Development 120, 1201-1211 (1994)
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Localization of vasa protein to the *Drosophila* pole plasm is independent of its RNA-binding and helicase activities

Lu Liang, William Diehl-Jones† and Paul Lasko*

Department of Biology, McGill University, 1205 Avenue Docteur Penfield, Montréal, Québec, Canada H3A 1B1

*Author for correspondence
†Present address: Department of Biology, University of Waterloo, Waterloo, Ontario, Canada N2L 3G1

SUMMARY

The *Drosophila* gene *vasa* encodes a DEAD-box protein, which is localized during early oogenesis to the perinuclear region of the nurse cells and later to the pole plasm at the posterior end of the oocyte. Posterior localization of vasa protein depends upon the functions of four genes: *capu*, *spir*, *osk* and *stau*. We have found that localization of vasa to the perinuclear nuage is abolished in most *vas* alleles, but is unaffected by mutations in four genes required upstream for its pole plasm localization. Thus localization of vasa to the nuage particles is independent of the pole plasm assembly pathway. Furthermore, electron-dense nuage particles are less abundant in the cytoplasm of nurse cells from *vas* mutants that fail to exhibit perinuclear localization, suggesting that the formation of the nuage depends upon *vas* function. Eight of nine *vas* point mutations cause codon substitutions in a region conserved among DEAD-box genes. The proteins from two mutant alleles that retain the capacity to localize to the posterior pole of the oocyte, vas^{O14} and vas^{O11}, are both severely reduced in RNA-binding and -unwinding activity as compared to the wild-type protein on a variety of RNA substrates including in vitro synthesized pole plasm RNAs. Initial recruitment of vasa to the pole plasm must consequently depend upon protein-protein interactions but, once localized, vasa must bind to RNA to mediate germ cell formation.

Key words: oogenesis, germ cells, DEAD-box, morphogenetic determinants, *Drosophila*

INTRODUCTION

Pole cells, the progenitors of the germ line, form at the posterior end of the *Drosophila* embryo from a region of specialized cytoplasm rich in ribonucleoprotein particles termed polar granules (Mahowald, 1968; Illmensee and Mahowald, 1974; Okada et al., 1974). The RNAs and proteins that make up polar granules assemble during the later stages of oogenesis (Illmensee et al., 1976). Mutations in genes that encode polar granule components have maternal-effect phenotypes, in that females lacking a wild-type copy of the gene produce embryos that fail to form pole cells and usually also carry somatic patterning deletions. The latter phenotype occurs because *nanos* mRNA localization depends on earlier steps of pole plasm assembly, and posterior localization of *nanos* mRNA is required in the wild-type embryo for posterior patterning (Boswell and Mahowald, 1985; Schüpbach and Wieschaus, 1986; Wang and Lehmann, 1991, Lehmann and Nüsslein-Volhard, 1991; Gavis and Lehmann, 1992).

In the initial stages of pole plasm assembly, the first molecules known to move to the posterior pole are *osk* mRNA and staufen protein (Kim-Ha et al., 1991; Ephrussi et al., 1991, St Johnston et al., 1991). Their localization is dependent on the functions of the *capu* and *spir* genes. The next pole plasm component to be localized is vasa protein, an event dependent upon the functions of *capu*, *spir*, *osk* and *stau* (Lasko and Ashburner, 1990; Hay et al., 1990). At least seven further mRNAs (*nos*, *pum*, *cyclin-B*, *gcl*, mtlrRNA, *Hsp83* and *orb*), and two proteins (tudor and fat facets), associate in the pole plasm (Gavis and Lehmann, 1992; Barker et al., 1992; Jongens et al., 1992; Kobayashi et al., 1993; Dalby and Glover, 1992; Fischer-Vize et al., 1992; Lantz et al., 1992; Ding et al., 1993; Bardsley et al., 1993). These molecules localize late in oogenesis, and the recruitment of most of them to the pole plasm has been shown to depend on *vasa* function. In addition to its role in pole plasm assembly, vasa has an earlier function in oogenesis, as females homozygous for null mutations of *vas* produce no eggs and terminate oogenesis in early vitellogenic stages (Lasko and Ashburner, 1988).

The components of pole plasm are functionally essential for formation of the embryonic germ line, since females mutant in genes encoding pole plasm components produce embryos that lack sex cells. Furthermore, mislocalization of the *oskar* mRNA to the anterior pole of the oocyte by replacing its 3′ untranslated region with that of bicoid results in the formation of ectopic, functional pole cells at the anterior end of the embryo (Ephrussi and Lehmann, 1992). Among the set of genes identified by mutation as involved in pole plasm formation, in addition to *osk* only *vas* and *tud* must be present in a functional copy for the formation of pole cells at the

anterior pole to occur in these embryos. This implies a central role for these three genes in pole cell determination.

We are interested in determining how vasa protein functions in this pathway. Vasa is a member of the DEAD-box family of putative RNA helicases similar to eukaryotic initiation factor 4A, and such proteins have been implicated in spliceosome assembly, ribosomal RNA processing, and translational control (Schmid and Linder, 1992). In this paper, we show that bacterially expressed wild-type vasa protein functions in vitro as an ATP-dependent RNA helicase. We further report the molecular lesions of nine *vas* point mutations and correlate these changes with localization and developmental phenotype. Finally, we investigate the interactions of wild-type and four mutant vasa proteins with various in vitro-synthesized mRNAs. Our results indicate that alteration of a number of individual amino acid residues abolishes localization of vasa protein to the perinuclear region of the nurse cells and to the posterior pole of the oocyte, but that two mutant vasa proteins, greatly reduced in RNA-binding and helicase activity, still localize to the pole plasm in living oocytes.

MATERIALS AND METHODS

Fly strains

Most of the *vas* alleles used in this study have been described previously: vas^{PD}, Schüpbach and Wieschaus, 1986; vas^{O11}, vas^{O14}, vas^{Q6}, vas^{AS}, vas^{D1}, vas^{D5} and vas^{Q7}, Tearle and Nüsslein-Volhard, 1987, Lasko and Ashburner, 1990; vas^{HE}, vas^{PW}, vas^{QS} and vas^{RG}, Schüpbach and Wieschaus, 1991. The vas^{3F} and vas^{4C} alleles were isolated as dominant suppressors of a dominant *Bic-D* allele, and were generously provided by F. Pelegri and R. Lehmann. osk^{54}, $stau^{G2}$, $stau^{HL}$, $capu^{RK}$ and $spir^{RP}$ have all been previously described (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991; Manseau and Schüpbach, 1989).

Protein over-expression and purification

The 0.7 kb *Eco*RI fragment from *vas* cDNA clone cv1.092 (Lasko and Ashburner, 1988) was subcloned into the *Eco*RI site of a pGEX-3X expression construct encoding amino acids 16-433 of vasa (Lasko and Ashburner, 1990). The resultant plasmid contained coding regions for the entire vasa protein except for the first 15 amino acids at the N terminus and was used to produce vasa. The starting plasmid encoding amino acids 16-433 was used to produce vasaδC. Fusion protein was expressed in *E. coli* strains DH5α or DH10b. The chimeric protein was purified on glutathione-Sepharose 4B (Pharmacia), and the vasa polypeptide was cleaved from the glutathione-S-transferase protein with factor Xa (Boehringer-Mannheim; 1% wt/wt fusion protein).

ATP-binding assay

Cross-linking reactions were performed as described by Pause and Sonenberg (1992). The reaction mixture contained 1 μg of wild-type or mutant protein, 30 mM Tris-HCl (pH 7.5), 5 mM MgOAc, 10% glycerol, 1.5 mM DTT, and 2.5 μCi α-[^{32}P]ATP in a 20 μl reaction volume. This was placed on ice and irradiated from a distance of 2 cm for 10 minutes at 254 nm with a UVGL-58 Mineralight Lamp (UVP, Inc.). Unlabelled ATP (4 mM) was added and the reactions were incubated for 10 minutes at 37°C. Samples were analyzed by SDS-PAGE and autoradiography.

ATPase assay

Briefly, wild-type or mutant protein was incubated at 37°C in a 20 μl reaction volume containing 20 mM Tris-HCl (pH 7.5), 70 mM KCl, 2.5 mM MgOAc, 1.5 mM DTT, 0.1 A_{260} units poly(U) (Pharmacia), 2.5 μCi of γ-[^{32}P]ATP and 0.1 mM unlabelled ATP. Aliquots were removed at various times and processed on ice by the successive addition of reagents such that inorganic phosphate was extracted into an upper organic phase (Abramson et al., 1987). 0.5 ml samples of the upper phase were added to 10 ml of scintillation fluid, counted and inorganic phosphate release calculated.

Preparation of radiolabelled RNA

Clones were transcribed using RNA polymerase Sp6 (for *nos*, *cyclin B*, mtlrRNA and pRP40), T7 (for *gcl*), or T3 (for *osk*) in the presence of 0.5 mM each of ATP, CTP and UTP, 5 μM GTP, and 50 μCi α-[^{32}P]-GTP (>3000 Ci/mmol). After transcription, 10 units of RNase-free DNase were added for 15 minutes at 37°C. RNA was purified by phenol/chloroform extraction and precipitated three times with ammonium acetate and ethanol.

RNA-binding assay

A filter assay for RNA binding was adapted from those previously reported (Grifo et al., 1982; Abramson et al., 1987). Wild-type or mutant vasa protein (1 μg) was incubated with 10 or 20 ng [^{32}P]RNA substrate in 40 μl of binding buffer (30 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM $MgCl_2$, 2 mM dithiothreitol, 5% glycerol, 0.01% BSA, 2 mM ATP) at 37°C for 20 minutes or as indicated in the text. The binding reaction was stopped by filtering through a nitrocellulose membrane (Millipore, 0.45 μm pore size), which was presoaked in wash buffer (30 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5% glycerol, 0.01% BSA) for 30 minutes before use. The reaction tubes were rinsed with 1 ml of wash buffer, which was also applied to the membranes and filtered. The filters were then washed with 1 ml of wash buffer and radioactivity retained on the membrane was determined by liquid scintillation counting.

RNA helicase assay

Radiolabelled duplex RNA substrate was generated by linearizing the pRP40 plasmid (kindly supplied by A. Pause and N. Sonenberg) with *Bam*HI followed by transcription with Sp6 polymerase using α-[^{32}P]CTP. After transcription, RNA was separated on an 8% sequencing gel, the correct band excised and eluted in 2× SSC/1% SDS overnight at 4°C, and further purified by phenol/chloroform extraction and ethanol precipitation. This yielded a spontaneously annealing RNA homoduplex comprised of a 10 bp duplex region with two 3′ and two 5′ terminal single-stranded tails. The assay included 1 μg recombinant vasa protein, 30 mM Tris-HCl (pH 7.5), 0.1 mM NaCl, 8 mM $MgCl_2$, 15 mM DTT, 20 U RNAsin (Promega Biotech), 2 mM ATP and 5% glycerol in a 40 μl volume containing 4.4 ng [^{32}P]RNA template. The reaction mixture was incubated for 30 minutes at 37°C and stopped by addition of 1/8 volume of stop buffer (3% SDS, 30% glycerol, 150 mM EDTA and 0.01% bromophenol blue). Reactions were then analysed by SDS-PAGE and autoradiography.

Sequencing of *vas* genomic DNA

Genomic DNA from adult flies *trans*-heterozygous for a mutant allele of *vas* and a deletion of *vas* (either *Df(2L)A72* or *Df(2L)A220*; Ashburner et al., 1982) was amplified by PCR using AmpliTaq (Perkin-Elmer). The *vas* gene was separated into two fragments for PCR due to the large 3.7 kb intron which interrupts the coding sequence. For sequencing, single-stranded DNA was then prepared by PCR using single amplification primers from sequence immediately internal to the ends of the amplified DNA fragments. Dideoxy-mediated chain termination sequencing was performed on the single-stranded DNA using the single-stranded amplification and other internal primers.

Fixation and immunostaining of ovaries

Ovaries were dissected into Ringer's solution and fixed in 4% paraformaldehyde in 0.1 M PIPES, 2mM $MgSO_4$, 1 mM EGTA (pH 6.9) for 30 minutes at room temperature. Immunostaining and affinity

purification of the antiserum was carried out as described by Lasko and Ashburner (1990). Affinity-purified primary antibody was used at a dilution of 1:40, secondary antibody was Texas Red-conjugated goat anti-rabbit IgG (Jackson Laboratories; diluted 1:200). Final washes were in PBS + 0.1% Tween-20 for 2 hours in three changes. Ovaries were mounted in 1:1 PBS/glycerol and viewed on a Leica confocal laser scanning microscope in the rhodamine channel. Photomicrographs were taken with Ektachrome film (ASA 400).

For electron microscopy, tissues were fixed for 1 hour at room temperature in 2% glutaraldehyde in 50 mM sodium cacodylate, pH 7.2, 1% DMSO, dehydrated, embedded in LR White (Polysciences) and sectioned. Silver sections were mounted on 300 mesh nickel grids and were rehydrated in PBS for 10 minutes, blocked in PBS + 1% Blotto at room temperature for 15 minutes, incubated in primary anti-vasa antiserum (1:50 dilution) for 1 hour in PBS + 1% Blotto and washed in PBS + 0.1% Tween-20. Vasa protein was detected by incubating with colloidal gold (12 nm)-conjugated anti-rabbit antibody (1:20 dilution, Jackson ImmunoResearch Laboratories), washing in PBS + 0.1% Tween-20, postfixing for 2 minutes in 2% glutaraldehyde in PBS and washing in double-distilled water. EM staining was with lead citrate/uranyl acetate.

Site-directed mutagenesis

Mutations were introduced into subcloned *vasa* cDNA fragments via oligonucleotide-directed in vitro mutagenesis using a single-stranded kit from Amersham (oligonucleotide-directed mutagenesis system version 2.1) for vas^{O14}, vas^{O11} and vas^{AS}, and a double-stranded mutagenesis kit from Stratagene (Doubletake™) for vas^{D5}. Oligonucleotides used were 19-22mers. Mutations were verified by sequencing and appropriate fragments subcloned back into the pGEX-3X vasa expression construct. Mutant proteins were purified as described for vasa above.

RESULTS

Bacterially produced vasa protein binds and hydrolyzes ATP

To begin our analysis of the relationship between the enzymatic activities of vasa and its developmental phenotype, we wished to assay the wild-type vasa protein for activities that have been reported for other DEAD-box proteins; namely, ATP binding, ATP hydrolysis, RNA binding and RNA unwinding. As a convenient source of vasa protein, we assembled a bacterial expression construct that produces large quantities of a fusion protein including the *S. japonicum* glutathione-S-transferase (GST) joined to amino acids 16-661 of vasa (Materials and Methods). The expressed protein was purified by binding to glutathione-Sepharose and the vasa polypeptide separated from the GST and eluted from the column by cleavage with factor Xa. By SDS-PAGE analysis, this procedure yielded essentially one homogeneous band of the molecular weight expected for the vasa polypeptide (Fig. 1).

This bacterially expressed protein, which we will subsequently term vasa protein, was then assayed for ATP-binding and ATPase activity (Fig. 2). Radiolabelled ATP was incubated with vasa protein, GST and a truncated vasa protein lacking amino acids 434-661 (vasaδC), and only the full-length vasa was found to be capable of binding ATP. Furthermore, vasa, but not GST or vasaδC, is capable of removing the terminal phosphate moiety from γ-[^{32}P]ATP. The ATP hydrolysis reaction is linear with time for at least 1 hour, and is approximately linear with the amount of vasa added up to 2 μg. The ATPase activities of three other DEAD-box proteins, eIF-4A, SrmB and p68, are stimulated to varying degrees by the addition of RNA (Pause and Sonenberg, 1992; Nishi et al., 1988; Iggo and Lane, 1989). We tested the following RNAs for ability to increase the ATPase activity of vasa protein: polyuridylic acid, total embryonic RNA and in vitro synthesized sense and antisense transcripts of *osk*, *nos*, *cyclin-B*, *gcl* and mtlrRNA, and determined that none of these RNAs increased the ATP hydrolysis activity of vasa under the reaction conditions used. GTP cannot substitute for ATP in the binding reaction.

Vasa is an ATP-dependent RNA helicase

We next investigated the RNA-binding capability of vasa in order to determine whether vasa, like other DEAD-box proteins, has this activity, and also to investigate whether

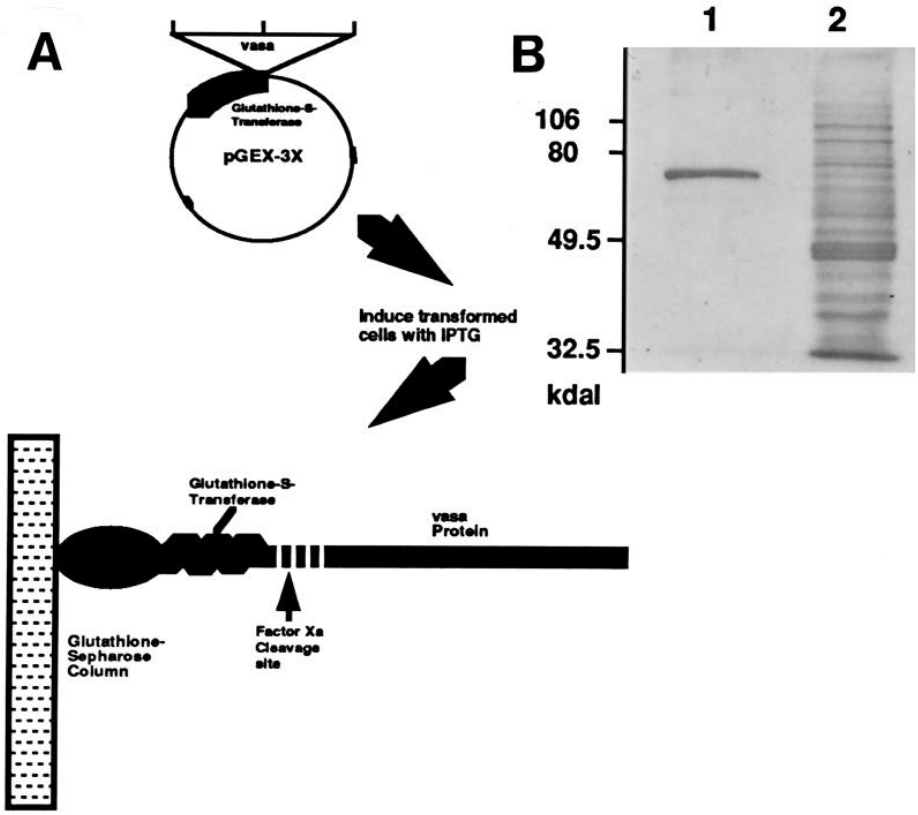

Fig. 1. (A) Schematic diagram of the procedure used to express and purify vasa protein from bacteria. (B) Coomassie-blue-stained SDS-PAGE gel loaded with: lane 1, purified vasa protein eluted from the GST-Sepharose column by cleavage with factor Xa; lane 2, crude bacterial lysate. Molecular weights were calibrated using commercial marker proteins.

purified vasa protein exhibits any sequence specificity or other preferences among potential substrate RNA molecules. To measure RNA binding, vasa protein was incubated with radiolabelled RNA, then filtered through a nitrocellulose membrane, washed, and membrane-bound radioactivity measured (Fig. 3A). In this assay vasa protein, but neither GST nor vasaδC, was capable of binding duplex RNA. We did not find that vasa had any preferences for binding among in vitro synthesized *nos* RNA, *cyclin-B* RNA, *gcl* RNA, mtlrRNA and an artificial 91-nt transcript (pRP40, see below), but the binding of vasa to *osk* RNA was very low. Omission of ATP from the binding buffer had no effect on vasa binding to RNA, indicating that this activity is ATP independent. Thermal denaturation of the RNA (100°C for 5 minutes, then chilling on ice) completely abolished its ability to bind vasa.

We then examined the ability of vasa protein to unwind duplex RNA, using a short RNA transcribed from the pRP40 plasmid (Jaramillo et al., 1990). The 91-nt RNA produced includes a 10-nt stretch of alternating C and G residues; at 37°C, two such RNA molecules base-pair to form a double-stranded molecule. Using this substrate RNA, we found that vasa is able to convert duplex RNA to single-stranded RNA (Fig. 3B). This reaction is dependent on ATP and on magnesium ions. Duplex DNA is not unwound by the vasa helicase activity (data not shown).

vas point mutations mainly affect residues conserved in the DEAD-box protein family

DEAD-box proteins contain extensive sequence similarity over an approximately 425 amino acid domain, and within this region there are eight nearly invariant motifs (Fig. 4; Schmid and Linder, 1992). In order to determine the residues in vasa that are required for its developmental phenotypes, we identified the lesions responsible for nine EMS-induced *vas* mutations by direct sequencing of PCR-amplified genomic DNA. Eight of the nine amino acid substitutions that we found map within the conserved 425 amino acid domain. The ninth, vas^{HE}, affects residue 170 in the amino-terminal unique region of vasa.

Of the amino acids changed by these mutations, two are glycines invariant in 34 DEAD-box proteins and all are extensively conserved within the family (Fig. 4). In only one case (out of 272 compared) does another wild-type DEAD-box protein carry the amino acid found in a mutant form of vasa: in vas^{AS}, His^{520} is replaced by tyrosine; the *S. cerevisiae* eIF-4A protein also has a tyrosine at this position (Linder and Slonimski, 1988). The two alleles that change the invariant glycines to glutamic acid residues, vas^{D5} and vas^{3F}, are also phenotypically the most severe. Both mutants lay very few eggs and vas^{D5}

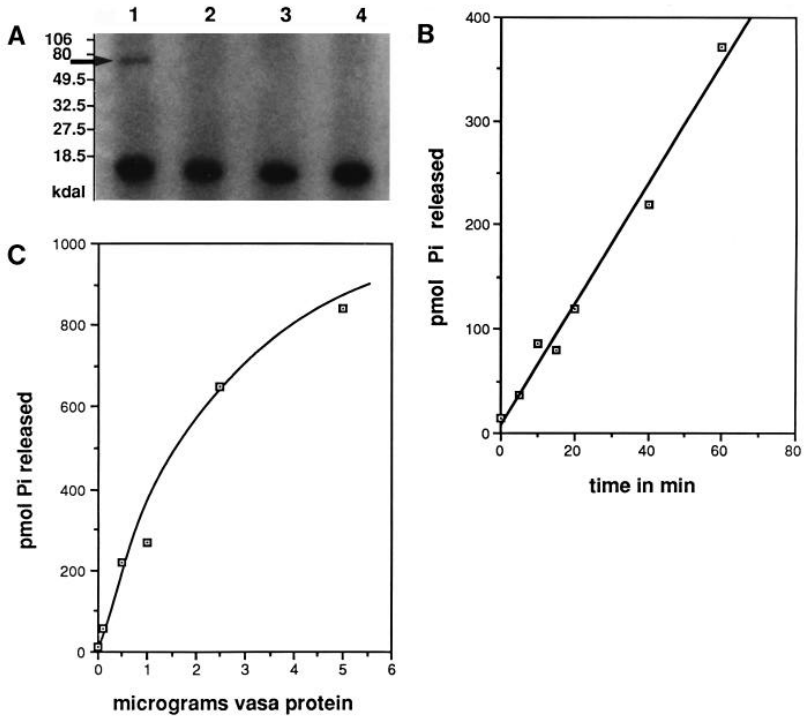

Fig. 2. (A) Autoradiograph of a dried SDS-PAGE gel loaded with protein samples incubated with α-[^{32}P]ATP and u.v.-crosslinked as described in Materials and Methods. Lane 1, 1 μg vasa protein; lane 2, no protein; lane 3, 1 μg vasaδC protein; lane 4, 1 μg bacterially produced GST. The radioactive material at the bottom of the autoradiograph migrates with the dye front and is free ATP. (B) ATPase activity of 1 μg of vasa as a function of time, assayed as described in Materials and Methods. (C) ATPase activity as a function of varying concentrations of vasa; all reactions proceeded for 60 minutes.

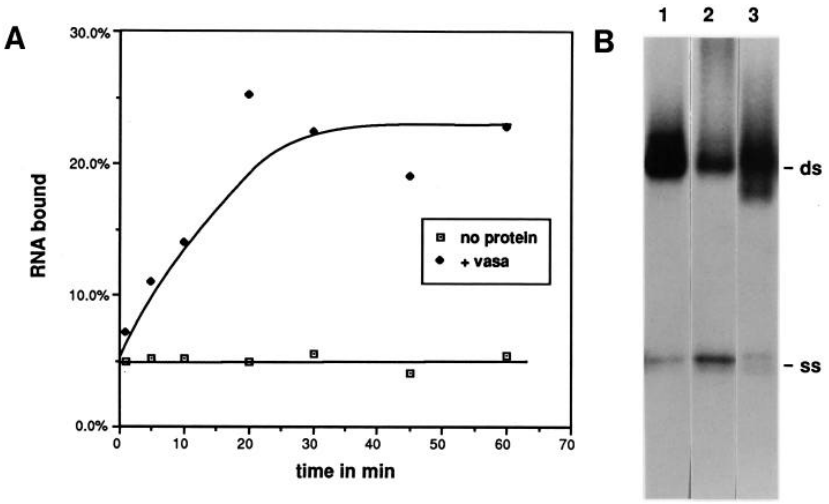

Fig. 3. (A) RNA-binding activity of vasa as a function of time, assayed as described in Materials and Methods. The y-axis gives the proportion of total radiolabelled RNA remaining bound to the nitrocellulose filter. The binding reaction included 20 ng of duplex pRP40 RNA. Removal of ATP from the binding buffer had no effect on RNA binding; however, heat denaturation of the template RNA completely abolished binding activity (see Results). (B) RNA-unwinding activity of vasa protein, assayed with 4.4 ng pRP40 RNA as described in Materials and Methods. The helicase activity is proportional to the ratio of single-stranded RNA to the sum of the single-stranded and double-stranded RNA. Lane 1, RNA incubated at 37°C with no added protein; lane 2, RNA incubated as in lane 1 with 1 μg of vasa protein and 2 mM ATP; lane 3, RNA incubated as in lane 1 but without ATP.

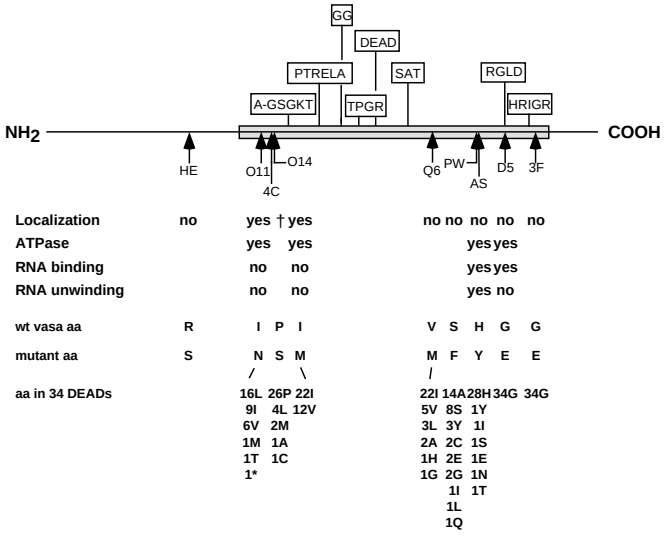

Fig. 4. Summary of the analysis of *vas* point mutations; protein localization data is presented in Figs 5 and 6, ATPase data in Fig. 7, and RNA-binding and unwinding data in Fig. 8. Nine EMS-induced mutations are marked on a schematic diagram of the vasa protein. The boxed and shaded region of the protein indicates the 425 amino acids that are highly similar among DEAD-box proteins, and the boxed motifs are nearly invariant (Schmid and Linder, 1992). The codon changes for the nine mutations are as follows: vas^{HE}, AGT for AGA; vas^{O11}, AAT for ATT; vas^{4C}, TCG for CCG; vas^{O14}, ATG for ATA; vas^{Q6}, ATG for GTG; vas^{PW}, TTC for TCC; vas^{AS}, TAT for CAT; vas^{D5}, GAA for GGA; vas^{3F}, GAA for GGA. **aa in 34 DEADs**: The frequencies of various amino acids at residues analogous to those affected by *vasa* mutations in vasa and 33 other DEAD-box proteins from bacteria, fungi, plants, and animals are listed for the eight *vasa* mutations that lie within the DEAD-family domain. Analogous residues were identified by multiple sequence alignment using the GeneWorks software package (IntelliGenetics). The asterisk in the I256N data indicates that the reported sequence of one DEAD-box protein, DbpA from *E. coli*, does not extend as far as this residue in the amino-terminal direction (Iggo et al., 1990). In addition to amino acid changes, we uncovered some sequence polymorphisms in the third base of codons or in the small introns, and one that alters codon 192 from GCC to GTC (alanine to valine). GTC is found in all but one of the alleles from the Tübingen and MIT mutageneses, while GCC is found in all the Princeton alleles. The other polymorphisms are also found in more than one allele. These different screens utilized independent wild-type chromosomes for mutagenesis (Tearle and Nüsslein-Volhard, 1987; Schüpbach and Wieschaus, 1986, 1991). This work led also to minor corrections in previously published versions of the *vas* sequence (Lasko and Ashburner, 1988; Hay et al., 1988b), and the complete sequence data have been submitted to the EMBL database (accession numbers X12945 and X12946).

ovaries frequently contain abnormal tumorous egg chambers (Lasko and Ashburner, 1990). The other amino acid substitution mutations (vas^{HE}, vas^{O11}, vas^{4C}, vas^{O14}, vas^{Q6}, vas^{PW} and vas^{AS}) lead to the posterior-group phenotype (Schüpbach and Wieschaus, 1986).

We found no amino acid substitutions in five other *vas* mutant alleles: vas^{PD}, vas^{D1}, vas^{Q7}, vas^{RG} and vas^{QS}. vas^{QS} carries a 4 bp insertion in the pyrimidine tract immediately upstream from the 3′ end of intron 4, which would be expected to affect processing of this intron. In all of these alleles, expression of vasa is greatly reduced (Hay et al., 1988a; Lasko and Ashburner, 1990, our unpublished results). Presumably the first four of these mutations disrupt *cis*-regulatory elements controlling *vas* expression or affect mRNA processing or stability; a detailed analysis in the wild-type and these mutant strains of the sequences that regulate *vas* expression will be required to determine this.

Effects of *vas* point mutations on protein localization

Wild-type vasa protein is only functional in pole cell formation when concentrated in the pole plasm. The inability of a mutant vasa protein to function in pole cell formation can therefore be explained solely on the basis of its failure to localize. We examined the distribution of vasa in the *vas* alleles carrying a single changed amino acid, using a new anti-vasa antiserum prepared against the full-length vasa protein and confocal laser scanning microscopy. Our results are shown in Fig. 5. Six mutant proteins, encoded by the vas^{HE}, vas^{Q6}, vas^{PW}, vas^{AS}, vas^{D5} and vas^{3F} alleles, fail to localize detectably either to the perinuclear region of the nurse cells or to the pole plasm. The other three mutant proteins, which all carry amino acid substitutions within a 16-amino-acid stretch of the amino-terminal end of the conserved DEAD-family region, retain some ability to localize. The Vas^{O14} protein localizes normally to the perinuclear region and to the pole plasm. The Vas^{O11} protein localizes normally within the nurse cells to the perinuclear region, and localizes at a somewhat reduced level to the pole plasm. The Vas^{4C} protein fails to localize to the pole plasm, but can be seen concentrated around the nurse cell nuclei. These data indicate that residues within a portion of the amino-terminal unique region and within the region between amino acids 465-587 are important for the localization of vasa, but that three amino acids that lie between residues 256-271 are partly dispensable for this function.

Since most *vas* mutations that abolish posterior localization also abolish perinuclear localization of the protein, we investigated whether mutations in genes upstream of *vas* in the pole plasm assembly hierarchy have any effect on perinuclear localization. Our results indicate that, unlike posterior localization, perinuclear localization of vasa is independent of the functions of *capu*, *spir*, *osk* and *stau*. Moreover, mutations in downstream genes such as *tud*, *vls*, *nos* and *pum* affect neither perinuclear nor posterior localization (Lasko and Ashburner, 1990).

The presence of abundant perinuclear nuage particles correlates with localized vasa

In order to investigate the perinuclear localization of vasa in more detail, we carried out EM immunocytochemistry on ovaries from wild-type, vas^{HE} and vas^{AS} ovaries, the latter being two alleles in which perinuclear localization is not observed. Our results for the wild type are consistent with those previously reported (Hay et al., 1988a). Commencing at about stage 1, most vasa protein is found in nuage material within a 200 nm band of the cytoplasmic face of the perinuclear zone. Much smaller amounts of vasa are also observed as diffuse labelling in the germ cell cytoplasm (Fig. 6A-D).

We analyzed vas^{HE} and vas^{AS} ovaries with EM immunohis-

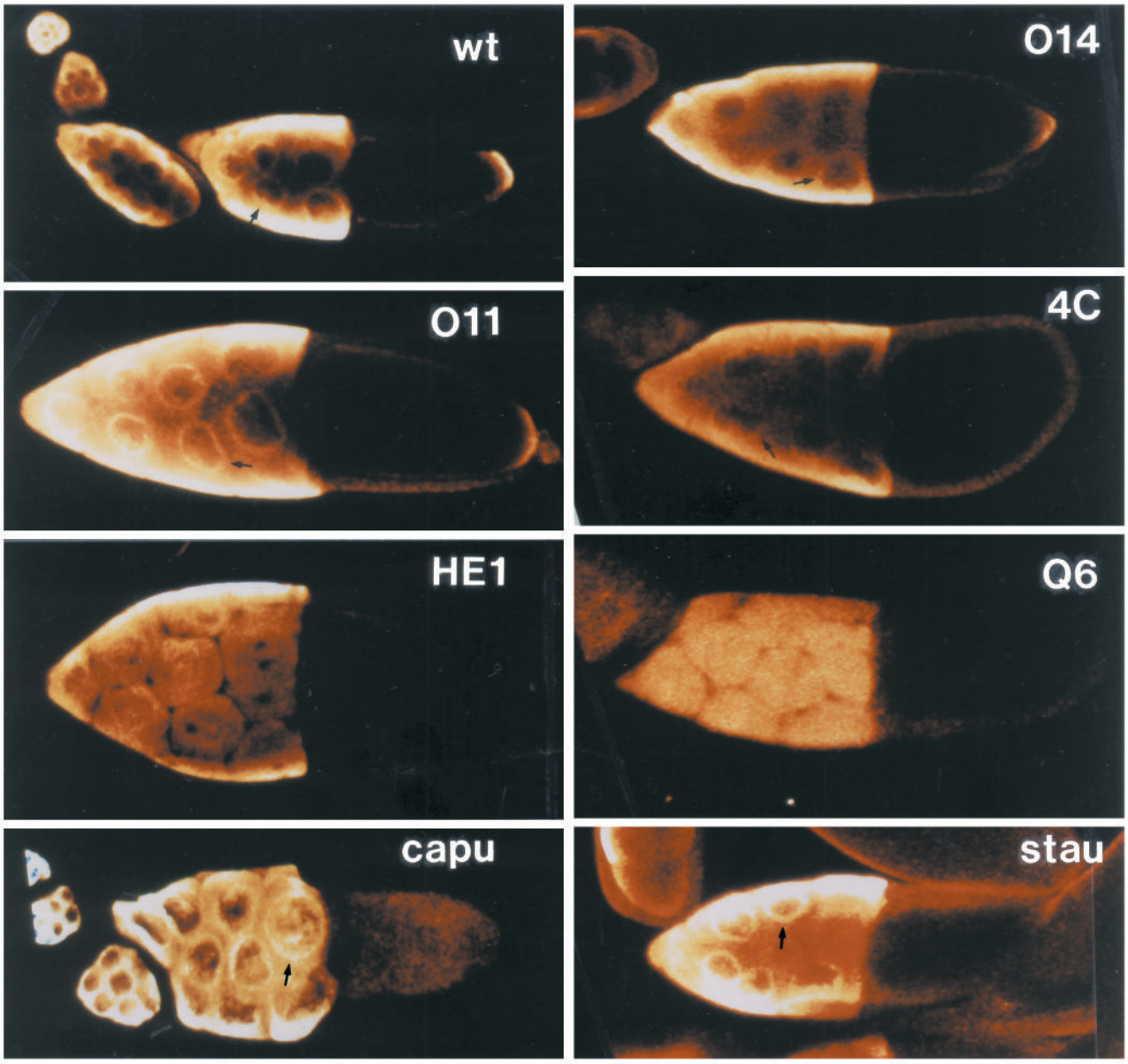

Fig. 5. Localization of vasa in wild-type and mutant stage-10 egg chambers as visualized by confocal laser scanning microscopy. Immunocytochemistry was carried out as described in Materials and Methods. The arrows indicate the high concentration of vasa in the perinuclear region of the nurse cells. The *vas* alleles were all *trans*-heterozygous with *Df(2L)A72*; similar results to those shown for vas^{HE1} and vas^{Q6} were obtained from vas^{AS}, vas^{PW}, vas^{D5} and vas^{3F}. The apparent reduction of perinuclear staining in vas^{O14} is a photographic artifact; focusing was optimized for posterior localization when present. Other genotypes are as follows: wt, Oregon-R; capu, $capu^{RK}/capu^{RK}$; stau, $stau^{G2}/stau^{HL}$. Identical results to those shown for *capu* and *stau* were obtained with osk^{54}/osk^{54} and $spir^{RP}/Df(2L)TW2$ ovaries (data not shown).

tochemistry, and observed that the delocalization of vasa protein observed in the confocal images is also evident at the ultrastructural level. vasa protein is observed as diffuse staining in both the nucleoplasm and the cytoplasm of the nurse cells (Fig. 6E,F). More importantly, the nuage particles, which are readily seen around the outer surface of the nuclear membrane in wild-type nurse cells, are very much less in evidence in the *vas* mutants that fail to exhibit perinuclear protein localization (contrast panel E with panels A and C). These results suggest that *vas* function is essential for the formation and/or structural integrity of perinuclear nuage particles of which vasa protein is a component.

The two mutant vasa proteins which still localize to the pole plasm are defective in RNA binding

The failure of pole cell formation in embryos from females bearing any of the seven *vas* alleles which are abrogated in posterior localization can be explained solely on the basis of

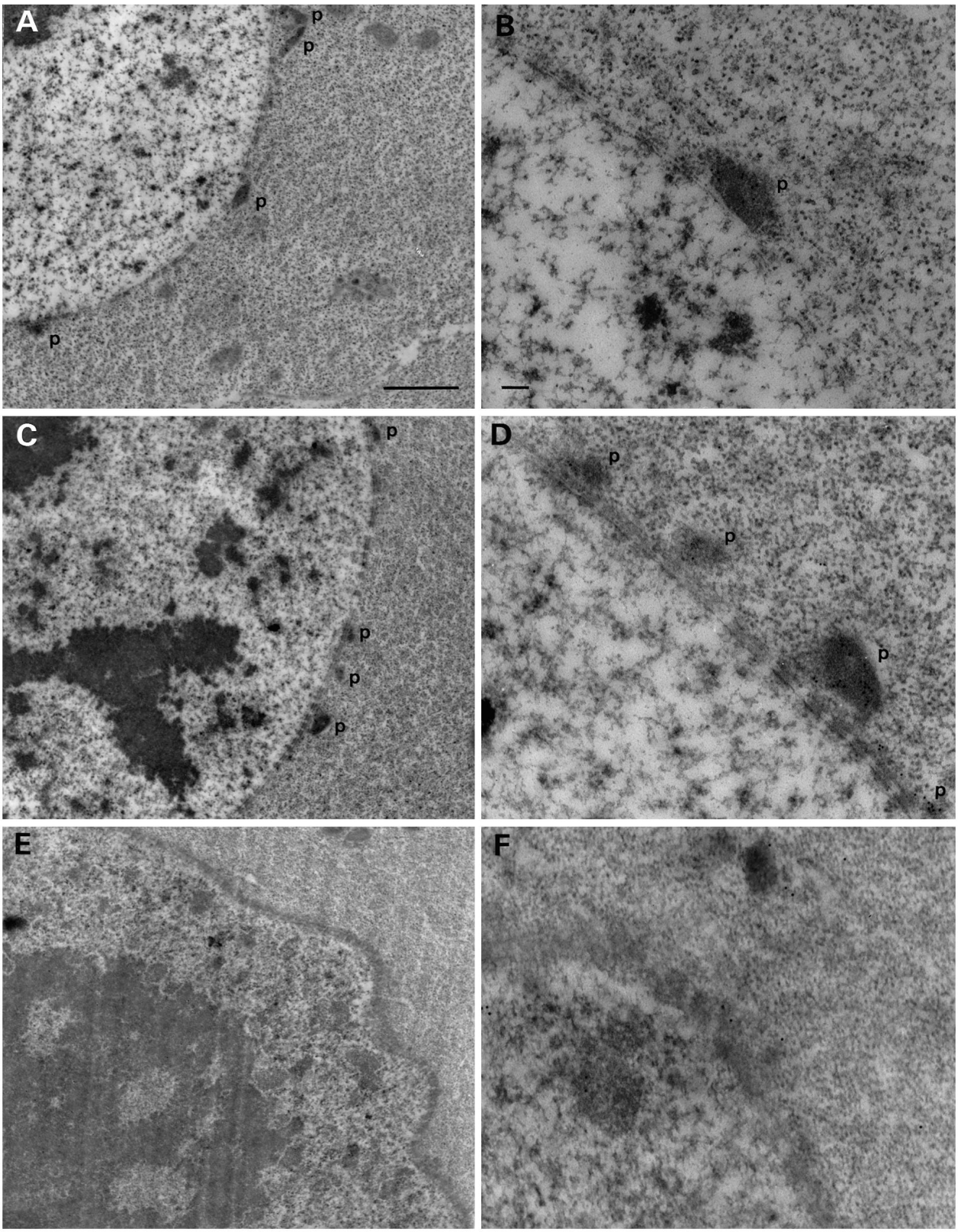

Fig. 6. Electron micrographs of thin sections of ovaries stained for vasa protein using anti-vasa antiserum and colloidal gold-conjugated secondary antibodies; p, nuage particle. (A,B) Wild-type stage 2 nurse cell. Nuage particles are apparent near the cytoplasmic face of the nuclear membrane, and most of the immunoreactive vasa protein is found in these particles. (C,D) Wild-type stage 6 nurse cell. (E,F) Nurse cell from a *vas*^{HE1}*/Df(2L)A72* ovary at a slightly later stage than C,D. Note the absence of perinuclear nuage particles, and the presence of scattered grains corresponding to vasa protein in both the nucleus and the cytoplasm. Bars (A,C,E), 1 μm; (B,D,F) 0.1 μm.

that failure to localize. However, the Vas^{O11} and Vas^{O14} proteins, which carry out normal localization, still fail to function in pole cell formation. Understanding the defects in these two proteins will identify activities of vasa that are essential in addition to posterior localization for mediating pole cell formation. To do this, we introduced the *vas^{O11}*, *vas^{O14}* and two other point mutations individually into the vasa expression construct, purified these mutant proteins and assayed them as above for ATPase activity, RNA-binding activity, and RNA helicase activity. In addition to the two localizing alleles, we analyzed *vas^{AS}*, which alters a conserved histidine residue at position 520, and *vas^{D5}*, which changes the invariant glycine residue at position 552 (Fig. 4).

None of the mutant proteins examined was compromised in ATP hydrolysis activity (Fig. 7). This is consistent with previous assignments of the ATPase A, DEAD and HRIGRXXR motifs to that function (Pause and Sonenberg, 1992); none of our mutations affect these motifs. The effects of the mutations were more striking with respect to RNA-dependent activities. Both the Vas^{D5} and Vas^{AS} proteins bind in vitro synthesized pRP40 RNA, *nos* mRNA and mtlrRNA at least as well as does the wild type; however, the Vas^{O14} and Vas^{O11} proteins show severely reduced RNA-binding activities on all these transcripts (shown for pRP40 and mtlrRNA in Fig. 8A,B). Of the two mutant proteins that retain RNA-binding activity, only Vas^{AS} functions as an RNA helicase (Fig. 8C,D). The mutant proteins that assayed negatively for RNA binding also fail to exhibit significant RNA-unwinding activity. We conclude from these results that the initial localization of vasa to the pole plasm is independent of its RNA-binding and -unwinding activities, but that subsequent interactions between localized vasa and RNA are essential for pole cell formation.

DISCUSSION

Functions of conserved DEAD-family residues

Our investigation of nine EMS-induced *vas* alleles has uncovered amino acids important for the biochemical function and intracellular localization of vasa protein. Mutant alleles of only one other DEAD-box protein, eIF-4A, have been biochemically characterized, and various highly conserved amino acid motifs have been implicated in ATP binding (AXXXXGKT), ATP hydrolysis (DEAD, HRIGR), RNA binding (HRIGR) and RNA unwinding (SAT) (Schmid and Linder, 1991; Pause and Sonenberg, 1992; Pause et al., 1993). As *vas^{D5}* alters the highly conserved ARGXD domain, the failure of the protein produced by that mutant to unwind RNA implicates this motif as essential for RNA helicase activity. Furthermore, the two conserved amino acids altered in Vas^{O14} and Vas^{O11} (Ile^{256} and Ile^{271}) are required for RNA-binding activity.

In addition to the residues implicated in interactions with RNA, other residues in the protein are required for in vivo function and for protein localization. Vas^{AS}, which is altered at His^{520}, retains all the enzymatic functions of vasa for which we assayed, so is presumably mutant in vivo solely because of its failure to localize. This mutation (and the nearby *vas^{PW}* mutation, which also produces a protein which fails to localize) may identify a structural feature of vasa required for protein-protein interactions leading to its localization (see below). Vas^{AS} and Vas^{D5} show somewhat higher activities in all our assays than the wild-type vasa protein. As we discuss below, vasa protein is found in vivo in multimolecular complexes. Other molecules present in these complexes may increase the activity and/or confer substrate specificity on the wild-type vasa protein; perhaps these two mutant proteins, which fail to associate in complexes, have a reduced dependence on such co-factors.

Nuage as a precursor to the polar granules

Vasa is a component of both perinuclear nuage and polar granules (Hay et al., 1988a, this work), and in *vas* mutants in which perinuclear localization of vasa does not occur, nuage is not observed. Furthermore, we observe no case in which

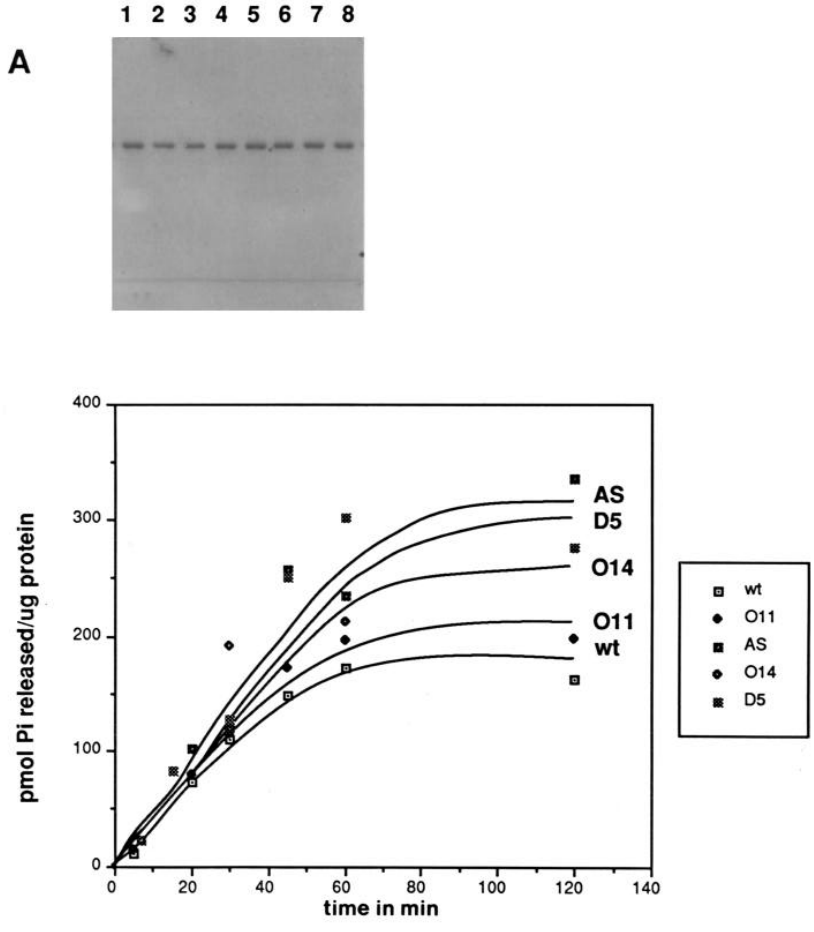

Fig. 7. (A) SDS-PAGE analysis of wild-type and mutant vasa proteins purified from expression constructs as in Fig. 1. Lane 1, wild-type; lanes 2 and 3, Vas^{O11}; lane 4, Vas^{O14}; lanes 5 and 6, Vas^{D5}; lanes 7 and 8, Vas^{AS}. (B) ATPase activities of wild-type and mutant vasa proteins, assayed as in Fig. 2.

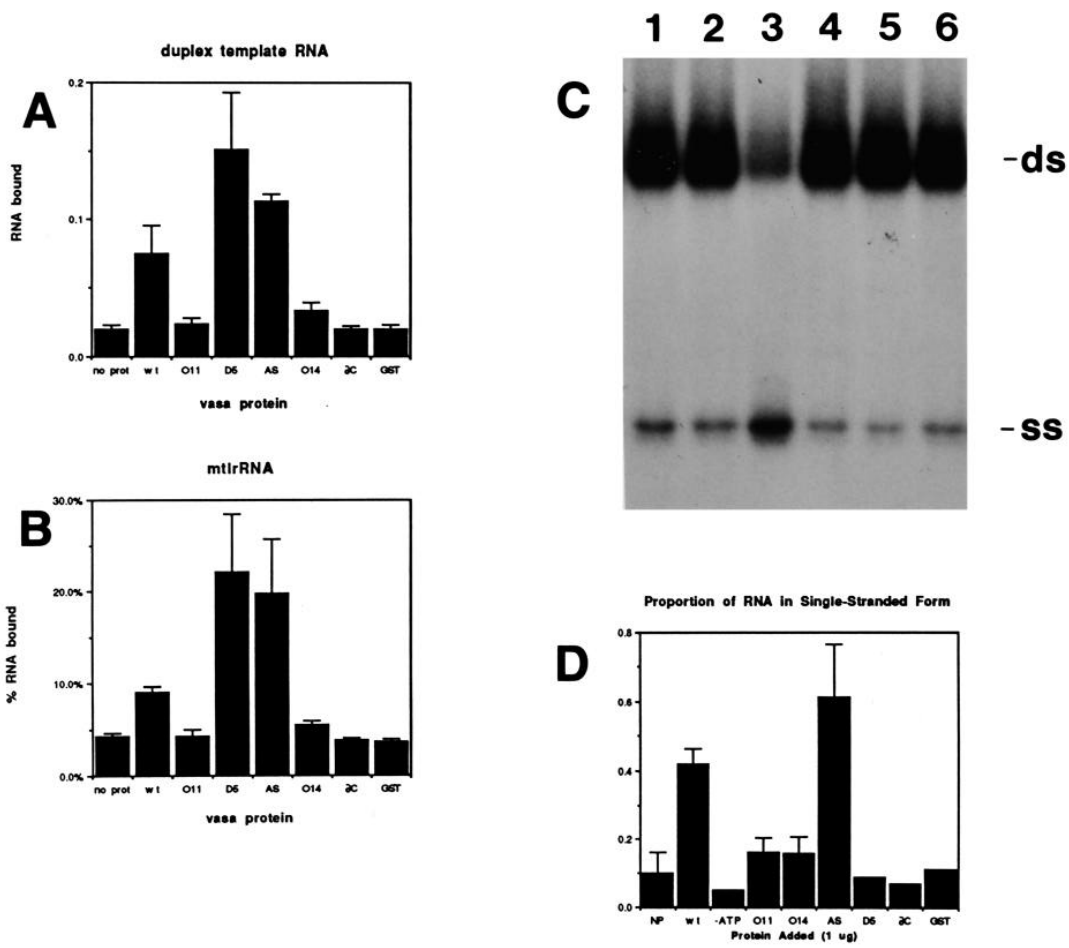

Fig. 8. (A) RNA-binding activity of wild-type and mutant vasa proteins assayed on the pRP40 construct as in Fig. 3. The y-axis gives the proportion of total RNA bound to the filter and the error bars indicate the standard deviations calculated from four determinations. δC, a truncated vasa construct lacking 227 amino acids at the C terminus; GST, glutathione-S-transferase control. (B) A similar set of RNA-binding assays as in A, but using in vitro-transcribed mitochondrial large ribosomal RNA, which is concentrated in the pole plasm (Kobayashi et al., 1993). (C) RNA-unwinding activity of the mutant vasa proteins, assayed as in Fig. 3 on pRP40 RNA. Lane 1, Vas^{O11}; lane 2, Vas^{O14}; lane 3, Vas^{AS}; lane 4, Vas^{D5}; lane 5, δC; lane 6, GST. Only the Vas^{AS} protein retains RNA-unwinding activity. (D) Quantitation of RNA helicase activity of the various vasa proteins by densitometric tracing of three experiments such as that pictured in C.

vasa is not concentrated in the perinuclear region of the nurse cells and yet posterior localization of vasa occurs. Thus it appears that the assembly of nuage, which is organized by vasa, is a necessary step in pole plasm assembly, and further that the posterior localization of vasa involves the translocation of vasa-containing nuage particles, not simply vasa itself, to the posterior pole of the oocyte. This is consistent with earlier observations that identified stages 9 and 10 as the initial phase of polar granule assembly and proposed a relationship between the assembly of nuage in the nurse cells and the formation of polar granules (Mahowald, 1962, 1971; Hay et al., 1988a). Our data show that the perinuclear localization of vasa early in oogenesis and its later localization to the pole plasm both depend on *vas* function. Furthermore, both of these localization events must be independent of RNA binding, as the Vas^{O11} and Vas^{O14} proteins, which we show to be greatly reduced in RNA binding, localize to the nuage and to the pole plasm.

The early perinuclear localization of vasa is independent of the functions of the four genes required for its later movement to the posterior pole; namely, *capu, spir, osk* and *stau*. Consistent with this, neither *osk* RNA nor staufen co-localizes with vasa in early oogenesis to the perinuclear region of the nurse cells although tudor protein does, at least at the level of light microscopy (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991; Bardsley et al., 1993; M. Mahone and P. Lasko, unpublished observations). Although *tud* mutations do not affect perinuclear localization of vasa (Lasko and Ashburner, 1990), it will be important to determine whether *vas* mutations affect the perinuclear localization of tudor and whether tudor co-localizes with vasa in the nuage particles. Although posterior localization of tudor depends on *vas* function and not the converse, tudor concentrates at the posterior pole of the oocyte at about the same time as vasa, suggesting coordinate movement of these two molecules, perhaps as components of nuage particles (Bardsley et al., 1993).

Posterior localization of vasa may depend on an interaction with oskar

Models for assembling the pole plasm consistent with the available data propose a stepwise association of the posterior-localizing molecules (Ephrussi and Lehmann, 1992). The first molecules to localize to the posterior pole, *osk* mRNA and staufen protein, do so during stages 8-9, followed a bit later by vasa and tudor proteins (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991; Hay et al., 1990; Lasko and Ashburner, 1990; Bardsley et al., 1993). Since vasa is an RNA-binding protein, the establishment of its posterior localization could involve its binding to a localized mRNA. Our results argue against such a model, as the Vas^{O11} and Vas^{O14} proteins localize to the pole plasm despite their inability to bind RNA. Consistent with this are earlier results showing that vasa fails

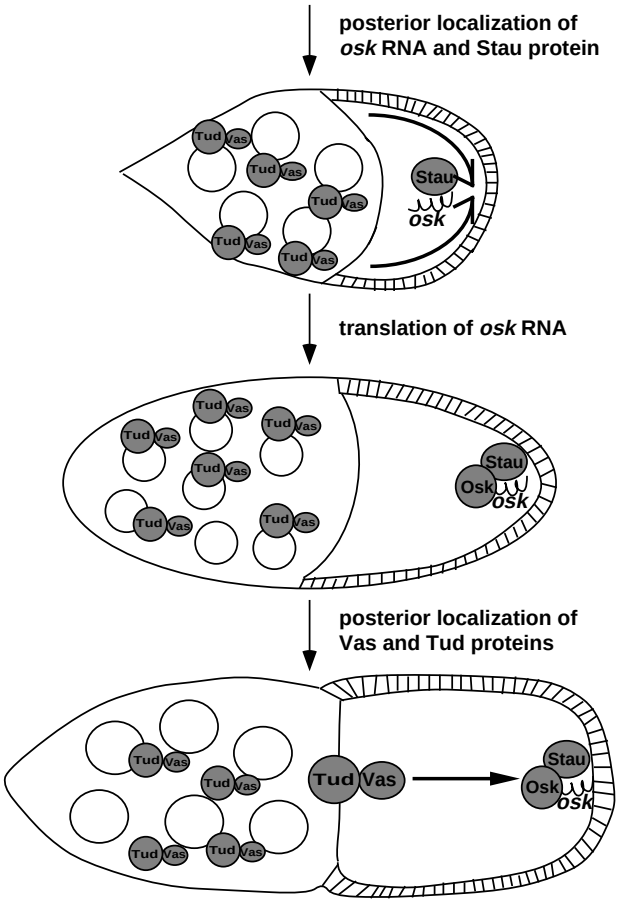

Fig. 9. A model for the initial stage of pole plasm assembly during stages 9 and 10 of oogenesis. Translation of a functional oskar protein in the pole plasm is required for an association with vasa protein. There is as yet no evidence indicating a direct interaction between vasa and tudor proteins; the model places them together on the basis of their similar subcellular localization in the nurse cells and the similar time of their posterior localization (Lasko and Ashburner, 1990; Hay et al., 1990; Bardsley et al., 1993).

to localize in mutant *osk* alleles which express a localized *osk* transcript (Hay et al., 1990; Lasko and Ashburner, 1990; Ephrussi et al., 1991; Kim-Ha et al., 1991).

We therefore favor an alternative model, which suggests that vasa localization to the pole plasm is dependent on an interaction between the vasa and oskar proteins (Fig. 9). Two pole plasm proteins, oskar and staufen, are themselves localized at the time of vasa localization (St Johnston et al., 1991; Lehmann, 1992), so either or both of these are candidate molecules for mediating vasa protein localization. For the following reasons, we think that oskar is the more likely possibility. First, vasa fails to localize in all *osk* mutant alleles; even those, like osk^{166} and osk^{301}, in which staufen is localized (Lasko and Ashburner, 1990; St Johnston et al., 1991). Second, *osk* and *vas* functions are required for the formation of ectopic pole cells in *osk-bcd*3′UTR embryos but that of *stau* is not (Ephrussi and Lehmann, 1992). Finally, the kinetics of vasa localization are more easily explained on the basis of an interaction with oskar; it is conceivable that the delay between the posterior localization of osk RNA to the pole plasm during stages 8-9 and the localization of vasa and tudor in stages 9-10a may be accounted for by the time necessary to translate *osk* mRNA in the pole plasm (Lehmann, 1992); staufen protein localization clearly precedes that of vasa (St Johnston et al., 1991; Lasko and Ashburner, 1990).

Our data do not exclude a role for staufen in the maintenance of vasa localization as has been proposed previously (St Johnston et al., 1991). Moreover, we believe that the potential oskar-vasa interaction only underlies the initial posterior localization of vasa. Interactions between vasa and downstream RNAs and proteins are essential for the maintenance of vasa localization and for specifying the germ line. This is supported by our results which indicate that RNA binding is required in addition to posterior localization for vasa to function in the pole cell determination pathway, and by our observations that posterior localization of vasa is not maintained after fertilization in vas^{O14} or vas^{O11} embryos. We expect that vasa interacts in the polar granules with specific RNA molecules and believe that our inability to demonstrate any specific vasa-mRNA interaction using purified bacterially expressed vasa in an in vitro assay system suggests that, in vivo, vasa operates in concert with other pole plasm proteins in carrying out its downstream functions. In this context, it is important to note that other DEAD-box proteins, such as eIF-4A and PRP5, primarily function in multisubunit complexes (Rozen et al., 1990; Pause and Sonenberg, 1992; Ruby et al., 1993). Interactions among vasa and other pole plasm proteins and RNAs, possibly analogous to those in the spliceosome or 43S initiation complex that involve PRP5 or eIF-4A, may underlie downstream regulatory events leading to pole cell formation. Further experiments to isolate and characterize the components of vasa-containing complexes directly from oocytes will be fundamental to an understanding of these events. As germ-line-specific nuage and structures related to polar granules are widely conserved throughout animal evolution (Smith, 1966; Eddy, 1975; Strome and Wood, 1983), and as a murine protein with extensive similarity to vasa and expressed in primordial germ cells has recently been reported (T. Noce, personal communication), this work should provide insight into basic mechanisms of germline development likely to be ubiquitous among metazoans.

We are very grateful to Mark Metzstein and Charles Wei for help in the early stages of the sequencing of the *vas* alleles, and to Beat Suter and Dana Lasko for helpful comments on the manuscript. We thank Ruth Lehmann, Francisco Pelegri and Trudi Schüpbach for fly strains, Arnim Pause and Nahum Sonenberg for the pRP40 clone, Brian Dalby and David Glover for the *cyclin-B* clone, Tom Jongens for the *gcl* clone, Satoru Kobayashi for the mtlrRNA clone, Anne Ephrussi for the *osk* clone and Charlotte Wang for the *nos* clone. Our research is supported with funds from the Canadian Cancer Society through an operating grant from the National Cancer Institute of Canada to P. L., and with operating grants to P. L. from the National Science and Engineering Research Council, the Medical Research Council (with N. Sonenberg) and the Fonds pour la formation de chercheurs et l'aide à la recherche du Québec. P. L. is a Research Scientist of the National Cancer Institute of Canada.

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(Accepted 27 January 1994)