Posterior localization of *vasa* protein correlates with, but is not sufficient for, pole cell development

Paul F. Lasko[1] and Michael Ashburner

Department of Genetics, University of Cambridge, Cambridge CB2 3EH UK

The protein product of the *Drosophila* maternal-effect posterior group gene *vasa* is localized to the posterior pole of the oocyte and is sequestered by the pole cells as they form. It is, however, present at easily detectable levels throughout the oocyte and pre-blastoderm embryo. The protein is present in the pole cells and their germ line derivatives throughout all stages of development. An antiserum against this protein recognizes a pole-cell-specific antigen in seven other *Drosophila* species. Of six other maternal-effect loci essential for embryonic pole cell development, none affects expression of *vasa*, mutations in four abolish *vasa* protein localization, and mutations in two, *tudor* and *valois*, have little, if any, effect on *vasa* expression or localization. This indicates that *vasa* protein, when properly localized, is not sufficient for induction of pole cell development, and that at least the *tudor* and *valois* wild-type functions are also required specifically for this process. These results are discussed with respect to the multiple functions of the *vasa* gene.

[*Key Words*: *vasa*; maternal-effect; posterior group; pole cells; germ line; *Drosophila*]

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Positional information in the *Drosophila* embryo requires the activities and correct regulation of about 60 maternal and zygotic genes (Akam 1987; Scott and Carroll 1987; Ingham 1988). The maternally active genes can be usefully grouped into five classes according to the phenotypes of embryos from mutant mothers: the anterior, posterior, and terminal groups, which are essential for specifying positional information along the anterior–posterior axis (Schüpbach and Wieschaus 1986; Nüsslein-Volhard et al. 1987; Manseau and Schüpbach 1989a), the dorsoventral group (Anderson 1987), and the bicaudal group (Bull 1966; Mohler and Wieschaus 1986). Genes of the anterior group are required for provision of head and thoracic information in the oocyte; genes of the posterior group are essential for abdominal development; and the genes of the terminal group are required for development of the extreme anterior and posterior ends of the embryo.

The bicaudal group is composed of four known loci: *bicaudal*, *Bicaudal-C*, *Bicaudal-D*, and *bicaudal-F* (Bull 1966; Mohler and Wieschaus 1986; Tearle and Nüsslein-Volhard 1987). Females with two mutant copies of either the *bicaudal* or *bicaudal-F* genes produce double-abdomen embryos at low frequency, and have normal fecundity. Females heterozygous or homozygous for most mutant *Bicaudal-D* alleles produce double-abdomen embryos; heterozygotes do so at low frequency; homozygotes do so at high frequency. However, females homozygous for the most phenotypically severe mutant allele of *Bicaudal-D*, *Bic-D*^{7134R}, completely fail to differentiate oocytes, and therefore lay no eggs. The *Bicaudal-D* gene has recently been cloned (Suter et al. 1989; Wharton and Struhl 1989). The *Bicaudal-C* gene is required for oocyte maturation; ovaries from females mutant for both copies of *Bicaudal-C* cease development at an early stage (Mohler and Wieschaus 1986). Females heterozygous for mutant alleles of *Bicaudal-C*, or for a deficiency including *Bicaudal-C*, give rise to variable proportions of bicaudal progeny.

In addition to their role in the determination of the body plan, five members of the posterior group of maternally active genes are also needed for pole cell formation in the embryo. These genes are *oskar*, *staufen*, *tudor*, *valois*, and *vasa* (Boswell and Mahowald 1985; Lehmann and Nüsslein-Volhard 1986; Schüpbach and Wieschaus 1986). Mutations at two other loci, *cappuccino* and *spire*, affect both dorsoventral and anteroposterior patterning, and also abolish pole cell formation (Manseau and Schüpbach 1989b). Pole cells are the progenitors of the germ line, and the polar nuclei are surrounded by cell membranes three mitotic divisions prior to somatic cellularization (Mahowald 1962; Warn et al. 1985). Pole cells contain ribonucleoprotein particles called polar granules, which themselves are localized to the posterior pole during oogenesis (Mahowald 1962, 1968, 1971; Counce 1963).

The *vasa* gene, a member of this posterior class, has

[1]Present address: Department of Biology, McGill University, Montréal H3A 1B1 Canada.

been isolated and characterized (Lasko and Ashburner 1988; Hay et al. 1988a). Its activity is required only in the female germ line, and females homozygous for the EMS-induced vas^{PD} mutant allele give rise to progeny that lack both pole cells and abdominal segments (Schüpbach and Wieschaus 1986). Females deleted for both copies of the *vasa* gene fail to complete oogenesis and lay no eggs (Lasko and Ashburner 1988); however, they are viable. Males deleted for *vasa* are both viable and fertile. The predicted *vasa* protein includes extensive sequence similarity to murine eukaryotic initiation factor-4A and helps define the D-E-A-D family of putative RNA-dependent ATPases conserved from *Escherichia coli* to humans (Linder et al. 1989).

Results

Localization of vasa *protein in wild-type gonads*

The localization of the *vasa* protein in ovaries and testes is shown in Figure 1. In ovaries taken from late third-instar larvae, the *vasa* protein is restricted to a set of round cells located in a wide band around the circumference of the ovary, extending from just basal to the apical–basal midline to nearly the apical tip (Fig. 1a). We believe that these cells are the central cells, precursors to the oogonia (King 1970) on the basis of their position within the ovary and the complementarity of the anti-vasa staining pattern with that of an antibody directed against fasciclin III, which stains cells of the larval ovary destined to become somatic cells (Brower et al. 1981; Patel et al. 1987). The pattern of *vasa* protein in the pupal ovary is consistent with this identification (Fig. 1b), as columns of oogonial cells, arranged in rudimentary germaria, stain heavily with the antibody. The terminal filament cells at the apical ends of the ovarioles do not label, but some individual cells at the basal end of the ovary contain the *vasa* protein. These cells are probably oogonial cells that have not yet migrated into the ovarioles, or else germ line cells that have been excluded from the ovarioles and will later be lost.

vasa protein is abundant in the germaria of adult ovaries, and is also abundant around the pronurse cell nuclei throughout early stages of oogenesis (Fig. 1c). The *vasa* protein begins to be transported to the oocyte during stage 8 [Fig. 1c; stages are as defined by King (1970)], and by stage 10a, the protein has clearly begun to accumulate at the posterior pole of the oocyte (Fig. 1d). The nurse cells continue to stain, most intensively around their nuclei, but also quite strongly throughout their cytoplasm. No antigen is detected in or around the oocyte nucleus. Accumulation of *vasa* protein in the oocyte continues throughout oogenesis; the protein collects at the posterior end in a cap, but is also present at lower but easily detectable levels throughout the entire oocyte (Fig. 1e).

Male germ line cells also express *vasa*. Larval spermatogonial cells label for *vasa* protein, most heavily around their nuclear membrane (Fig. 1f,g). In the adult testes (Fig. 1h), cysts containing cells in the early stages of spermatogenesis contain the antigen. These cells are located mostly at the apical tip, but extend some distance along the concave face of the testis (Lindsley and Tokayasu 1980). Spermatids and mature sperm do not label.

The antibody reaction is dependent, in both ovaries and testes, on the presence of the *vasa* gene. Figure 1, i and j shows the result of staining germ line tissues from flies of the genotype *Df(2L)TE116-GW18/Df(2L)A267*, which lack all but a small part of one copy of the *vasa* gene (Lasko and Ashburner 1988). The mutant tissue completely fails to elicit a reaction, as do ovaries from homozygotes for any of three strong mutant alleles of *vasa* (vas^{D1}, vas^{Q7}, vas^{P808}). The total absence of *vasa* protein is correlated with major morphological changes in the ovary; very few oocytes complete development, most stopping at stages 8 or 9. Testes from *Df(2L)TE116-GW18/Df(2L)A267* males, however, appear to be morphologically normal, and these males are fertile, as are males of three other homozygous $vasa^-$ deletion genotypes [*Df(2L)TE146-GV5/Df(2L)TE116-GW18*, *Df(2L) fn30/Df(2L)TE116-GW18*, and *Df(2L)TE36-GW29/Df(2L)TE116-GW18*; Lasko and Ashburner 1988].

Localization of vasa *protein in the wild-type embryo*

The *vasa* transcript is abundant, but not localized, within the cleavage embryo, and essentially disappears by the cellular blastoderm stage (Lasko and Ashburner 1988; Hay et al. 1988a). The distribution of *vasa* protein, however, is strikingly different (Fig. 2). In early cleavage embryos (Fig. 2a) *vasa* protein is found in a shallow posterior–anterior gradient, with its highest concentration at the posterior pole, a decreasing gradient from about

Figure 1. (*a*) Fixed whole third-instar larval ovary stained with affinity-purified anti-vasa antiserum, as described in Materials and methods. Labeled cells are the central cells, precursors of the oogonia. (*b*) Fixed whole ovary taken from a pupa aged ~24 hr after puparium formation, and stained as in *a*. Stained cells are now grouped mostly into the columnar precursor germaria. Outside the focal plane in the basal region of the ovary are a small number of scattered stained cells, marked with an arrow. (*c*) Freeze-substituted Canton-S ovarian tissue stained as in *a*. Early stages of oogenesis from germaria to stage 9 are visible. (*d*) Freeze-substituted stage-10a egg chamber from Canton-S female, stained as in *a*. Posterior localization of *vasa* protein in the oocyte is now apparent. (*e*) Sectioned stage-14 oocyte from Canton-S female, stained as described in Materials and methods. (*f*) Freeze-substituted testis taken from a third-instar larva, stained as in (*a*). Only germ line cells contain the *vasa* protein. (*g*) High magnification view of a 16-cell spermatogonial cyst from a third-instar larva, stained as in *a*. The *vasa* protein is most abundant around the nuclei of the spermatogonial cells (*h*) Canton-S testis freeze-substituted and stained as in *a*; antibody labels cells at the apical tip. (*i*) Freeze-substituted ovarian tissue from a *Df(2L)TE116-GW18/Df(2L)A267* female, stained as in *a*. (*j*) Freeze-substituted testis from a *Df(2L)TE116-GW18/Df(2L)A267* male, stained as in *a*. Affinity-purified immune serum was used at dilutions of 1 : 400 (1 : 1000 for *a*, *b*, *f*, and *g*).

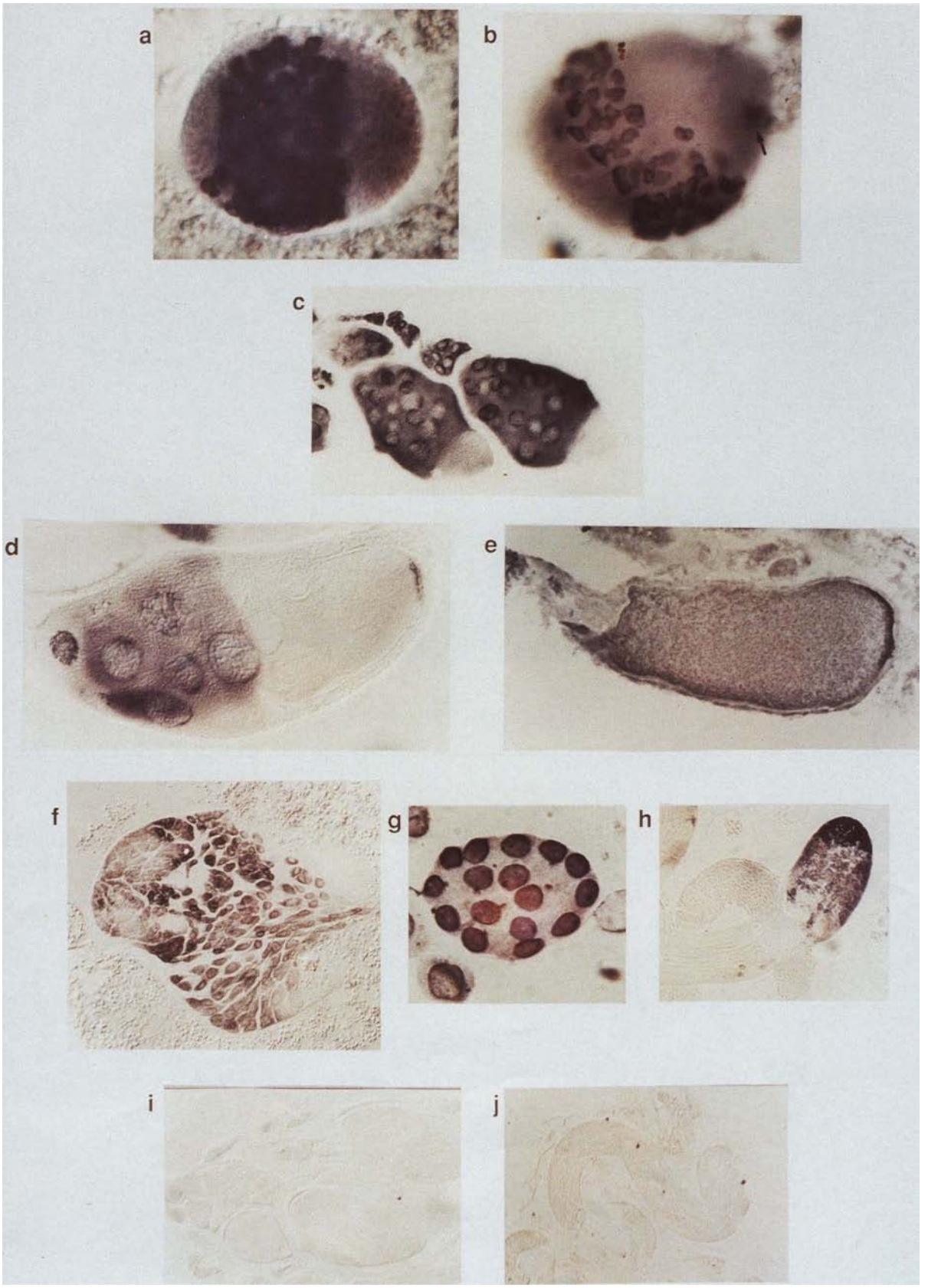

Figure 1. {*See facing page for legend.*}

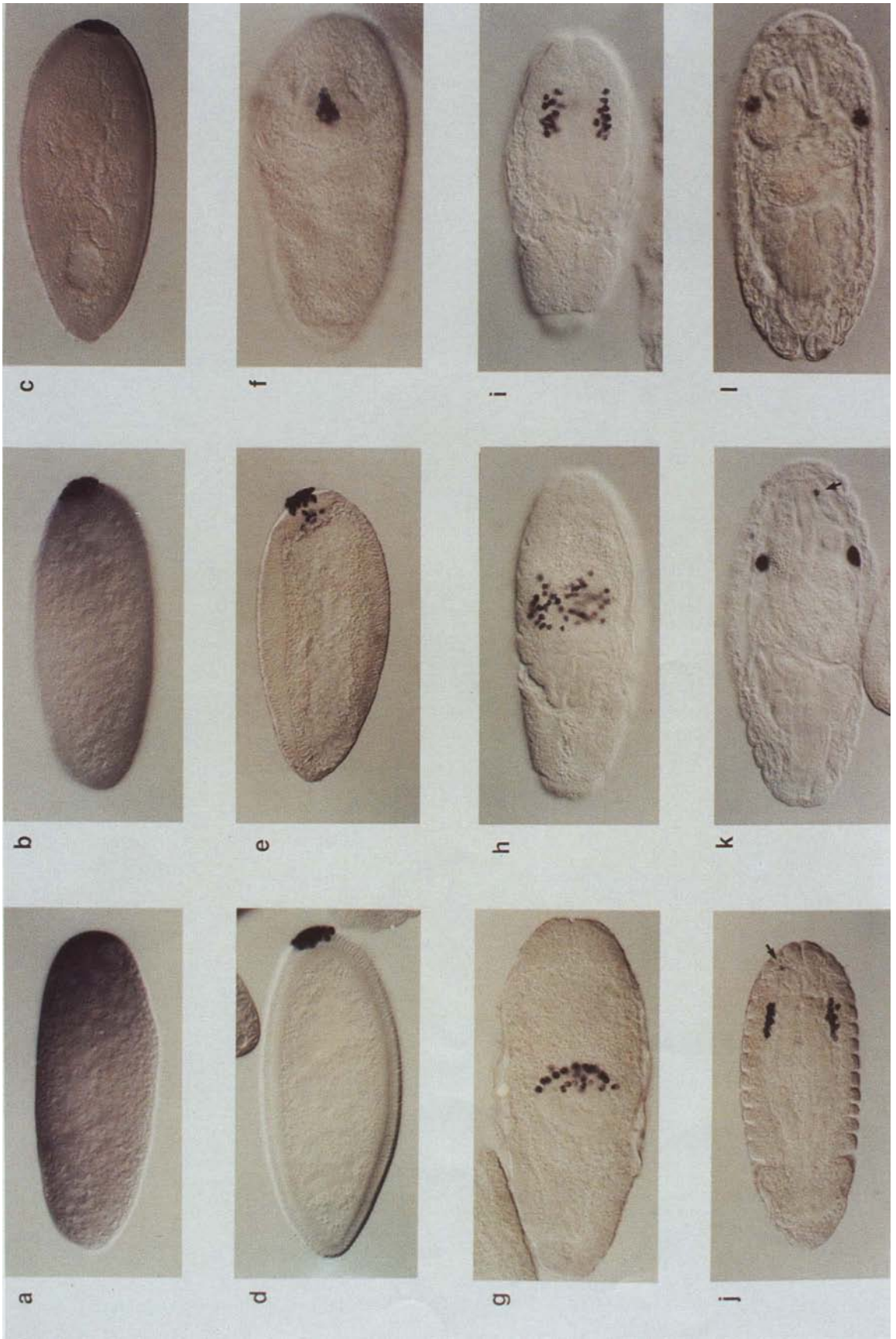

Figure 2. Canton-S embryos stained with affinity-purified anti-vasa antiserum as described in Materials and methods. Stages are as defined by Campos-Ortega and Hartenstein (1985). (*a*) Cleavage embryo, stage 2; (*b*) syncytial blastoderm, stage 4; (*c*) later syncytial blastoderm, stage 5; (*e*) early gastrulation, stage 6. Note the onset of pole cell migration in the embryo in *e*. (*f*) Germ-band elongation, stage 8, dorsal view; (*g*) stage 9, dorsal view. Pole cells are beginning to migrate away from posterior midgut primordium. (*h*) Late stage 10, dorsal view; (*i*) early stage 12, dorsal view; (*j*) late stage 12, dorsal view; (*k*) stage 14, dorsal view; (*l*) stage 16, dorsal view. Lost pole cells are marked with an arrow in *j* and *k*.

0–20% egg length, and a constant, easily detectable amount throughout the remainder of the embryo. At mitotic cycle 9 the posterior staining begins to localize to the pole buds, and by the syncytial blastoderm stage, the pole cells stain heavily (Fig. 2b,c). Throughout the cellular blastoderm stage, the pole cells stain very heavily (Fig. 2d).

vasa protein persists throughout the embryo, but rapidly decreases in concentration, until the onset of gastrulation (Fig. 2e), when the protein can only be seen in the pole cells. Labeling continues specifically in the pole cells, and later the gonads, throughout embryogenesis (Fig. 2 f–l). The antigen is cytoplasmic, rather than nuclear, within the pole cells. Pole cells can be seen to be lost during migration (Fig. 2j,k; Hay et al. 1988b).

The data presented in Figure 3 extend the characterization of the antiserum. Neither the preimmune serum nor the immune serum affinity-purified against glutathione-*S*-transferase protein gives any localized staining pattern (Fig. 3a,b). The immune serum recognizes a pole-cell-specific antigen in the following *Drosophila* species: *D. simulans*, *D. mauritiana*, *D. sechellia*, *D. yakuba*, *D. erecta*, *D. hydei*, and *D. virilis* (Fig. 3c–f; S. Frenk, unpubl.).

Expression and localization of vasa *protein in ovaries mutant for* vasa

As mentioned above, females deleted for both copies of *vasa*, or carrying a P-element-induced mutation, vas^{P808}, fail to complete oogenesis and lay no eggs. Females homozygous or hemizygous for either of two EMS-induced

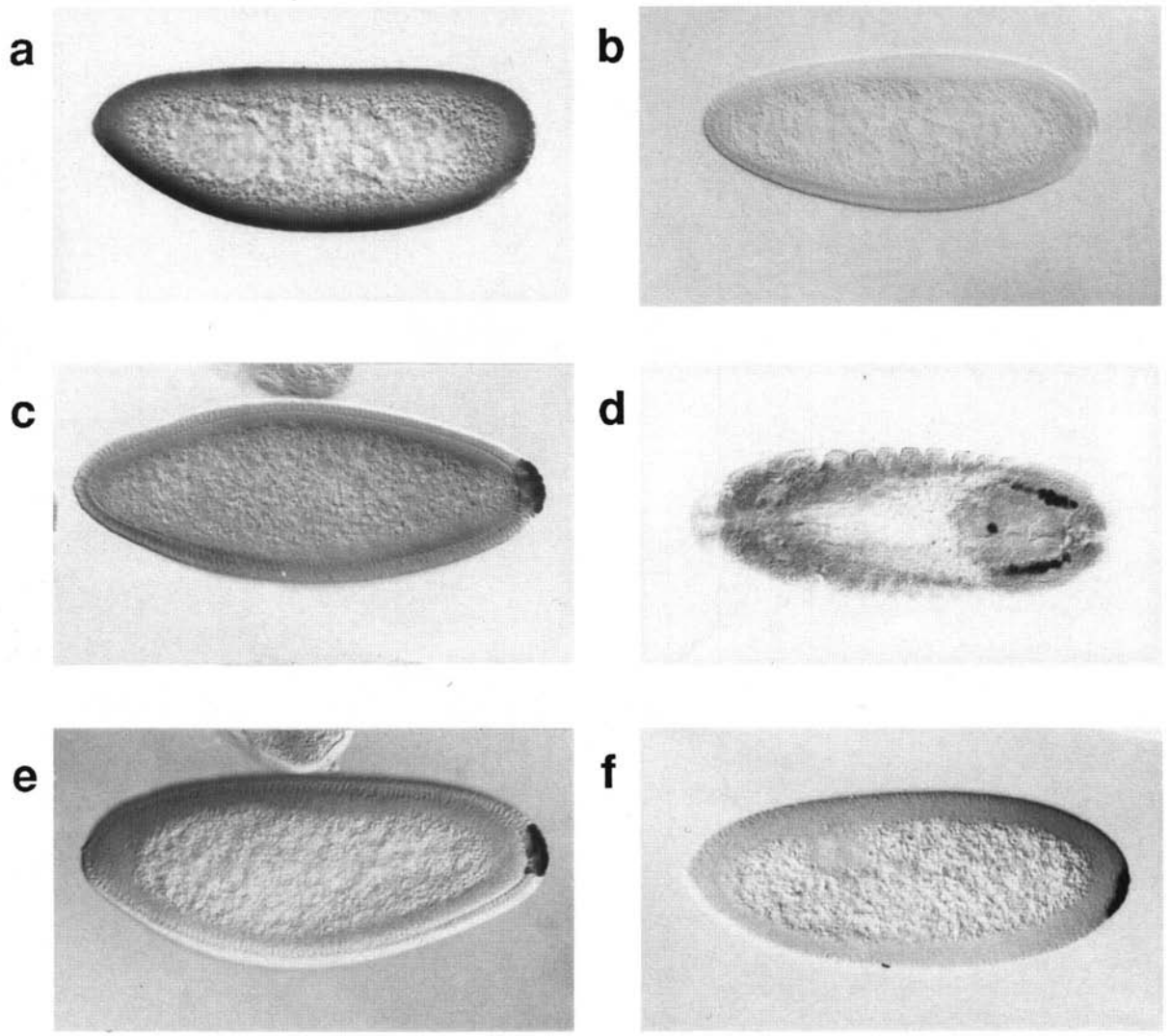

Figure 3. (*a*) Canton-S cellular blastoderm embryo treated with preimmune serum. Crude serum was used at a dilution of 1 : 200. The preimmune serum reacts with several bands on Western blots, not including the *vasa* protein, and gives some generalized background on fixed embryos. Secondary antibody alone gives no reaction. (*b*) Canton-S cellular blastoderm treated with immune serum affinity-purified against glutathione-*S*-transferase, diluted 1 : 400. (*c*) Embryos from four *Drosophila* species treated with anti-vasa antiserum as in Fig. 2. (*c*) *D. mauritiana*; (*d*) *D. simulans*; (*e*) *D. yakuba*; (*f*) *D. virilis*.

alleles, vas^{D1} or vas^{Q7}, also have undetectable amounts of *vasa* protein in their ovaries. These females must retain some residual activity of the gene, however, as they lay eggs at low frequency, some of which develop into embryos with abdominal deletions and no pole cells.

The expression and localization of *vasa* protein in other mutant alleles is examined in Figure 4. Four mutant alleles express the gene at normal levels throughout oogenesis (Fig. 4a,c). These include vas^{O14}, a weak allele, which, at 18°C, gives progeny that show no abdominal defects, but still lack pole cells, and three other alleles that exhibit the typical posterior-group phenotype. One of these latter alleles, vas^{AS}, shows greatly reduced posterior localization of the protein in the oocyte, whereas the other three localize *vasa* protein normally (Fig. 4b,d).

Despite its typical posterior-group phenotype, the

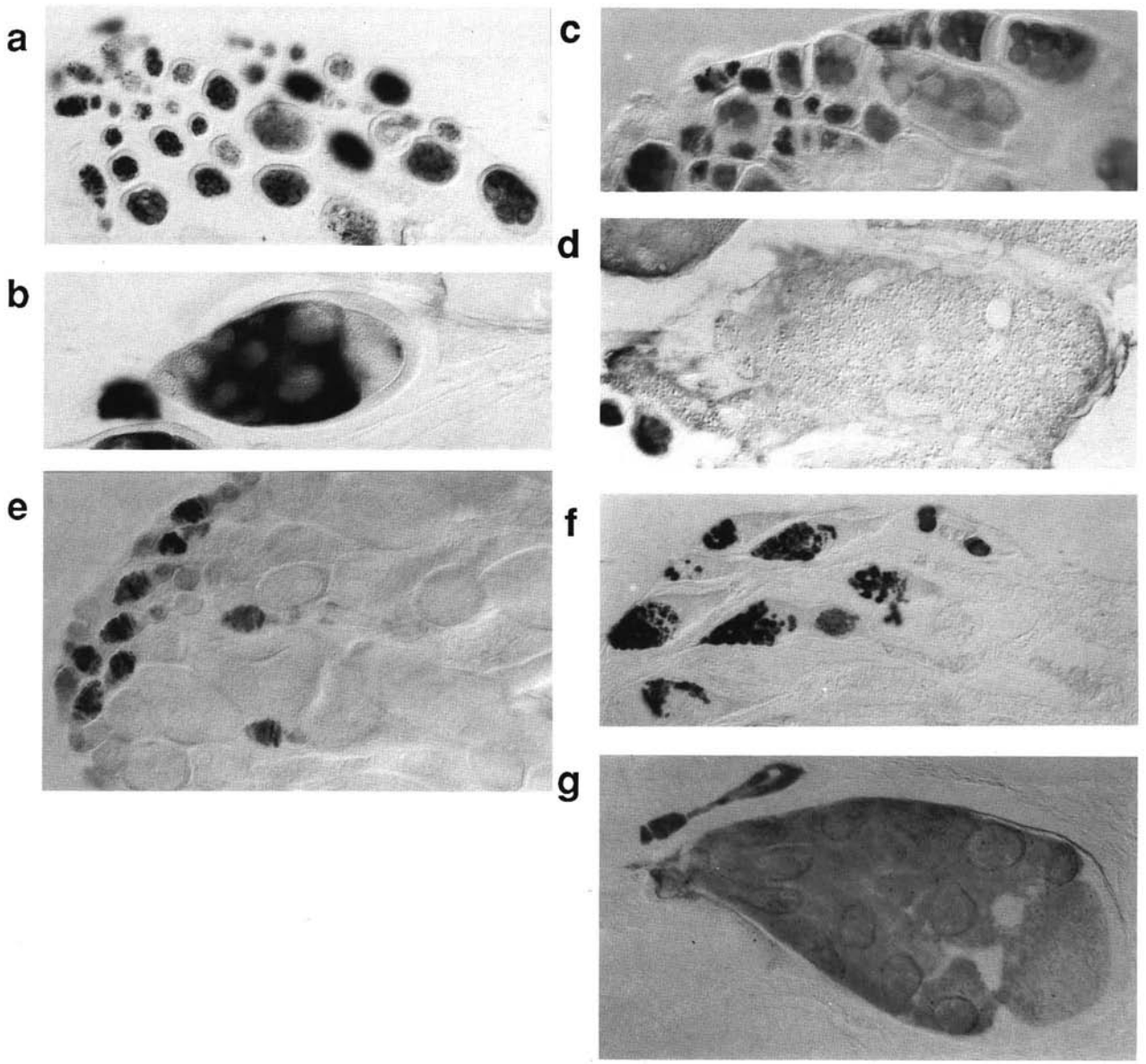

Figure 4. The effect of EMS-induced mutations at *vasa* on protein expression and localization. All tissue treated with affinity-purified anti-vasa antiserum, at a dilution of 1 : 400, as in Fig. 1. (*a*) Freeze-substituted ovarian tissue from a $vas^{O14}/Df(2L)A72$ female, showing normal pronurse cell expression. (*b*) Freeze-substituted stage-8 egg chamber from a $vas^{O14}/Df(2L)A72$ female, showing normal posterior localization of *vasa* protein in the oocyte. (*c*) Freeze-substituted ovarian tissue from a $vas^{AS}/Df(2L)A72$ female, showing normal pronurse cell expression. (*d*) Sectioned stage-14 oocyte from a $vas^{AS}/Df(2L)A72$ female. Posterior localization of *vasa* protein is very weak. (*e*) Freeze-substituted ovarian tissue from a $vas^{PD}/Df(2L)A72$ female. Only the germarium stage shows the presence of *vasa* protein. (*f*) Freeze-substituted ovarian tissue from a $vas^{D5}/Df(2L)A72$ female. Note the structures resembling abnormally large germaria that stain with the antibody. Also note the degeneration of the ovarioles. (*g*) Freeze-substituted ovarian tissue from a $vas^{D5}/Df(2L)A72$ female. The oocyte can be seen to be partly separated from the nurse cells and exhibits no posterior localization of antigen. All panels except *d* are reproduced at identical magnification.

vas^{PD} allele shows a unique pattern of expression. As is illustrated in Figure 4e, *vasa* protein is only detectable in this allele in the germarium stage of ovarian development. Females homozygous or hemizygous for this allele, however, lay wild-type numbers of eggs which, at high frequency, develop into embryos lacking abdominal segments and pole cells.

One final allele, vas^{D5}, leads to a different phenotype. Many vas^{D5} ovarioles consist only of a structure resembling an abnormally large germarium, containing supernumerary cells that stain heavily with the *vasa* antiserum (Fig. 4f). Other ovarioles develop egg chambers; however, these apparently have poor cell–cell adhesion, and are variable in morphology (Fig. 4g). The *vasa* protein is present at a constant low level throughout these egg chambers, with no posterior localization in the rudimentary oocytes, and only weak perinuclear localization in cystocytes and nurse cells. Homozygous or hemizygous vas^{D5} females lay no eggs.

The effects of mutations at other posterior-group genes on localization of vasa *protein*

The posterior group comprises six maternally-active genes in addition to *vasa*: *nanos*, *oskar*, *pumilio*, *staufen*, *tudor*, and *valois* (Boswell and Mahowald 1985; Lehmann and Nüsslein-Volhard 1986, 1987a; Schüpbach and Wieschaus 1986; Lehmann 1988). Progeny of females lacking the wild-type function of any of these genes lack abdominal segments. Embryos derived from *oskar*, *staufen*, *tudor*, and *valois* mothers completely lack pole cells, as do *vasa* embryos. Most, but not all, *vasa* embryos also fail to undergo cellularization of somatic nuclei (Schüpbach and Wieschaus 1986, 1989). The functions of the *nanos* and *pumilio* genes are not required for pole cell formation.

The phenotypic similarities seen in mutants of the various posterior group genes led us to consider the possibility of direct interactions among their wild-type products; examination of the effects of these other mutations on *vasa* expression and protein localization may give evidence for a temporal hierarchy of their functions. The expression pattern of *vasa* protein in ovaries lacking wild-type function of each of these six genes and in embryos derived from mutant mothers is shown in Figure 5. None of these mutations eliminates expression of *vasa*, as all the mutant ovaries express large amounts of *vasa* protein in early stages of oogenesis (Fig. 5a). As in wild-type cystocytes, the antigen is most highly concentrated in mutant cysts around the nuclear membranes but is present throughout the cytoplasm. Mutations in *staufen* may reduce perinuclear localization, in favor of additional protein in the cytoplasm. In *tudor*, *valois*, *nanos*, and *pumilio* oocytes, *vasa* protein localizes to the posterior pole, at the same stage of egg development as in the wild type, but in *oskar* or *staufen* oocytes, *vasa* protein, though exported to the oocyte, fails to distribute asymmetrically (Fig. 5a,b).

The difference in the distribution of *vasa* protein between *oskar* and *staufen* mutants and mutants in the other four genes continues after fertilization. Cleavage embryos from *tudor*, *valois*, *nanos*, or *pumilio* females exhibit posterior concentrations of *vasa* protein indistinguishable from that seen in the wild type, but *oskar* or *staufen* embryos stain uniformly with the antibody (Fig. 5c). By the blastoderm stage (Fig. 5d), pole cells are prominent in the *nanos* and *pumilio* embryos, and these stain heavily, like wild-type pole cells. In the four mutants that fail to form pole cells, *vasa* protein disappears by early gastrulation, like the unlocalized *vasa* protein in the wild-type embryo. This suggests that *vasa* protein located outside pole cells decays during blastoderm formation. Pole cell migration is normal in *nanos* or *pumilio* embryos until the time of germ-band shortening, after which the pole cells often fail to aggregate, scattering instead throughout the posterior half of the embryo (Fig. 5e). The effect of mutations at *bicoid* on *vasa* protein expression and localization is also shown in Figure 5. Like *nanos* and *pumilio*, *bicoid* is not required for *vasa* protein localization although, at germ-band shortening, the pole cells of embryos from *bicoid* mothers do not coalesce correctly into the gonads.

The effects of mutations at the Bicaudal-C *and* Bicaudal-D *genes on expression of* vasa *protein*

We examined the effect of these mutations on *vasa* expression and localization for two reasons. First, the duplication of abdominal information in the bicaudal embryos could be associated with an alteration in *vasa* protein localization. Secondly, the defects in oocyte maturation found in null alleles of *Bic-C*, and/or the failure of oocyte differentiation found in the most severe allele of *Bic-D*, may involve changes in *vasa* expression. The effects of mutations at these two bicaudal loci on *vasa* expression and localization is shown in Figure 6. Phenotypically wild-type embryos from *Bic-C*/+ females localize *vasa* protein normally throughout embryogenesis (Fig. a,b). Embryos with head defects do so as well, and form normal gonads (Fig. 6c,d). In phenotypically bicaudal embryos, *vasa* protein is never found concentrated at the anterior pole, and pole cells are found only in one abdomen. These pole cells mostly fail to coalesce into gonads (Fig. 6e). Also among the progeny of *Bic-C*/+ females are embryos that fail to cellularize, but which include scattered round structures that stain intensely with *vasa* antibody. In ovaries from *Bic-C* homozygotes, *vasa* protein is produced in the nurse cells as in the wild type (Fig. 6f). Antigen can sometimes be detected in the rudimentary oocytes that form in these females, but posterior localization of *vasa* protein in the oocyte is not seen. However, the oocytes cease development at approximately the stage that posterior localization of *vasa* protein would be first evident in the wild type.

Mutations at *Bic-D* have no effect on *vasa* protein expression. Both normal and bicaudal embryos from heterozygous or homozygous *Bic-D* females stain normally with the *vasa* antibody (Fig. 6g–i). Posterior localization of *vasa* protein in late oocytes of homozygous *Bic-D* fe-

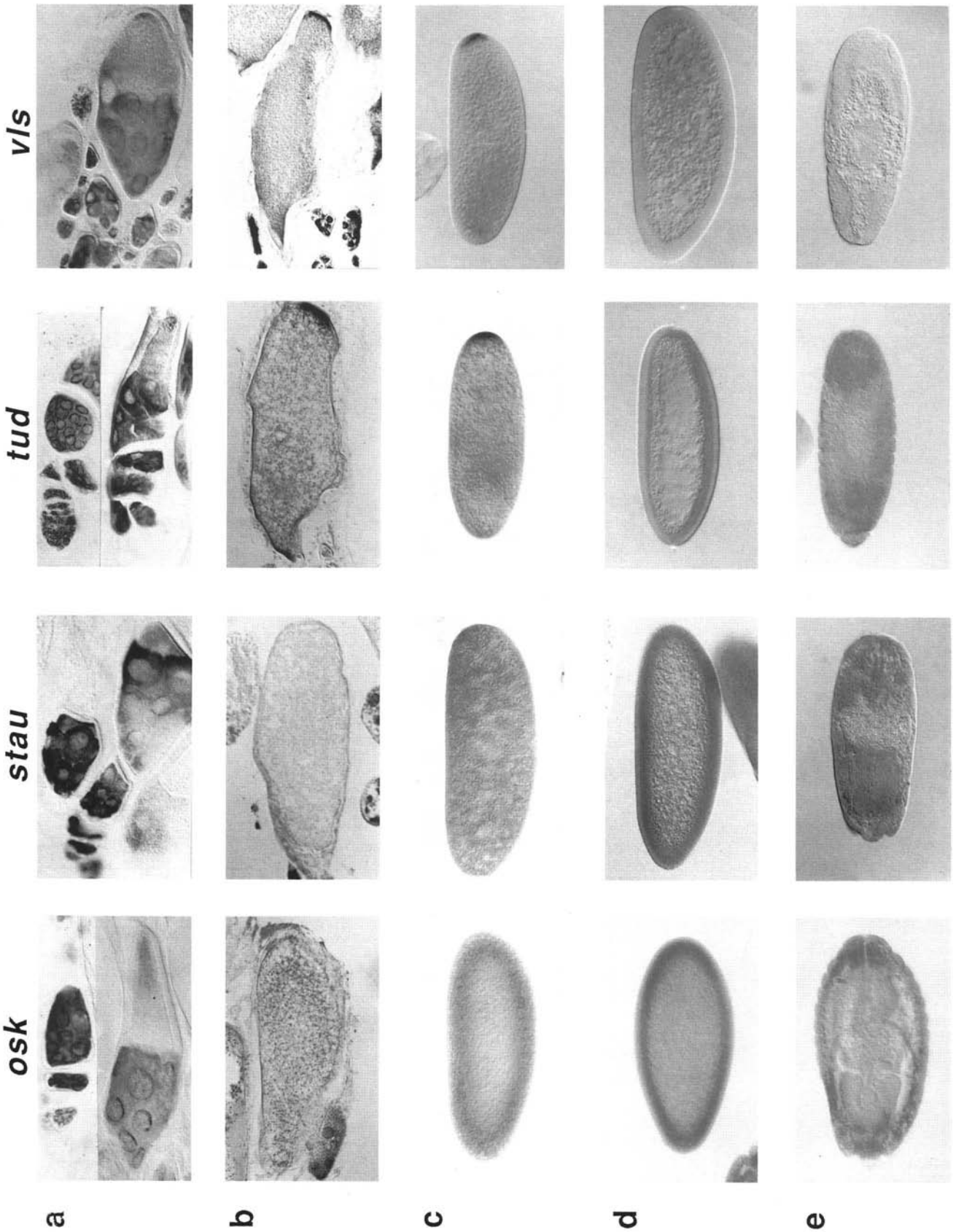

Figure 5. (*See facing page for remainder of figure and legend.*)

males is also normal (Fig. 6j), as is the expression in the pronurse cells of ovaries of *Bic-D*^{7134R} females (Fig. 6k). Concentration of antigen at the anterior pole of the oocyte or embryo is never observed. Similar results with *Bicaudal-D* mutants have recently been reported by Wharton and Struhl (1989), using a different antibody.

The effects of other oogenesis mutations on expression of vasa *protein*

The genes *cappuccino* and *spire* affect both dorsoventral and anterioposterior patterning; mutations in these genes also abolish pole cell and polar granule formation

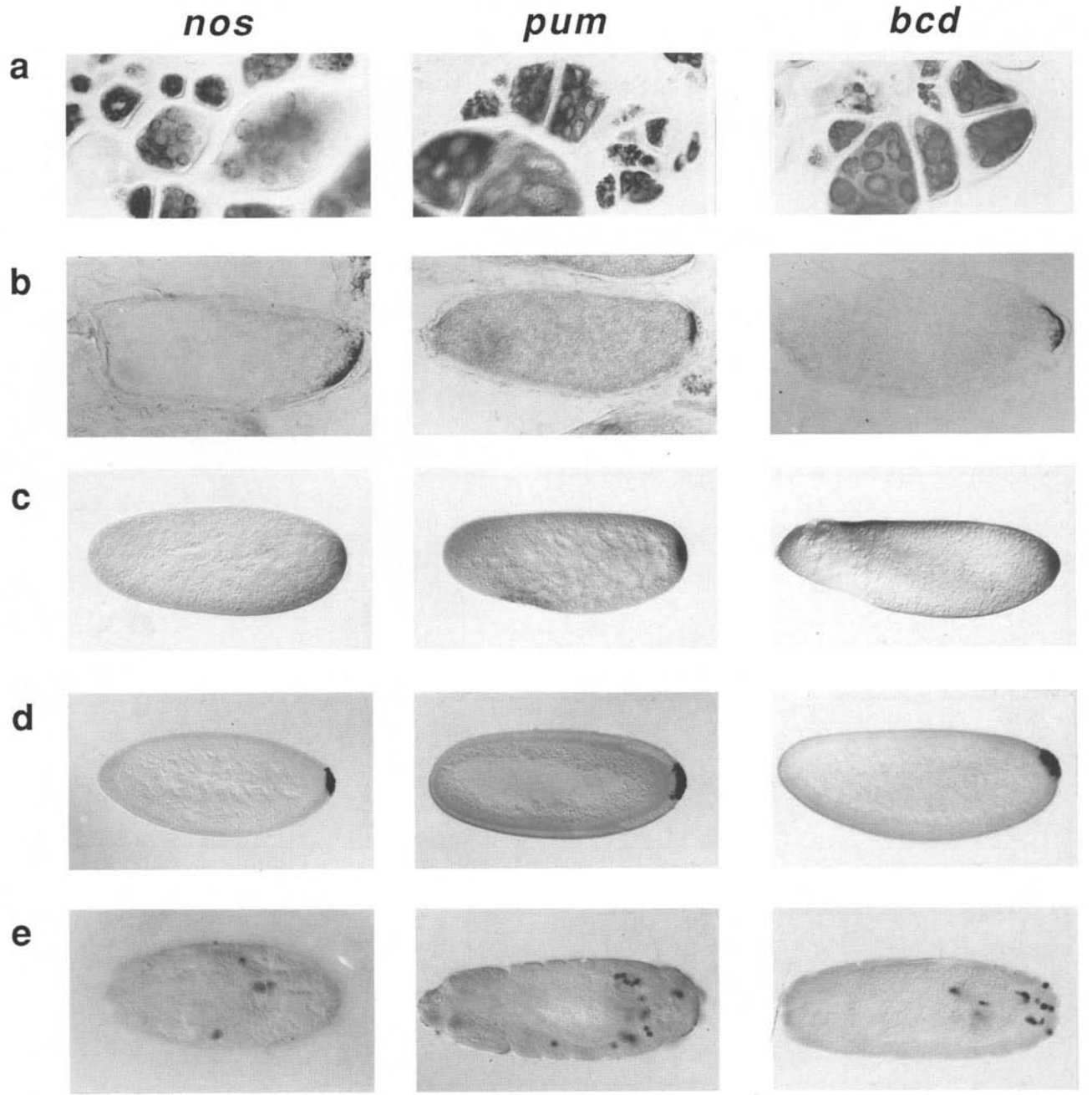

Figure 5. The effect of maternal-effect mutations on *vasa* expression and localization. All tissue treated with anti-vasa antiserum is as in Figs. 1 and 2. (*a*) Freeze-substituted ovarian tissue, as in Fig. 1a. (*b*) Sectioned late-stage oocytes, as in Fig. 1d. (*c*) Cleavage-stage embryos, as in Fig. 2a. (*d*) Blastoderm-stage embryos, as in Fig. 2d. (*e*) Late-stage (stage 10–14) embryos, as in Fig. 2, j and k. (*osk*) Illustrated material is from *osk*^{301}/*Df(3R)p*^{XT26} flies; *osk*^{166}/*Df(3R)p*^{XT26} gives identical results. (*stau*) Illustrated material is from *stau*^{G2}/*stau*^{HL} flies; *stau*^{G2}/*Df(2R)Pcl7B* gives identical results. (*tud*) Illustrated material is from *tud*^{WC} *homozygotes; tud*^{WC}/*Df(2R)Pu-rP133* gives identical results. (*vls*) *vls*^{PE}/*Df(2L)TW2* flies. (*nos*) Illustrated material is from *nos*^{L7} homozygotes; *nos*^{53} homozygotes give identical results. (*pum*) *pum*^{680} homozygotes. (*bcd*) Illustrated material is from *bcd*^{E1} homozygotes; *bcd*^{E1}*pum*^{680} females give similar results. Similar results to those illustrated have also been obtained with this antiserum in the following genotypes: (*osk*) *osk*^{301} homozygotes, raised at 18 or 29°C; (*stau*) *stau*^{G2}/*stau*^{C8}; (*tud*) *tud*^{WC}/*tud*^{B36}; (*vls*) *vls*^{RB} homozygotes (J. Raff, pers. comm.).

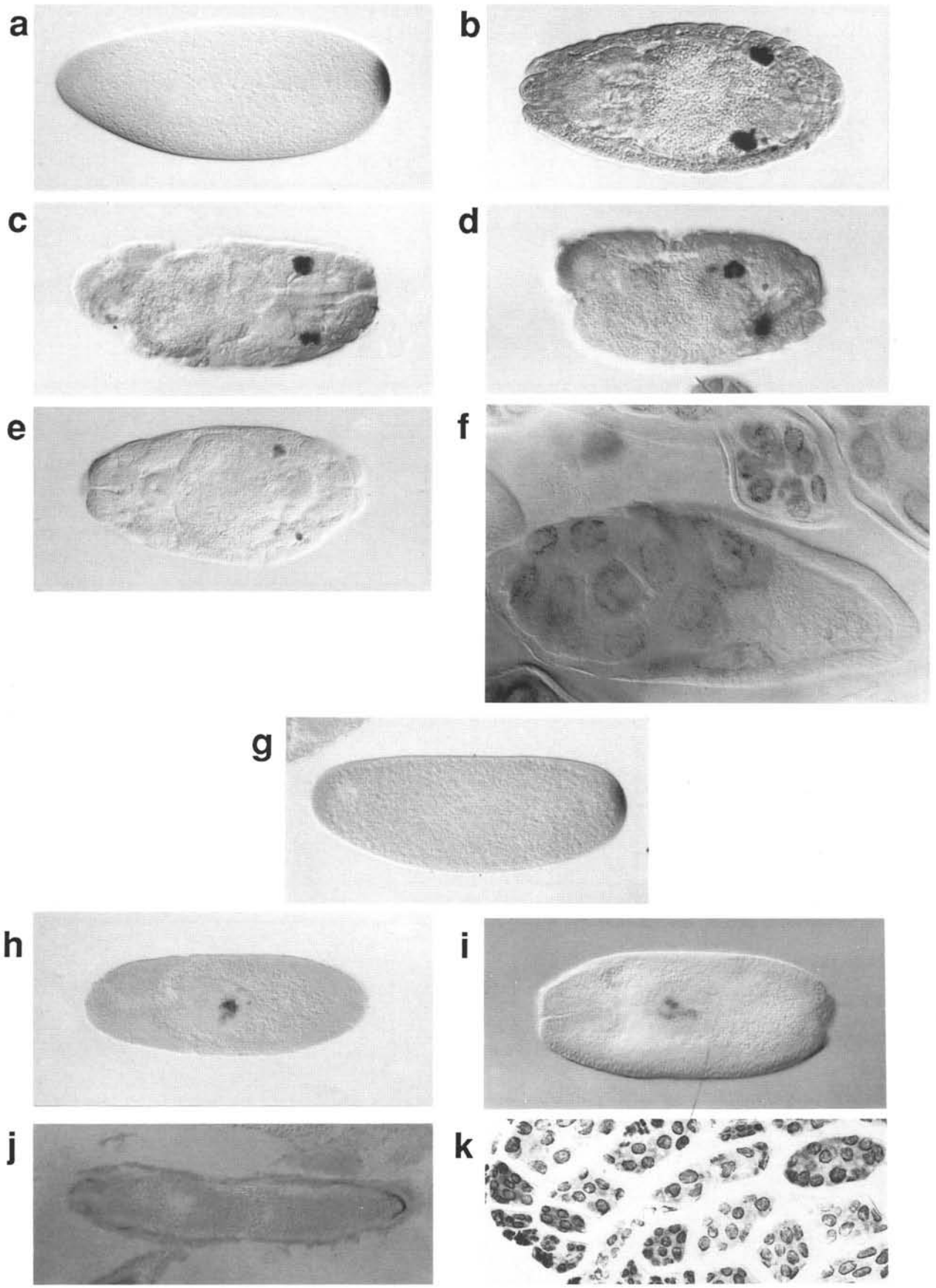

Figure 6. *(See facing page for legend.)*

(Manseau and Schüpbach 1989b). These authors also found that these mutations abolish localization of *vasa* protein to polar granules and pole cells, using a monoclonal antibody that recognizes *vasa* protein (Hay et al. 1988b; Manseau and Schüpbach 1989b). We confirmed their results, showing failure of posterior localization of *vasa* protein in late oocytes from these mutants (data not shown).

We also investigated a number of other mutations affecting oogenesis at different stages for changes in *vasa* protein localization, including *egalitarian*, *tiny ovaries*, *variable size and shape-1*, *fs(2)Y12*, *morula*, *spindle-C*, *bicaudal-F*, and *quail*. With the exception of *tiny ovaries*, which fails to produce any structures expected to contain *vasa* protein, all of these mutant ovaries express *vasa*. Those mutants that reach stage 8 of oogenesis also localize *vasa* protein to the posterior pole of the oocyte. A mutation of particular interest is *egalitarian*, which leads to the production of long chains of stage 6-like egg chambers, a phenotype similar to that of *Bic-D*^{7134R} homozygotes (Mohler and Wieschaus 1986). Like the strong *Bic-D* allele, early expression of *vasa* is normal in *egalitarian* ovaries.

Discussion

We isolated an antiserum that is monospecific for the product of the *vasa* gene and used it to study the effects of mutations at a variety of related loci on *vasa* expression pattern. It is striking that germ line cells, with the exception of mature sperm, contain the *vasa* protein at all stages of development. The *vasa* protein present in embryonic pole cells is maternally derived, as there is no zygotic transcription of *vasa* until the end of stage 12 of embryogenesis, around the time of primordial gonad formation (Hay et al. 1988a).

Our results support the conclusion that the major antigen recognized by the monoclonal antibody 46F11 (Hay et al. 1988b) is the *vasa* protein. However, the antiserum we have raised against the glutathione-*S*-transferase–vasa fusion protein does not recognize a smaller, 45-kD protein in ovaries, nor does it recognize a generally distributed nuclear antigen in late embryos. These further reactions seen with mAb46F11 could be the result of cross-reaction with another protein or proteins that perhaps share the extensive structural similarities of the D-E-A-D family with vasa (Linder et al. 1989).

Both mAb46F11 and our antiserum react with an antigen in the male germ line, and Hay et al. (1988a) have localized transcripts of *vasa* in testes by in situ hybridization. Testes taken from males deficient for the *vasa* gene completely lack this antigen, confirming the presence of *vasa* protein in the male germ line. Furthermore, this indicates that the testes-specific protein cannot be due to persistence of maternal *vasa* protein, and must result from zygotic *vasa* transcription in the male germ line because the *vasa*$^-$ deficiency homozygotes are themselves derived from heterozygous *vasa*$^+$ mothers. Because these males are fertile, and their testes are morphologically normal, the biological role of *vasa* in males, if any, is obscure.

Expression of vasa *is unaffected by most maternal-effect mutations*

Our results indicate that *vasa* acts very early in the hierarchy of gene interactions necessary for oocyte differentiation, as most mutations that lead to aberrant egg formation have no effect on *vasa* expression or localization. Genes required as early as the initial differentiation of the oocyte, such as *egalitarian* and *Bicaudal-D*, do not affect expression of *vasa* in the pronurse cells.

No single posterior-group mutation affects early ovarian expression of *vasa*. However, the wild-type activities of at least four genes, *cappuccino*, *spire*, *oskar*, and *staufen*, are required for *vasa* protein to be correctly localized at the posterior pole of the oocyte. The *tudor* and *valois* gene activities appear not to be required for localization of *vasa* protein; however, this conclusion must be tempered by the fact that we cannot be certain that any of the available mutant alleles of *tudor* and *valois* are amorphic. Although the tested alleles of *tudor* and *valois* are the most severe known, ultimately, vasa localization in females carrying overlapping deficiencies for these genes (or mutant lesions shown at a molecular level to be amorphic) will need to be investigated.

vasa and its possible interactions with other gene products

The *vasa* protein has been identified as a component of polar granules (Hay et al. 1988b), structures that migrate to the posterior pole of the oocyte and are taken up by the developing pole cells. It is therefore somewhat surprising that *vasa* protein is normally localized in *tudor* and *valois* oocytes because these two mutations abolish the formation of morphologically identifiable polar granules (Boswell and Mahowald 1985; Schüpbach and Wieschaus 1986). Consistent with this result is the conclusion that *vasa* protein itself is the component of polar

Figure 6. All tissue stained with affinity-purified anti-vasa antiserum as in Figs. 1 and 2. (*a*) Cleavage embryo from a *Bic-C*^{YC33}*/CyO* female. (*b*) Morphologically unaffected stage-14 embryo from a *Bic-C*^{YC33}*/CyO* female. (*c* and *d*) Embryos with increasingly severe head defects from a female of the same genotype, still exhibiting normal gonad formation. (*e*) Bicaudal embryo from a female of the same genotype. Note the abnormally low number of pole cells in the gonads; many individual pole cells are outside the focal plane in the immediate area of the gonads. (*f*) Freeze-substituted ovary from a *Bic-C*^{WC45}*/Df(2L)osp29* female. (*g*) Cleavage embryo from a *Bic-D*^{7134}*/CyO* female. (*h*) Morphologically unaffected extended germ-band embryo from a *Bic-D*^{7134}*/CyO* female, dorsal view. (*i*) Extended germ-band bicaudal embryo from a female of the same genotype, dorsal view, again with stained pole cells. (*j*) Sectioned stage-13 oocyte from a *Bic-D*^{7134}*/Df(2L)H68* female, showing only posterior localization of vasa protein in oocyte. (*k*) Freeze-substituted ovary from a homozygous *Bic-D*^{7134R} female.

granules required for posterior localization and the conclusion that a reaction between the *tudor* and *valois* products and the *vasa* product is an essential intermediate step in polar granule formation.

It is possible that *vasa* protein binds directly to the transcript of one or more of the genes necessary for its localization, as the protein sequence of *vasa* suggests RNA binding activity. Such a model would predict that the ligand RNA would have a similar distribution to that described here for *vasa* protein. Two pole-cell-specific transcripts are known: cyclin-B (Whitfield et al. 1989) and the posterior-group transcript *nanos* (R. Lehmann, pers. comm.). There has been no mutation isolated thus far in the cyclin-B gene, and our data show that two independent mutant alleles of *nanos* (nos^{L7} and nos^{53}) have no effect on the localization of *vasa* protein. However, mutations at *vasa* abolish *nanos* transcript localization (R. Lehmann, pers. comm.). This suggests that the interaction that localizes the *vasa* protein does not require *nanos* activity, but the localization of *nanos* mRNA may occur by virtue of its association with *vasa* protein.

Whereas *oskar* and *staufen* wild-type activities, as well as those of *cappuccino* and *spire*, are required for the proper localization of *vasa* product, it is clear that the wild-type activities of the *tudor* and *valois* genes are required, along with that of *vasa*, for pole cell formation. Although it is true that pole cells never form (and in fact, oocytes never fully develop) in the absence of *vasa* protein, the results with *tudor* and *valois* mutations demonstrate that the presence of wild-type *vasa* protein at the posterior pole of the embryo is itself not sufficient for the differentiation of pole cells. It is possible that the role of *valois* in pole cell formation is related to its role in somatic cellularization (Schüpbach and Wieschaus 1986, 1989), and it is interesting that mutations in *cappuccino* (Manseau and Schüpbach 1989b), *Bicaudal-C* (this paper), and three terminal-group genes also give rise to general cellularization defects [*fs(1)N*, Degelmann et al. 1985; *fs(1)ph*, Perrimon et al. 1986; *l(1)ph*, Perrimon et al. 1985].

The functions of vasa

Mutations of *vasa* were originally isolated as a consequence of the abdominal gap phenotype of embryos from homozygous mutant mothers. In common with many, but not all, other posterior group mutations, *vasa* embryos also lack pole cells. However, the loss-of-function phenotype of *vasa* shows that this gene is required as well for the process of oocyte maturation. It is important to consider separately these three processes.

In vas^{PD} ovaries, expression of *vasa* is aberrantly regulated, so that, in adult ovaries, *vasa* protein is only detectable in the germaria. This indicates that the requirement for *vasa* in oocyte maturation is fulfilled well before oocyte differentiation, as vas^{PD} females lay normal numbers of eggs. It will be important to determine whether the protein produced in vas^{PD} is also altered in sequence, or whether the phenotype results strictly from improper regulation of the wild-type gene.

vasa appears to play a direct role in the determination of pole cells. The *vasa* protein is found in the germ line throughout development. Hay et al. (1988a,b) showed that *vasa* protein is associated with polar granules, and no *vasa* mutant makes pole cells. Pole cells never form except at the posterior end of the embryo, and then only in the presence of high levels of *vasa* protein; they are not duplicated in the anterior abdomens of bicaudal embryos, which do not contain high levels of *vasa* protein. Nor are they formed in the normal-abdomen progeny produced by various weak posterior-group alleles. A view of the temporal sequence of events leading to pole cell formation, and the gene activities involved therein, is given in Figure 7. Expression of *vasa* continues throughout female germ line development. The *egalitarian* and *Bic-D* genes are required for oocyte differentiation, and the *Bic-C* and *vasa* genes for oocyte maturation. The *oskar*, *staufen*, *cappuccino*, and *spire* wild-type products are required for the localization of *vasa* protein and polar granules to the posterior end of the developing oocyte. The *vasa* protein may bind specifically to the transcript of one or more of these genes, which itself may be localized to the posterior pole of the oocyte. The *Bicaudal-C* gene product may also be involved in localizing polar granules to the posterior pole of the oocyte. The activities of the *tudor* and *valois* genes are essential for polar granule assembly and pole cell formation.

The role of pole cells in the determination of the abdominal segments is clear—there is none. For example, mutations of *nanos* and *pumilio* lead to a typical posterior-group phenotype but produce pole cells. Conversely, some mutant alleles of *oskar* (osk^{301}; Lehmann and Nüsslein-Volhard 1986), *tudor* (tud^{WC}; Schüpbach and Wieschaus 1986), and *vasa* (vas^{O14}; this paper) completely lack pole cells but form a normal abdomen. Finally, none of the bicaudal mutations duplicate pole cells at the anterior pole of the embryo (Nüsslein-Volhard 1977).

The posterior segmentation phenotypes of *vasa*, and of similar posterior group mutations (e.g. *oskar*), are best interpreted as being a secondary consequence of an interaction between their products and that of *nanos* (Nüsslein-Volhard et al. 1987). If the *nanos* gene product is not correctly localized to the posterior pole, then this will result in the failure of inactivation of the maternal *hunchback* product and a consequent failure of activation of the zygotic gap gene *knirps* (Nauber et al. 1988; Hülskamp et al. 1989; Irish et al. 1989; Struhl 1989; Lehmann and Frohnhöfer 1989). Cytoplasm from the anterior of *Bicaudal-D* embryos can rescue the abdominal phenotype of mutant *oskar* embryos (Lehmann and Nüsslein-Volhard 1986). This is presumably due to the ectopic localization of *nanos* activity in *Bicaudal-D* embryos (Nüsslein-Volhard et al. 1987; Wharton and Struhl 1989).

The distribution of *vasa* protein in *Bicaudal-D* embryos is normal (this work, Wharton and Struhl 1989).

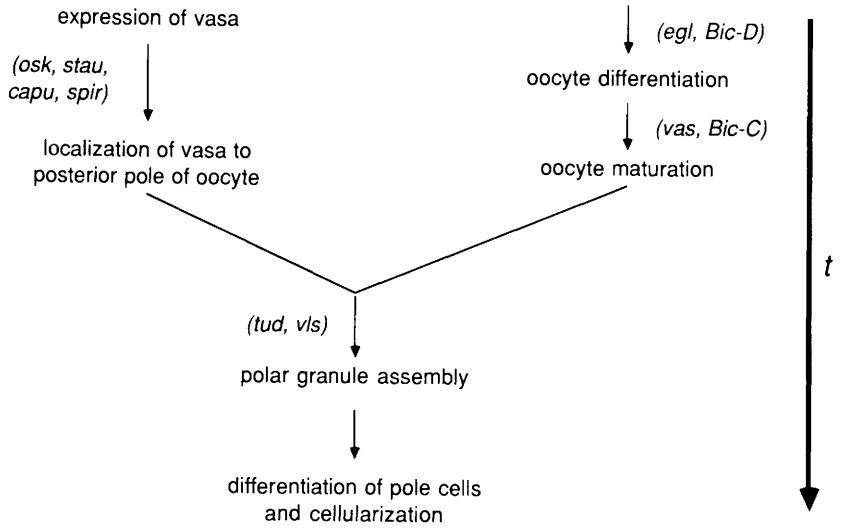

Figure 7. A schematic representation of the possible hierarchy of gene interactions leading to pole cell formation.

Despite these data, there is evidence that *vasa* is required for the ectopic localization of *nanos* activity in *Bicaudal-D* oocytes because mutations or deletions of *vasa* act as dominant suppressors of *Bicaudal-D* mutations (Mohler and Wieschaus 1986). The most obvious interpretation of these data is that, normally, the amount of *vasa* protein generally distributed in the embryo is sufficient to localize *nanos* activity ectopically in the presence of a mutant *Bicaudal-D* product. The reduced amount of *vasa* protein to be expected in a heterozygous $vasa^-$ female is insufficient to localize *nanos* activity anteriorly in the *Bicaudal-D* oocyte and therefore suppresses the bicaudal phenotype.

It could be argued then that the function of *vasa* in abdominal segmentation in the wild-type is mediated by its interaction with the *nanos* product and is fulfilled by the component of the *vasa* protein generally distributed in the oocyte. This model would also be consistent with the finding that *vasa* protein is not localized in osk^{301} oocytes, despite the ability of flies bearing that mutation to produce progeny with normal abdomens but no pole cells. The vas^{O14} mutation of similar phenotype further indicates that the pole cell and abdominal segmentation functions of the *vasa* gene are separable by mutation.

In summary, *vasa* is required for three distinct functions in the female germ line. Its earliest role is in the growth and maturation of the oocyte after its differentiation, dependent on its expression prior to ovarian cyst formation. Pole cell determination appears to depend directly on the presence of high levels of the *vasa* protein, which are normally present at the posterior pole. Finally, the role of *vasa* in abdominal segmentation is likely to be an indirect one mediated through the *nanos* product.

Materials and methods

Fly strains

The fly strains used are listed in Table 1.

Plasmids

BlueScript was purchased from Stratagene (La Jolla, California), and pGEX-3X was obtained from Amrad Corporation (Melbourne, Australia).

Protein gel electrophoresis

The 10% SDS-polyacrylamide slab gels were run as described by Hames (1981).

Overproduction of the chimeric glutathione-S-transferase–vasa protein in E. coli

A 1416-bp *Nar*I fragment from *vasa* cDNA clone cv1.092 (Lasko and Ashburner 1988) was subcloned into the *Acc*I site of BlueScript to give the plasmid pNN1.4. This was then digested with *Eco*RV and *Eco*RI to isolate a 1270-bp fragment containing the open reading frame of *vasa* from the *Nar*I site at position 156 to the *Eco*RI site at position 1404, as well as 22 nucleotides from the BlueScript polylinker. This fragment was subcloned into pGEX-3X. The resulting plasmid encodes a chimeric protein consisting of amino acids 16–433 of vasa, representing virtually all of the unique amino-terminal region of the *vasa* polypeptide and the putative ATP-binding site, but not the more carboxy-terminal motifs conserved in the D-E-A-D family of proteins, fused to the *Schistosoma japonicum* glutathione-*S*-transferase (Smith and Johnson 1988). *E. coli* strain TG1 carrying this plasmid expressed a novel fusion protein of the predicted size (73 kD) at a level of about 20% of total cell protein after a 3-hr induction with IPTG at 37°C.

Preparative electrophoresis of fusion protein

Protein bands were visualized by incubating SDS-polyacrylamide gels in 0.25 M KCl at 0°C for 15 min. The band containing the fusion protein was excised with a razor blade and minced. Protein was eluted in SDS running buffer using a Biotrap elution chamber (Schleicher & Schuell); electrophoresis was at 200 volts for 2–4 hr. The protein was then dialyzed against two changes of PBS, and stored at −20°C.

Immunization of rabbits

Rabbits were immunized subcutaneously at multiple sites,

Table 1. *Mutant fly strains used in this study*

Strain	Source	Reference
vas^{PD} cn bw/CyO	R. Lehmann	Tearle and Nüsslein-Volhard 1987
b vas^{O11} pr cn/CyO	R. Lehmann	Tearle and Nüsslein-Volhard 1987
vas^{O14} cn/CyO	R. Lehmann	Tearle and Nüsslein-Volhard 1987
b vas^{Q6} pr cn/CyO	R. Lehmann	Tearle and Nüsslein-Volhard 1987
b vas^{AS} pr cn bw/CyO	R. Lehmann	Tearle and Nüsslein-Volhard 1987
b vas^{D5} pr cn sca/CyO	R. Lehmann	Tearle and Nüsslein-Volhard 1987
b vas^{D1} cn/CyO	R. Lehmann	Tearle and Nüsslein-Volhard 1987
b vas^{Q7} pr/CyO	R. Lehmann	Tearle and Nüsslein-Volhard 1987
vas^{P808}/CyO	S. Halsell	S. Halsell and H. Lipshitz (pers. comm.)
Df(2L)A267, b cn bw/CyO		Ashburner et al. 1982a
Df(2L)A72, b cn bw/CyO		Ashburner et al. 1982a
Df(2L)osp29, Adh^{uf3} pr cn/CyO		Ashburner et al. 1982b
Df(2L)TE116(R)GW18, al dp b sp/CyO	S. Roth	Lasko and Ashburner 1988
Df(2L)H68, dp b M/CyO	C. Nüsslein-Volhard	Nüsslein-Volhard et al. 1984
Df(2L)TW2/CyO	C. Nüsslein-Volhard	Wright et al. 1976
Df(2R)Pcl7B/CyO	A. Martinez-Arias	Duncan 1982
Df(2R)Pu-rP133, c px sp/SM1	C. Nüsslein-Volhard	Mackay et al. 1985
Df(3R)p-XT26, ru st e ca/TM3	C. Nüsslein-Volhard	Lehmann and Nüsslein-Volhard 1987a
th st in ri roe p^{p} osk^{166}/TM3, Sb	C. Nüsslein-Volhard	Lehmann and Nüsslein-Volhard 1986
osk^{301} e/TM3, Sb	C. Nüsslein-Volhard	Lehmann and Nüsslein-Volhard 1986
vls^{PE} cn bw/CyO	C. Nüsslein-Volhard	Schüpbach and Wieschaus 1986
tud^{WC} bw sp/CyO, $l(2)100^{DTS}$	C. Nüsslein-Volhard	Boswell and Mahowald 1985
b pr $stau^{G2}$/CyO	C. Nüsslein-Volhard	Schüpbach and Wieschaus 1986
cn $stau^{HL}$ tud^{WC} bw/CyO	C. Nüsslein-Volhard	Schüpbach and Wieschaus 1986
st nos^{L7} e/TM3	R. Lehmann	Lehmann 1988
st nos^{53} e/TM3, Sb	C. Nüsslein-Volhard	Tearle and Nüsslein-Volhard 1987
st pum^{680}/TM3, Sb	C. Nüsslein-Volhard	Lehmann and Nüsslein-Volhard 1987b
th st ri bcd^{E1} roc p^{p}/TM3, Sb	C. Nüsslein-Volhard	Frohnhöfer and Nüsslein-Volhard 1986
st bcd^{E1} ri pum^{680}/TM3, Sb	C. Nüsslein-Volhard	Lehmann and Nüsslein-Volhard 1987b
cn bw $capu^{RK12}$/CyO	T. Schüpbach	Manseau and Schüpbach 1989
cn $spir^{RP48}$ bw/CyO	T. Schüpbach	Manseau and Schüpbach 1989
$B^{S}Y$; tov^{012}, ru st e ca/TM3, Sb	C. Nüsslein-Volhard	Tearle and Nüsslein-Volhard 1987
egl^{PV27} cn bw/CyO	T. Schüpbach	Mohler and Wieschaus 1986
fs(2)Y12, b pr/CyO	C. Nüsslein-Volhard	Tearle and Nüsslein-Volhard 1987
$B^{S}Y$; $vss\text{-}1^{675}$, ru st e ca/TM3, Sb	C. Nüsslein-Volhard	Tearle and Nüsslein-Volhard 1987
qua^{WP}, cn bw sp/CyO	C. Nüsslein-Volhard	Tearle and Nüsslein-Volhard 1987
ru $spnC^{094}$ st e ca/TM3	T. Schüpbach	Tearle and Nüsslein-Volhard 1987
b cn mr^{2}/ln(2L)Cy ln(2R)Cy, al^{2}Cy pr Bl cn^{2} L^{4} bw sp	B. Reed	King 1959
+/CyO, $l(2)513^{DTS}$; bic-F/TM3, Sb	C. Nüsslein-Volhard	Tearle and Nüsslein-Volhard 1987
Bic-C^{WC45}, cn bw sp/CyO	T. Schüpbach	Mohler and Wieschaus 1986
Bic-C^{YC33}/CyO	T. Schüpbach	Mohler and Wieschaus 1986
Bic-D^{7134} pr cn/CyO	T. Schüpbach	Mohler and Wieschaus 1986
dp b Bic-D^{7197R}/CyO	T. Schüpbach	T. Schüpbach (pers. comm.)

with an initial inoculum of 200 μg of fusion protein in complete Freund's adjuvant, followed by boosts of 50–200 μg in incomplete Freund's adjuvant every 2 to 4 weeks. All immunizations were carried out commercially (ABC Ltd., Cambridge).

Immobilization of proteins on nitrocellulose filters

This was carried out as described (Towbin et al. 1979); transfer buffer was 25 mM Tris-HCl, 150 mM glycine, 20% methanol (pH 8.3); transfer proceeded overnight at 200 mA.

Binding of protein to affinity column

Protein was purified as described above, and dialyzed against 0.1 M HEPES-KOH (pH 7.5) for 1 hr. The protein was then incubated for 1 hr on a rolling platform with 1 ml of Affigel-10 beads (Bio-Rad), which had been prewashed with 20 ml of cold deionized H_2O. The supernatant was removed, and the remaining binding sites on the beads were blocked by treatment for 1 hr with 0.2 M glycine in 0.1 M HEPES-KOH (pH 7.5). The beads were then washed with 20 ml of PBS, 10 mM NaN_3, transferred to a 2-ml syringe plugged with glass wool, and stored at 4°C. Typical binding efficiencies were 50–75%, as determined by analysis of the supernatant by SDS-PAGE before and after binding. All manipulations were carried out at room temperature.

Affinity purification of antisera

Serum was pretreated with an acetone powder from *E. coli*

strain TG1 (prepared by the method of Harlow and Lane 1988) for 2 hr at 4°C, spun in a microcentrifuge at 12,000*g* for 10 min, and the supernatant was passed over an Affigel-10 column (Bio-Rad) bound with 200–400 μg of purified fusion protein. The column was washed five times with two volumes of PBS, then the bound antibodies were eluted in 0.1 M glycine (pH 2.5). Eluted fractions were neutralized with 0.25 volume of 2 M Tris-HCl (pH 7.4), BSA was added to 1%, and the samples were dialyzed against PBS. Affinity-purified sera were stored at 4°C in the presence of 10 mM NaN_3.

Characterization of anti-vasa antiserum

Immune serum, preabsorbed against an acetone precipitate of *E. coli* proteins, and affinity-purified against the fusion protein affixed to nitrocellulose (Robinson et al. 1988) or to Affigel-10 beads, recognizes a single band in protein extracted from *Drosophila* female adults. This is of the size predicted for the *vasa* protein (Fig. 8). The reactive protein is present in females only in the ovaries, and is not recognized by preimmune serum.

Freeze-substitution of ovaries and testes

Adult ovaries or testes from larvae and adults were dissected in Ringer's solution, then pressed between two gelatin-coated slides, frozen on dry ice, separated from each other with a razor blade, fixed through −70°C acetone (3 min) and −70°C methanol (5 min), and rehydrated through an ethanol series (100%, 95%, 80%, 60%, 30%) into PBS + 0.1% Triton X-100 (PTX).

Fixation of larval and pupal ovaries

Larval and pupal ovaries were dissected in Ringer's solution and fixed in 4% paraformaldehyde in 0.1 M PIPES, 2 mM $MgSO_4$, 1 mM EGTA (pH 6.9) for 30 min at room temperature.

Cryostat sectioning of ovaries

Ovaries were dissected in Ringer's solution, then transferred directly to O.C.T. embedding fluid (Tissue-Tek) and frozen in place on dry ice. Sections were cut in a Slee cryostat at −18°C at a thickness of 10 μ. The sections were collected on gelatin-coated microscope slides, fixed for 5–10 min in 4% paraformaldehyde in PBS, and transferred directly into PTX + 0.1% bovine serum albumin (PBTX) for blocking.

Fixation of embryos for antibody staining

Embryos were collected in microcentrifuge tubes, washed in PTX, dechorionated for 2 min in 50% commercial bleach, washed once in PTX and once in distilled water. They were then fixed in heptane saturated with 4% paraformaldehyde in 0.1 M PIPES, 2 mM $MgSO_4$, 1 mM EGTA (pH 6.9) for 40 min at room temperature on a rolling platform, devitellinized by shaking in absolute methanol, taken through three additional changes of absolute methanol, and stored at −20°C.

Antibody staining of tissues

Blocking was in PBTX for 1–4 hr at room temperature. Affinity-purified primary antibody was diluted 1 : 300 to 1 : 1200 in PBTX, and reactions were incubated overnight at room temperature (embryos on a rolling platform; sectioned tissue in Coplin jars). Washes were in PBTX for 2–3 hr using at least three changes. The secondary antibody was biotinylated horse anti-rabbit IgG (Vector Laboratories; Peterborough, England, 1 : 400). Washes were then in PBS + 0.1% Tween 20 (PT), for 1.5–2 hr in at least three changes. Samples were then incubated with avidin–biotin complex (ABC Elite, Vector Laboratories), washed through five changes of PT in 30 min, and stained in the following solution: 100 μl 6% nickel ammonium sulfate, 25 μl 10 mg/ml diaminobenzidine (Sigma), 1 μl commercial hydrogen peroxide solution (6%; 20 volumes available oxygen; Boots), and PT to 1 ml (Adams 1981). Secondary antibodies were preabsorbed against similar tissue for 1–2 hr at room temperature.

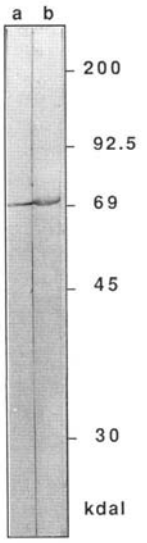

Figure 8. Total protein, after electrophoresis on a 10% SDS-PAGE gel, from (*a*) Canton-S total females and (*b*) Canton-S ovaries, transferred to nitrocellulose and reacted with affinity-purified anti-vasa serum, at a dilution of 1 : 100. Tissue from three individuals was used for each track.

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Posterior localization of vasa protein correlates with, but is not sufficient for, pole cell development.

P F Lasko and M Ashburner

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