization for *grk* RNA and BIC-D/EGL?

In vas^{PH165} ovaries, *grk* RNA and the BIC-D/EGL complex still localize efficiently to the oocyte, but these molecules are concentrated in a tight focus rather than being distributed more widely in the oocyte cytoplasm. This suggests that in wild-type egg chambers, localization of these molecules may occur in two steps: a *vas*-independent step, which concentrates them in the oocyte, and a second step which distributes them to a specific region within the oocyte, depending directly or indirectly on *vas* activity. Alternatively, loss of *vas* function results in the formation of a novel structure or structures in the oocyte cytoplasm which trap *grk* RNA and the BIC-D/EGL complex. The distribution of BIC-D and EGL is unaffected in grk^{HK36} ovaries, indicating that the abnormal localization of these two proteins in vas^{PH165} oocytes is not a consequence of the failure to accumulate GRK in vas^{PH165} (data not shown). We have found that *vas*-null mutations affect some of the same processes as do *Bic-D* and *egl* mutations; namely, oocyte determination (in rare 16-nurse-cell and two-oocyte egg chambers) and establishment of dorsal-ventral polarity (Swan and Suter, 1996; Mach and Lehmann, 1997; this paper). In this context, it is possible that these *vas* phenotypes are a result of the altered distribution of BIC-D/EGL we observed. The altered distribution of *grk* RNA within the early oocyte is similar to that observed in *maelstrom* (*mael*) mutants which affect the organization of oocyte microtubules (Clegg et al., 1997). However, unlike in *vas*-null egg chambers in which many RNAs, such as *osk*, are poorly transported from the nurse cells to the oocyte (Fig. 4), *mael* only affects RNA distribution within the oocyte (Clegg et al., 1997). Furthermore, posterior localization of KHC-βGAL in *vas*-null oocytes (Fig. 7) suggests that the organization of the microtubule cytoskeleton is at least partially maintained.

Is VAS a direct activator of GRK translation?

In the yeast *S. cerevisiae*, a protein highly similar to VAS, DED1p, has recently been implicated in translation initiation (Chuang et al., 1997; de la Cruz et al., 1997). While the loss-of-function experiments presented here cannot distinguish whether VAS is required directly or indirectly for GRK accumulation, a simple model which takes into account the molecular nature of VAS and explains the reduction of GRK protein expression in *vas*-null ovaries is that VAS interacts directly with *grk* RNA and activates its translation. This is consistent, as well, with the striking reduction we observed in GRK accumulation in *vas*-null oocytes despite the high level of *grk* RNA in these oocytes. Importantly, other RNAs, such as *Bic-D* and *orb*, whose localization to the oocyte is more severely perturbed in *vas*-null ovaries, are still translated at levels comparable to wild-type in *vas*-null oocytes. Nevertheless, demonstration of a direct effect of VAS on *grk* translation must await more detailed experimentation. Bacterially expressed VAS has been demonstrated to bind RNA in vitro (Liang et al., 1994). However, no specificity for RNA binding by VAS in vitro has thus far been demonstrated, though *grk* was not among the RNAs tested in these in vitro binding
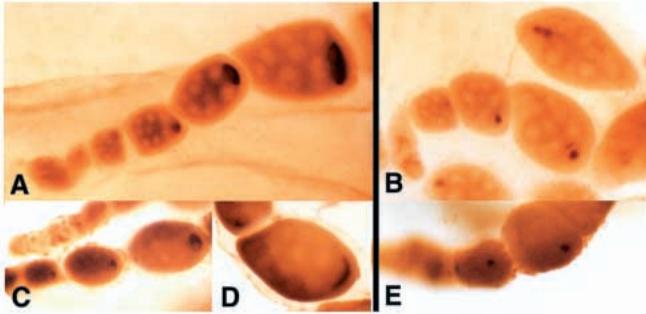
Fig. 5. (A,B) Ovaries from (A) wild-type and (B) vas^{PH165}/vas^{PH165} females stained with a monoclonal antibody recognizing BIC-D protein. In the *vas* mutant, BIC-D protein is localized in a tight focus adjacent to the oocyte nucleus throughout previtellogenic stages of oogenesis. (C-E) Ovaries from (C,D) wild-type and (E) vas^{PH165}/vas^{PH165} females stained with an antiserum recognizing EGL protein. The distribution of EGL is identical to that of BIC-D in both the wild-type and the *vas* mutant.
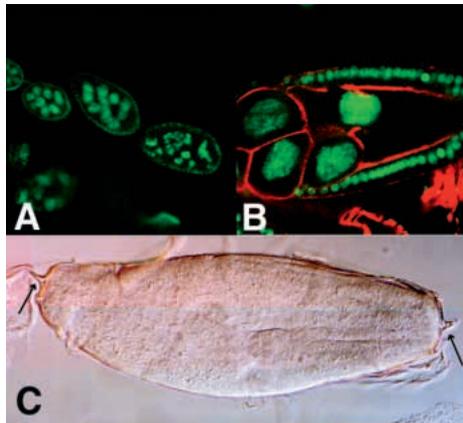
Fig. 6. Phenotypes of *vas*-null egg chambers affecting later stages of oogenesis. (A) Confocal micrograph of a vas^{PH165}/vas^{PH165} ovariole, stained with the nuclear dye Oli-Green, showing pycnosis and degeneration after stage 6. (B) Confocal micrograph of a $vas^{PH165}/Df(2L)A267$ egg chamber, stained with Oli-Green and Texas Red-phalloidin, illustrating a nurse cell nucleus invading the anterior of a stage-10 oocyte. (C) Nomarski optics image of a $vas^{PH165}/Df(2L)A267$ egg chamber with duplicated micropyles (arrows). The phenotypes depicted in B and C are also observed in vas^{PH165}/vas^{PH165} ovaries, at a similar frequency to $vas^{PH165}/Df(2L)A267$.

experiments. There appears to be elaborate posttranscriptional regulation of GRK in the developing oocyte, involving the activities of numerous genes. In addition to *vas*, the genes *aubergine* and *encore* have been implicated as essential for efficient GRK accumulation (Wilson et al., 1996; Hawkins et al., 1997).

Reduction of GRK activity explains only a subset of *vas* phenotypes

Duplication of anterior vitelline membrane structures occurs in strong *grk* mutant alleles, and GRK signalling has been implicated in the specification of posterior polar follicle cells (González-Reyes et al., 1995; Roth et al., 1995). We
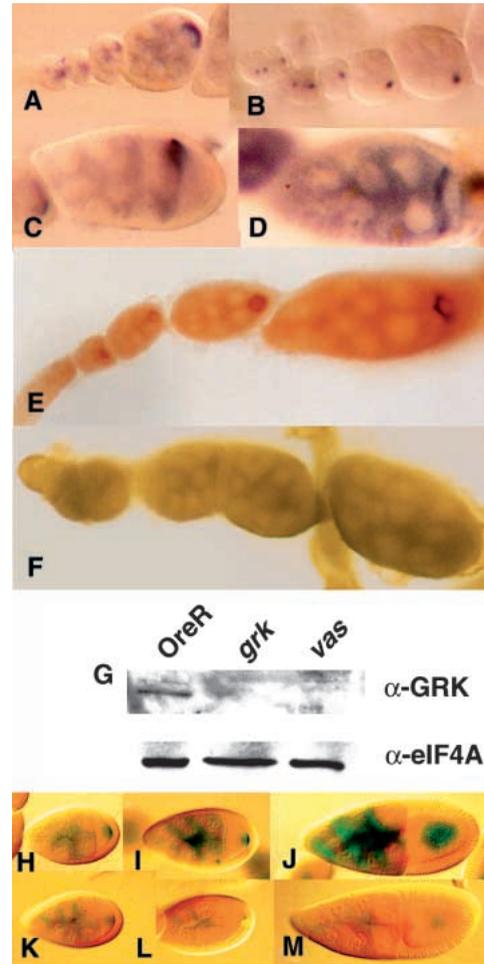

Fig. 7. (A-D) In situ hybridizations illustrating the distribution of *grk* RNA in (A,C) wild-type and (B,D) vas^{PH165}/vas^{PH165} ovaries. In the *vas* mutant, *grk* RNA is localized in a tight focus within the oocyte throughout pre-vitellogenic stages of oogenesis. This distribution resolves into an anterior ring at stage 8, with a variably higher concentration at the anterodorsal corner, but not so sharp an asymmetry as is evident in the wild-type. (E-F) Ovaries from (E) wild-type and (F) vas^{PH165}/vas^{PH165} females stained with an antiserum recognizing GRK protein (Neuman-Silberberg and Schüpbach, 1996). Essentially no localized signal is observed in the *vas* mutant. For both the in situ hybridization and antibody staining reactions, the control and vas^{PH165}/vas^{PH165} stainings were processed in parallel, and color reactions proceeded for identical lengths of time. (G) Western blot prepared from extracts of ovaries dissected from OreR, wild-type; *grk*, grk^{HK36}/grk^{HK36}; *vas*, vas^{PH165}/vas^{PH165} females, and probed with the antiserum directed against GRK (top panel) or with an antiserum directed against eIF4A (Lavoie, 1995) as a loading control (bottom panel). GRK protein level is greatly reduced in the grk^{HK36} and vas^{PH165} extracts. (H-M) Distribution of β-galactosidase activity in ovaries bearing the *khc:lacZ* transgene (Clark et al., 1994). H-J) Stage-8, -9, and -10 egg chambers from CyO/vas^{PH165}; *khc:lacZ* individuals. (K-M) Stage-8, -9, and -10 egg chambers from vas^{PH165}/vas^{PH165}; *khc:lacZ* individuals. The control and vas^{PH165}/vas^{PH165} stainings were processed in parallel for identical lengths of time.

demonstrate in this paper that many of the oocytes that complete oogenesis in a loss-of-function *vas* mutant have duplicated micropyles and/or fused or reduced dorsal

appendages, and four other *vas* alleles (*PW72, QS17, AQB3, RG53*) have previously been shown to produce eggs with fused and reduced dorsal appendages when hemizygous over *Df(2L)osp29* (Schüpbach and Wieschaus, 1991). All of these phenotypes resemble those observed in *grk* mutants, making it likely that these aspects of the *vas* mutant phenotype are brought about by a reduction of GRK activity, particularly since we show that GRK accumulation is negligible in *vas*-null oocytes. However, aspects of the *vas*-null phenotype which manifest themselves earlier in oogenesis, such as the reduced number of developing cysts, the defects in RNA localization to the oocyte, and the aberrant localization of BIC-D and EGL within the oocyte, are not found in *grk* mutant ovaries (Fig. 2; S.S. and P.L., unpublished results). This suggests that VAS may regulate the activity of numerous genes. Evidence similar to that presented here implicates VAS in translational activation of Short OSK in the pole plasm (Markussen et al., 1995). Furthermore, it is possible that VAS regulates translation of other, presently unknown, RNAs whose products are involved in establishing cytoskeletal polarity in the early cyst and/or in localizing RNAs to the oocyte.

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