Title
Elements required for germ line determination and abdomen specification in Drosophila embryogenesis

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Publication Date
1989

Peer reviewed|Thesis/dissertation

ELEMENTS REQUIRED FOR GERM LINE DETERMINATION AND ABDOMEN SPECIFICATION IN *DROSOPHILA* EMBRYOGENESIS.

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

To my parents,whose friendship and support has made all the work described here possible

ACKNOWLEDGEMENTS

Graduate school is something nobody does alone. Along the way a number of people have helped to give me the strength, the reagents and most of the ideas that have allowed me to carry out the work described below. In particular I thank my parents for their friendship and and financial support during the long and costly process of my education--to what end it is still not clear.

Also, I owe a particularly large debt of gratitude to both Yuh Nung and Lily Jan for taking me back into their lab after I had earlier left--and spent a lot of money just before leaving--, for their support and constructive criticism along the way, and for their keen sense of an important biological question, which allowed me to take up a project in which they had no particular initial interest or stake.

The most important requirement for getting through graduate school is not necessarily having a particularly exciting project, being in an exciting lab or having a particular lot of smarts, but rather having friends to help you through whatever it is you do end up working on. In this regard I am particularly indebted to Chauncey Bowers, Kay Logan, Eve Perara, Karla Neugebauer, and Dianne Parry for their companionship throughout various phases of this work. Their help, both in and out of lab, has been in large measure responsible for my making it through graduate school, and actually enjoying some of it.

There are a number of other people who have made the process of graduate school much smoother and more pleasent:

The many, many members of the Jan lab for their help over the years, in particular:

Larry Ackerman for his help with the antibody experiments described in chapter one, for his help with all the photography and printing that went into all this work, and for just keeping the lab running smoothly,

Sandra Barbel, and Yuh Nung Jan and Larry Ackerman, respectively, for generating the monoclonal antibody that is the foundation of this work, and for recognizing that it might be interesting and saving it until I came along,

the members of room U212, both past and present, Mike Caudy, Rolf Bodmer, Gabrielle Boulliane, Harald Vaessin, Kevin Lee, Marc LosHuertos, Ethan Bier, and Yi Rao for their help, advice and gifts of reagents (both known and unbeknownst to them),

Yi Rao, who simply by being himself, has "drawn fire" away from myself on numerous occasions,

Harald Vaessin and Mike Caudy for the Northern blots in chapter two,

Rabiya Tuma and Denise Mulhrad for their help with sequencing the vasa gene and making this data presentable,

the genetics group, Susan Sheperd, Elsworth Grell, Kimberly McCall and Jacinto Villeneueva, for their advice, help maintaining (and gently reminding me to transfer) the fly stocks used in these studies, and the other members of the "hop and sniff" group, Ethan Bier, Harald Vaessin, Kevin Lee, Sandra Barbel, Larry Ackerman,

Robert Caretto and Tadashi Uemura, for making it possible for me to screen through the P-element lines for female sterile and grandchildless mutants,

Tom Jongens, for his collaboration on experiments described in chapter four on the grandchildless mutations,

some members of the Lingappa lab, Ray Chavez, Charlie Lopez, Rich Rothman, Francis Ip, and Clara Calayag for their help, some good times and an interesting experience,

the members of my thesis committee, Pat O'Farrell, Bruce Alberts, Kathryn Anderson and Louis Reichardt, for their comments and criticism of various aspects of this work,

Louis Reichardt, chairman of my thesis committee, for his gentle reminders now and then that I really ought to finish, and his help in speeding along the process when I finally decided to listen.

Writing this thesis has been a long process. It has been made significantly longer, though more pleasent, by the late night company of Mike Caudy and Tom Jongens, whose vast knowledge of cheap beer is surpassed only by the amount of it they consume in a single sitting. Also, special thanks to Tina Cheung for her help with the photography and artwork for the figures contained herein.

ELEMENTS REQUIRED FOR GERM LINE DETERMINATION AND ABDOMEN SPECIFICATION IN DROSOPHILA EMBRYOGENESIS.

Bruce A. Hay

ABSTRACT

Information necessary for the formation of pole cells, precursors of the germ line, and for proper abdomen specification, is provided maternally and localized to the posterior pole of the Drosophila egg. Polar granules may be determinants, or be associated with determinants, of these embryonic structures. The goal of the present work was twofold: first, to address the question of whether polar granules are important for the function of these localized determinants, and if so, what this role might be, and secondly, to identify and characterize new genetic loci which are required for the function of these determinants.

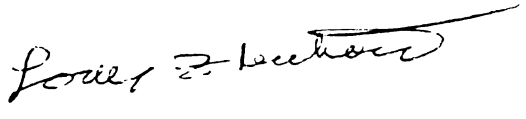
A monoclonal antibody (Mab46F11) was isolated that recognizes a 72kd protein component of Drosophila polar granules as well as other germ line-specific structures (Hay et al. 1988a). With this antibody cDNA and genomic clones were isolated corresponding to the gene for this antigen (Hay et al. 1988b), which maps to the vasa locus. Embryos from homozygous $vasa^{PD}$ females lack the Mab46F11 antigen, lack localized polar granules, fail to form pole cells and have

large abdominal deletions (Schupbach and Wieschaus, Roux's Arch. 195:302; Hay et al., Cell 55:577). Thus polar granules, or at least the vasa product as a component of polar granules, are required for the formation of germ cells and abdomen specification.

The predicted vasa protein has a large internal domain with homology to ATP-dependent RNA helicases (proteins which bind single RNA and unwind double stranded regions). This homology is particularly notable with eukaryotic translation initiation factor 4A (eIF-4A), which is thought to bind to, and unwind mRNA. The significance of this homology is discussed in light of the observation that specific mRNAs (that for cyclin B for example) become localized to the posterior pole in a vasa-dependent manner (B. Hay and T. Jongens, unpublished).

To identify stages in the process in the process of polar granule function, antibodies against the vasa product have been used to follow the fate of this granule component in mutants which fail to localize polar granules. The results suggest that polar granule localization involves multiple steps, including an initial localization step and a second stabilization step following egg activation.

In a genetic approach to identifying components required for germ cell determination, I screened about 4000 lines in which P-element hops had been carried out, for female sterility and grandchildlessness. Three mutations which appear to represent new genetic loci affecting germ cell formation have been identified and are described.



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INTRODUCTION

Mechanisms of development can be divided into two broad categories. In the first, assymetrically localized cytoplasmic components, by virtue of their unequal distribution into the embryonic blastomeres (blastomeres here being defined as cells which are not yet committed to a particular fate) , play an instructive role in determining the fate of cells in which they are concentrated. In the second, that of induction, blastomeres acquire different fates as a result of differential interactions with each other or with the external environment. The idea that there exist localized cytoplasmic determinants is particularly interesting because it implies that molecules triggering specific programs of development are present in the oocyte or early embryo. It also poses a number of other interesting questions, including the molecular nature of the morphogenetic agents and the mechanisms by which they regulate cell fate, the means by which they become distributed to specific regions of the egg or early embryo, and the ways in which they become anchored at these sites.

Historical Introduction to the concept of cytoplasmic determinants

The idea that the egg is a mosaic, in which different tissues derive from different regions of the egg, is an old one, which can be traced back to at least the 18th century and the preformationist school of thought. A strong form of this thinking,

put forward by Bonnet (1762, described in Wilson, 1896) held that development was only the unfolding of that which already existed, and that organized bodies preexist in the egg from the beginning. Thus, each egg contains a complete embryo; and this egg must itself contain eggs for the next generation, and so on ad infinitum. Early direct observations of the embryo quickly put an end to this simple form of preformationism. Wolff (1759) first showed that chick blood vessels and gut developed from apparently undifferentiated tissue. Later workers, notably Pander (1817), von Baer (1828, 1837), and Kowalevsky (1867) extended and generalized these observations. Out of their work came the germ layer theory of development, one of the foundations of modern embryology, in which complexity in the embryo is thought to derive epigenetically from the interactions of primitive germ layers with each other.

In the 1870s a more subtle form of preformationism was put forward, most notably by Wilhelm His and his coworkers. He suggested that the epigenetic character of development is only superficial; the forces that underly the behavior of cells in development are the result of the action of microscopic preformed differences in the constituents of the egg:

> It is clear, on the one hand, that every point in the embryonic region of the blastoderm must represent a later organ or part of an organ, and on the other hand, that every organ developed from the blastoderm has its preformed germ in a definitely localized region. The material of the germ is already present, but is not yet...directly recognizable (His, 1874; translated by Wilson, 1896).

Careful lineage analysis of the development of a number of organisms, first carried out by Whitman (1878) in the leech Clepsine, and subsequently by others in a variety of organisms (cf Reverberi, 1971a), supported this hypothesis. He observed that complex structures, such as the nerve cord, derived from identified sets of stem cells, which themselves could be traced back to early blastomeres that arose only in certain regions of the egg. The implications of these observations to the early workers were that perhaps the regions of cytoplasm from which the early blastomeres derived were important in determining the subsequent fate of these blastomeres. A particularly striking example for the early workers was provided by studies of the ascidian Cynthia (styela) (Chamby, 1887; Conklin, 1905a). In the uncleaved eggs of this organism five pigmented areas of cytoplasm could be distinguished which subsequently gave rise to distinct, different, embryonic structures. Summarizing his results Conklin stated:

> As early as the close of the first cleavage...these strikingly different ooplasmic substances...are differentiated for particular ends, and...they give rise to organs of a particular kind. These materials are, therefore, "organ forming substances," and the areas of the egg in which they are localized are "organ forming regions"(Conklin,1905; quoted from Davidson, 1986; p.416).

The idea that the egg is a mosaic was further substantiated by two sorts of experiments. In the first, embryonic blastomeres were dissociated into individual cells and their developmental potential determined. In a number of organisms these blastomeres carried out a program of division and differentiation

that was identical to that of the same blastomeres in the intact embryo. Thus as Wilson said of his experiments on the mollusc Patella coerulea:

> The history of these cells gives indubitable evidence that they possess within themselves all the factors that determine the form and rhythm of cleavage, and the characteristic and complex differentiation that they undergo, wholly independently of their relation to the remainder of the embryo (Wilson, 1904).

In a related series of experiments Driesch and Morgan (1895), Wilson (1896) and others (reviewed in Wilson, 1925) showed that removal of cytoplasm from a particular region of the molluscan egg prior to first cleavage resulted in a failure of the embryo to form those structures which would derive from cells originating in that region of the egg. The definitive demonstration that these determinants of cell fate are cytoplasmic, and not due to some qualitative differences in the nuclei which become incorporated into the blastomeres, came from experiments carried out by Driesch and Wilson (1896). They used treatments which would redistribute nuclei in early embryos such that they would end up in inappropriate blastomeres. These embryos went on to develop normally, however. These results demonstrate that the differences in fate displayed by certain cell lineages must be due to the presence of differentially localized cytoplasmic factors. Lillie (Chaetopterus, 1909), and later Morgon and Spooner (sea urchin, 1909) and Conklin (Crepidula, 1917), showed that these determinants were in some way anchored within the cell. Thus while centrifuging

the eggs would stratify the microscopically visible components such as pigment granules and yolk platelets, the cleavage planes of the egg and the developmental capacities of the resultant daughter blastomeres remained unaffected.

At the same time as these results were being obtained it was also being found that some embryos showed a large capacity for regulation of blastomere fate. Driesch first showed that in the sea urchin isolated blastomeres up to the 4 cell stage could give rise to a perfect larvae of reduced size. This was soon followed by similar observations in a number of other organisms (reviewed in Wilson, 1925). These sorts of observations led some, including Driesch, to the strong conclusion that self-differentiation did not occur at all: "The blastomeres of the sea urchin are to be regarded as forming a uniform material, and they may be thrown about, like balls in a pile, without in the least degree impairing thereby the normal power of development" (Driesch, quoted in Wilson, 1925; p. 1056). An important series of experiments which provided a way out of these seemingly contradictory observations, and that provided an important insight into the way in which embryonic development could display both mosaic (self-determination) like qualities, as well as inductive (or regulative) features, involved the study of the development of egg fragments. In the first of these sorts of experiments, carried out by Boveri (1902), sea urchin eggs were shaken to pieces, these pieces fertilized, and their development followed. Eggs of the species used, Paracentrotus lividus, display distinct colored strata. Boveri found that egg fragments deriving

from each of these different strata gave rise to characteristic sorts of embryonic structures. These results demonstrate that the sea urchin egg is not at all isotropic. Different regions of the egg have quite different developmental potentials. The difference between sea urchins on the one hand, and overtly mosaic organisms like molluscs and ascidians on the other, has to do with the way in which the components of the egg are segregated during the early cleavages. To quote Wilson,

> For, in the sea urchin egg, the first two cleavages cut the three strata of the egg symmetrically (both passing through the egg-axis and at right angles to each other); both, therefore, are purely quantitative. The first two or four cells thus receive identical portions of each of the three zones, and are consequently equipotential and totipotent. In the mollusc or annelid, on the other hand, at the first cleavage the lower stratum (lower polar area) passes bodily into one of the two cells. This division, therefore, is qualitative, allotting different combinations of ooplasmic materials to the first two blastomeres; hence the mosaic-like character of cleavage from the beginning. We may therefore conclude that one important difference between the so-called "mosaic eggs" and the "regulative eggs" lies merely in a different relation of the c leavage-pattern to the ooplasmic materials. (Wilson, 1925; pp. 1067-68).

By 1925, then, an essentially modern conception of cytoplasmic determinants was being put forward, based on experimental evidence. It was clear that determinants of cell fate are localized assymetrically in eggs, that it is the unequal segregation of these materials to the daughter blastomeres which is required for bringing about different cell states, and that ultimately these components exert their effects by acting on totipotent nuclei in which are contained the elements of

heredity. It was also clear that components of the egg cell, presumably attached to the cytoskeleton are required for setting up the initial assymetries and for maintaining them. In the 60 years since Wilson published his book "The Cell in Heredity and Development", in which the current state of knowledge about the role of cytoplasmic determinants in specifying cell fate was summarized, our understanding of the mechanism of action and segregation of these components has progressed only slightly. In only one case, the specification of anteriorness in anteroposterior axis specification in the insect Drosophila melanogaster by the product of the bicoid gene, is anything known about the nature of the molecules involved and their mechanism of action (Driever and Nusslein-Volhard, 1988a,b). A particularly intriguing system in which there exists a large body of evidence demonstrating the presence of cytoplasmic determinants is the specification of the germline. In animals as phylogenetically distant as nematodes, insects, and anuran amphibians, germ cell formation from localized regions of the egg occurs. A number of lines of evidence, described below, indicate that germline determination in these animals is due to the action of localized cytoplasmic determinants.

Examples of early germline determination

Germline determination in nematodes.

Evidence for the determination of the germ cell lineage by cytoplasmic products in nematodes was first obtained by Boveri (1899, 1910) in the parasitic nematode Ascaris. In Ascaris, as in

Caenorhabditis elegans (discussed below), the germline precursor cell is the product of a series of unequal cleavages. In Ascaris "chromosome diminution", loss of major parts of the chromosomes during mitosis occurs in all but the future germline. At the first cleavage only one of the daughters retains a complete chromosome set. During each of the next four cleavages this cell produces one daughter which becomes a somatic cell and one which can give rise to the germline. Boveri showed that whether a cell retained a complete genome complement or not depended on the presence of a particular polar region of egg cytoplasm. In eggs in which abnormal cleavage patterns were induced (by polyspermy or centrifugation), nuclei which would not normally occupy the polar region of egg cytoplasm were present in this region and would be spared chromosome loss at mitosis. Other nuclei, including the prospective germ cell nucleus, underwent the somatic cell-specific loss. In related experiments, UV irradiation of the polar plasm, but not other regions of the egg, was shown to prevent the formation of the germ cell lineage. Boveri's experiments do not provide a complete answer to the question of germ cell determination since he could not follow the fate of these cells in the adult (do they form functional gametes?). However, they do demonstrate that an obviously crucial first step in this pathway--the maintenance of the complete genome--requires the function of localized cytoplasmic components.

In the free-living soil nematode C. elegans, a similar pattern of early cell cleavages takes place (Sulston et al. 1983), though

chromosome diminution does not occur. Using monoclonal antibodies against germline-specific granules ("P granules"; Strome and Wood, 1983; Yamaguchi et al. 1983), it was found that in the unfertilized egg large numbers of P granules are present, scattered throughout the cytoplasm. During the first cleavage the granules become localized to the posterior pole of the egg and segregated into the P1 cell. The initial segregation probably requires microfilaments since it can be blocked by cytocholasin D treatment (Strome and Wood, 1983). During subsequent cleavages these granules become precociously segregated to that region of the cell which, during cleavage will give rise to a cell with the potential to contribute to the germ cell lineage. Ultimately the P granules are found exclusively in the germline progenitor cells of the embryo, and subsequently in the gonadal germ cells of the larvae. With the electron microscope these granules appear as electron-dense bodies in unfertilized eggs. By midcleavage stages what appear to be derivatives of these granules appear as tufts of electron-dense material on the cytoplasmic face of the nuclear envelope (Wolf et al. 1983).

Are P granules important in C. elegans germline determination? Schierenberg (1988) has carried out experiments in which polar cytoplasm of the uncleaved egg was allowed to leak out and the fate of the egg remnant left inside the shell followed. These cells carried out only soma like cleavages, indicating that the germline-like cleavage potential is localized in the posterior region of the zygote. A concentration of P granules in this region of cytoplasm is apparently not

necessary for the germline-like cleavage pattern, however. Schierenberg allowed about 20% of the posterior pole cytoplasm to leak out of the egg at a stage prior to the formation of pronuclei. At this time the P granules are not yet localized to the posterior pole cytoplasm. Therefore the extruded fragments must have received only small proportions of the P granule material. These fragments passed through a typical pattern of unequal germline-like cleavages, however. The potential for germline like cleavages must, therefore already be localized to the posterior pole, independently of the localization of the P granules. Whether the P granules that normally get segregated to the posterior pole are important for other aspects of germ cell determination or differentiation in nematodes is unclear from the data described above, since these fragments do not complete development (see the discussion of Drosophila polar granules below, however).

Though their function is unknown, it is interesting to note that structures similar in appearance and distribution to C. elegans P granules have been described in a variety of organisms. They have been found in the germ cells of nematodes, crustaceans, annelids, ascidians, teleosts, anuran amphibians and mammals (reviewed in Davidson, 1986). In animals in which the germline segregates early in development, polar granules are found in that region of the egg cytoplasm in which the germ cells will ultimately arise. In others, in which the germ line is not determined until later, and probably inductively (such as mammals), polar granules or their derivatives are not apparent

until the definitive germ cells can be identified (Eddy et al. 1981).

Germline determination in amphibians

In anuran amphibians it is now well established that the determination primordial germ cells in the normal embryo occurs early in development a is associated with the segregation of a distinctively staining cytoplasm, "germinal plasm", into these cells. Bounoure (1934) first noted that densely staining islands were present in the vegetal cortical region of the frog egg : that by late cleavage stages they were restricted to about four cells in the floor of the blastocoel. Later in development, following some divisions, thes cells migrate from the endoderm to their final position in the genital ridges In the normal embryo these cells would form the functional germline. At tl stage they are not irreversibly determined to do so, however, since if they are transplanted back into the blastocoel of a late blastula stage embryo, tl can give rise to a variety of different cell types (Wylie et al., 1985).

Electron microscopy shows that electron-dense structures, similar in morphology to C. elegans P granules, are present in the cortical, vegetal cytoplasm of the fertilized egg, and are segregated to cells that will form the germ cell lineage (Balinsky, 1966; Czolowska, 1969; Mahowald and Hennen, 1971). Three sorts of experimental approaches have been taken implicating a role for the "germ plasm" (containing polar granules) in the formation of

formation of primordial germ cells: removal of germ plasm, transplantation of vegetal cytoplasm or cytoplasmic extracts, into somatic blastomeres whose fate is then followed, and destruction of the germ cell-inducing capacity of this cytoplasm by UV irradiation. In the first case the most complete experiments have been carried out by Buehr and Blackler (1970), who made incisions into the vegetal pole of 2 or 4 cell embryos. About a third of the blastulae that developed lacked cells containing germ plasm, and correspondingly about one third of the tadpoles which developed were completely sterile. In the second approach, Ikenishi et al. (1986) tested directly for the presence of germ cell determinants in vegetal pole cytoplasm by injecting vegetal cytoplasm or fractions of this cytoplasm into somatic (animal pole) blastomeres at the 32 cell stage, which had been cultured in ^{3}H-thymidine for future identification. These cells were then transplanted into unlabeled hosts and allowed to develop to the tadpole stage. Some of the labeled cells differentiated as primordial germ cells, suggesting that vegetal pole cytoplasm contains elements that promote germ cell determination.

UV irradiation has been a major technique for demonstrating the developmental potential of cells originating from vegetal cytoplasm. Bounoure (1937) and Bounoure et al. (1954) showed that UV irradiation of the vegetal region of cytoplasm of frog eggs results in a decrease in number of germ cells or, in some cases, completely agametic gonads. A large body of subsequent work supports these basic observations (reviewed in Smith and

Williams, 1979). These experiments were carried one step further when it was shown that vegetal, but not animal pole cytoplasm, was able to rescue the ability of UV irradiated vegetal hemispheres to give rise to germ cells (Smith, 1966). More recent experiments have shown a more complex effect of UV irradiation. In Xenopus eggs irradiated vegetally, fewer divisions were found in the surviving germ cells and there was a dramatic delay in their migration to the germinal ridges (Ikenishi and Kotani, 1979). Several labs have observed that primordial germ cells eventually reappear in the germinal ridges of tadpoles raised from irradiated eggs, though on a delayed schedule. Though the germinal ridges from irradiated eggs lack germ cells at stages when they would normally be found, several weeks later they are present, and are ultimately functional (Zust and Dixon, 1977; Smith and Williams, 1979; Williams and Smith, 1984). These results do not affect the interpretation of the removal and transplantation studies discussed above; they do, however, suggest that the UV target in vegetal cytoplasm is important for some aspects of germ cell behavior (division and migration perhaps) other than initial specification.

Evidence that in anurans, polar granules, as components of the vegetal cytoplasm, are important for germ cell determination, is fairly weak. They are present in unfertilized eggs at the vegetal pole cortex (Smith and Williams, 1975, 1979). Thus they would be expected to be accessible to UV irradiation damage. The polar granules also have ribosomes associated with them during early cleavage stages, providing a potential UV

target. UV sensitivity is lost at about the 8 cell stage. At this time the polar granules move in from the cortex to a point where they would no longer be accessible to UV irradiation. Also, if 2 cell embryos are centrifuged at low speed, which might be expected to move large, dense structures like polar granules into the interior early, UV sensitivity is lost (Tonabe and Kotani, 1974). Thus, the data is consistent with the possibility that polar granules play some role in anuran amphibian germline determination, but they do not provide any definitive evidence in favor of the hypothesis.

In a related group of amphibians, the urodeles, the path to germ cell determination is not so clear. There are no early cytological markers, such as polar granules, that allow early identification of regions of egg cytoplasm or blastomeres that will give rise to the germline. Cells identifiable as primordial germ cells are first detected experimentally in the lateral plate mesoderm during gastrulation. Since mesoderm is a product of an inductive interaction of the vegetative yolk mass with the animal cap ectoderm, this has led Nieuwkoop to suggest that the primordial germ cells in urodeles arise epigenitically from indeterminate somatic cells of the blastula animal cap. This view has been supported by transplantation experiments in which the ventral yolk mass of a urodele blastula was combined with portions of the animal cap from the same or different species and the source of germ cells arising determined (Boterenbrood and Nieuwkoop, 1973; Sutasurya and Nieuwkoop, 1974). More recently, these grafting experiments have been repeated

(Michael, 1984), but the animal cap was divided up into several regions prior to combining with vegetal tissue. In this situation primordial germ cells were found only in combinations that included the equatorial, marginal zone of the animal cap. This observation becomes relevant for the question of the role of polar granules since electron-dense bodies similar to the polar granules of anurans have been detected in the marginal zone of the fertilized, uncleaved eggs of the urodele A. mexicanum, used in these transplantation experiments (Williams and Smith, 1971). To address the question whether the primordial germ cells are formed from this region of the animal cap under the inductive influence of the vegetative yolk mass, or whether they are already set aside, but restricted to the marginal zone of the animal cap, Michael removed the yolk mass at various times during development. Removal as early as stage six and a half still allowed the formation of primordial germ cells. Previous work had demonstrated that the inductive influence of vegetative yolk begins about stage six and decreases by stage eleven (Nieuwkoop and Ubbels, 1972). The time interval between stages six and six and a half, where the embryos are capable of developing primordial germ cells, is less than 1hr. Thus it is possible that little or no inductive influence is required for germ cell differentiation in A. mexicanum. Whether or not germ cell differentiation occurs only in cells containing specific elements (such as polar granules), or whether cells, perhaps restricted to the marginal zone, are induced to form germ cells remains to be unequivocally answered, however. On the basis of the above

evidence it is also possible that in urodeles the elements responsible for the formation of germ cells are distributed throughout the animal cap (in contrast to the vegetal localization in anuran amphibians), but that cells only differentiate as germ cells when they come to occupy a specific site, the marginal zone.

Germline determination in insects

The best case for a role of cytoplasmic determinants in germ cell determination is found in insects. Germ cell determination was first studied insects by Hegner (1911,1914), working with chrysomelid beetles. During early embryogenesis in these insects dividing nuclei present in the interior the egg migrate out to the posterior pole periphery where several of them enter a morphologically distinct region of cytoplasm and bud off. He showe that these cells were germ cells by ablating them and observing that surviving adults were sterile. He showed that this posterior pole cytoplasm necessary for the formation of these cells by destroying this cytoplasmic region prior to the peripheral movement of the nuclei. This resulted in a failure of the embryo to form germ cells, though otherwise normal adults were obtained.

In the dipteran Drosophila a similar process occurs. The first eight nuclear divisions of the Drosophila embryo occur without cellularization. The nuclei then migrate to the embryo periphery and most undergo additional divisions prior to becoming cellularized. However, those that migrate into the posterior pole

region become enclosed by polar buds which divide twice before cells, known as pole cells, pinch off. These cells will later become the germ cells of the adult (Huettner, 1923). A large number of experiments have shown that this posterior pole cytoplasm is required for the formation of pole cells. Thus removal of the posterior pole cytoplasm during early cleavage stages (Huettner, 1923; Frohnhofer et al. 1986), or UV irradiation of the posterior pole during this same time period (Geigy, 1931; Togashi and Okada, 1983), prevents pole cell formation. Nuclei which would have become incorporated into pole cells instead contribute to the somatic blastoderm. Similar observations have been made with a number of other insect species (Kalthoff and Rebagliati, 1988).

Experiments by Illmensee and Mahowald (1974) and, more recently, by Niki (1986), have shown that this posterior pole cytoplasm is not only necessary, but also sufficient for the determination of the germ cell lineage. Thus, Illmensee and Mahowald were able to take polar cytoplasm from cleavage stage embryos and inject it heterotopically into similarly staged embryos which were genetically marked. Following cellularization in these embryos, they observed that cells with a morphology similar to that of pole cells had been induced at the site of injection. They were able to show that these were in fact functional germ cells by transplanting them into the posterior pole of a blastoderm stage host, marked with a different set of genetic markers, and looking for germline mosaicism by doing the appropriate crosses. The factor(s) present in polar cytoplasm

that are required for germ cell determination are localized late during oogenesis. Using an experimental paradigm similar to that described above, Illmensee et al. (1976) showed that posterior pole cytoplasm becomes competent for germ cell induction late during oogenesis, during stages 13 and 14 (stages are those of King, 1970). Following fertilization the active components of polar plasm have a short half life. Thus if nuclear migration into the posterior pole is delayed by ligation for even 20 minutes, posterior pole cytoplasm loses its ability to induce pole cell formation. This loss of ability to respond is a function of the cytoplasm, not the nuclei entering it, since these embryos can be rescued by transplanting posterior pole cytoplasm from younger donor embryos into the posterior pole (Okada, 1986).

What is it in posterior pole cytoplasm that confers germ cell determination? Polar cytoplasm in the activated or fertilized egg is ultrastructurally distinct. It is relatively yolk free and contains large numbers of 0.1-0.5um electron-dense granules (polar granules), similar to those discussed previously in nematodes and anuran amphibians (Mahowald, 1962; Counce, 1963). When nuclei migrate into the posterior pole cytoplasm and become enclosed in the polar buds, most polar granules become incorporated into these buds. Subsequently, during pole cell formation and mitotic divisions, polar granules are segregated into these cells. Pole cells at this stage also contain large, electron-dense, nuclear structures, distinct from nucleoli, known as nuclear bodies (Mahowald et al.1976). Later during embryogenesis, the polar granules and nuclear bodies fragment

and small bits of electron-dense material (presumably derived from these structures) are found associated with the cytoplasmic face of the nuclear envelope. This material is called nuage (Mahowald, 1971a; Allis et al. 1979). Nuage is also seen in germline cells in larval and adult stages in both males and females and appears to be a germline-specific structure. In species in which the germline is determined late, such as mammals and urodele amphibians, it is this form of the polar granule material that is evident.

Several observations implicate polar granules as playing a role in germline determination. The first among these is simply that they are localized at the right place at the right time. In Drosophila they become localized to the posterior pole from about stage 10 of oogenesis onwards (Mahowald, 1962, 1971a), and remain concentrated in posterior pole cytoplasm during cleavage stages of embryogenesis. Secondly, both polar granules and pole cell determinants are associated with nucleic acid, probably RNA. Thus, the action spectrum of UV inhibition of pole cell formation is similar to the UV absorption spectrum of RNA, and the UV inhibition is photoreversible at longer wavelengths, consistent with an action of UV irradiation on nucleic acids (Togashi and Okada, 1983). Similar results have been reported for other insects as well (Kalthoff and Rebagliati, 1988). In Drosophila Togashi et al. (1986) found that polyA+ RNA from late stage oocytes or cleavage stage embryos (but not blastoderm stage embryos in which posterior pole cytoplasm is no longer competent to induce ectopic pole cell formation) can rescue the

ability of UV irradiated posterior poles to form pole cells. These cells complete their migrations to the embryonic gonad but disappear at some later point, and thus the adults are sterile. Whether the failure of polyA+ RNA to rescue germ cell function reflects simply a quantitative insufficiency in amount of the relevent RNA or the presence of a second UV sensitive target which is not included in the injections, remains unclear.

Polar granules are also associated with RNA, as assayed by histochemical criteria (Mahowald, 1971b). With this technique the granules are only found associated with RNA, however, during those stages of oogenesis and embryogenesis in which posterior pole cytoplasm is capable of inducing germline development. Electron microscopy of the granules at these competent stages indicates that the RNA is not ribosomal (ribosomes have a distinct, punctate structure), but perhaps mRNA. This latter point derives from the observation that following fertilization polysomes are found associated with the granules. Together these observations have suggested the hypothesis that polar granules function by storing and localizing specific mRNA to the posterior pole during oogenesis, and that this RNA subsequently becomes translated following fertilization, the translation products playing some important role in germ cell determination (Mahowald, 1971b). Consistent with this hypothesis, experiments in which cleavage stage Drosophila embryos are labeled with either ^{3}H-uridine or ^{3}H-valine show that much more translation occurs in the posterior pole cytoplasm during pole cell formation than in the rest of the

embryo, whereas RNA synthesis is relatively decreased with respect to the rest of the embryo (Zalokar, 1976). Similar observations have been made in the insect Musca (Pietruschka and Bier, 1972).

Recently several specific mRNAs have been found to be concentrated to the posterior pole early during embryogenesis. The transcript for cyclin B becomes concentrated in posterior pole cytoplasm between cleavage stages 7-10, perhaps independently of the nuclei which migrate into the posterior pole (Whitefield et al. 1989). This transcript then becomes concentrated into pole cells as they form. The transcript for the nanos product, required for proper abdomen specification, also becomes concentrated to the posterior pole and concentrated into pole cells. Whether this occurs during late oogenesis or early embryogenesis is unclear (Lehmann, unpublished). The nanos product is apparently not required for germ cell determination, since females which are homozygous mutant in nanos still form pole cells, though they lack most abdominal segmentation (Lehmann, unpublished). Whether cyclin B is required in a direct way for germ cell determination or pole cell formation is unknown. There is no direct evidence that either of these transcripts is localized to the polar granules. Their localization does require polar granule function, however, since neither transcript becomes localized in vasa mutants, which fail to localize polar granules (Lehmann, unpublished; Hay and Jongens, unpublished). Since cyclin B does not become localized to the posterior pole until following fertilization, yet polar plasm is

competent to induce pole cell formation when isolated from late stage oocytes, it must be the case that if granule associated RNA is important for pole cell formation and function, the cyclin B transcript is not the whole story. There is then no direct evidence implicating a role for the RNA associated with polar granules in germ cell determination.

The third and strongest piece of evidence implicating a role for polar granules in germ cell determination comes from the isolation of a group of maternal-effect mutations which alter the ability of the embryo to form pole cells. Seven mutants have been isolated, all of which have a similar phenotype: the progeny of homozygous females fail to form germ cells (tudor, Boswell and Mahowald, 1985; oskar, Lehmann and Nusslein-Volhard, 1986; vasa, valois, staufen, Schupbach and Wieschaus, 1986 and cappuchino and spire Manseau and Schupbach, 1989). Embryos from homozygous females also have abdominal deletions of varying degree, as well as other defects depending on the allele. At the EM level polar granules are not apparent at the posterior pole of strong alleles of any of the mutants. In experiments described in the results section it is shown that the protein product of one of these loci, the vasa locus, is a polar granule component (Hay et al. 1988b). From this fact one can say that polar granules, or at least the vasa product as a component of polar granules, is required for pole cell formation. Also, as described in the results, the homology of the vasa product with the helicase family of proteins, which function to unwind either DNA or RNA, is consistent with the evidence described above implicating a role

for maternal RNA in germ cell determination. In particular the homology of the vasa product with translation initiation factor 4A (eIF-4A), which is thought to function by unwinding secondary structure at the 5'end of mRNA, suggests a potential role of vasa in the regulation of protein translation. Mutation of conserved residues thought to be important in aspects of helicase function should allow testing of this hypothesis (Rozen et al., 1989).

It should be noted that other mutants have also been isolated in which the ability to form pole cells is altered. The first mutant isolated, gs (grandchildless), was isolated by Spurway in 1948. This maternal-effect mutation produces viable, but agametic offspring (Spurway, 1948). Polar granules are reduced in number or absent from the posterior pole of embryos from homozygous mothers and pole cells do not form. The mutant shows other defects as well. In particular, cleavage nuclei never migrate into the posterior pole cytoplasm (Mahowald et al. 1979). This defect, as detailed above, is sufficient to account for the observed sterility, though what the underlying defect responsible for this delay in migration is, is unknown. Another grandchildless mutation isolated in Drosophila melanogaster, gs(1)N26, also disrupts nuclear migration into the posterior pole cytoplasm but has no effect on polar granule disposition (Niki and Okada, 1981; Niki, 1984). The mutant gs(1)N441 shows a grandchildless phenotype and no other embryonic defects. Several other female-sterile mutants which show a grandchildless phenotype have been reported (fs(1)nasA, Counce and Ede, 1957; gs87, Thiery-Mieg et al. 1972; Thiery-Mieg, 1976; fs(1)1103 and fs(1)1122, Gans et al.

1975). However, the phenotypes of these mutants have not been characterized as extensively as those of the grandchildless-knirps group described above. Finally, the mutant agametic (ag) is interesting because, though pole cells form and migrate to the gonad, they die late during embryogenesis, giving rise to sterile adults (Engstrom et al. 1982).

Whether any of these loci encode elements which might be thought of as germ cell determinants is unknown. Certainly mutants such as gs(1)N441, which appear to affect only the germline are candidates for such elements. However, as discussed in chapter 4 with regard to the role of the products of the loci identified by the grandchildless screen on the second chromosome, from analysis of only one allele it is impossible to draw inferences about whether the phenotypes of the mutants are close to the null phenotype. And in any case there is, a priori, no reason to assume that factors required for germ cell determination do not also play some other essential role during other stages of the fly lifecycle. Therefore it is premature to speculate as to which of these genes may be more "interesting" than the others.

How many elements are required for polar granule function?

The seven genetic loci, tudor, oskar, vasa, valois, staufen, cappuchino and spire described above, all result in a common phenotype, the failure to show localized polar granules. Recently, in a screen carried out in the Jan lab, three new maternal-effect mutants have been isolated which have a similar phenotype in that embryos from homozygous females fail to give rise to a

germline (They are different in other respects, as described below). Thus it is clear that the path to polar granule function is fairly complex, involving at least 10 different gene functions. From consideration of some basic cell biology, however, it is clear that a number of different kinds of genes which affect this process should be identified. Localization of any organelle requires, in addition to those components which are purely structural, elements for coupling the organelle to the cytoskeleton, the cytoskeleton and its transport machinery, receptors which bind to the transported organelle, as well as perhaps other elements required for the stabilization of this localization. It is important to note that null mutants for any of these elements may well prove to be zygotic lethals. Hypomorphic alleles, though, may first show developmental lesions in pole cell formation and germ cell determination, since both the amount of material localized, its specific location, and the timing of contact with incoming cleavage nuclei are all likely to be important. Finally, it is also expected that genes which are required for the initial establishment of cell (in this case oocyte) polarity, will be identified. This last point is discussed further below.

Establishment of polarity during Drosophila oogenesis.

Drosophila oogenesis begins with a germline stem cell which divides four times without complete cytokinesis to give rise to a cluster of 16 cells interconnected by cytoplasmic bridges. The oocyte acquires the most posterior position among these. The fifteen anterior cells form the nurse cell cluster. They grow and

become polyploid until late stage 10, when they begin injecting their cytoplasm into the oocyte. This process continues until stage 14, when the nurse cells are completely absorbed. Study of the mutant dicephalic suggests that the position of the nurse cells with respect to the oocyte is important in determining anteroposterior polarity, including whether pole cells form (Lohs-Schardin, 1982). Embryos from homozygous dicephalic mothers sometimes show two anterior ends, in mirror image symmetry. In ovaries from dicephalic mothers the oocyte is sometimes found located such that nurse cell clusters are present at both anterior and posterior embryo poles. In normal oogenesis determinants required for the formation of anterior structures, in the form of the bicoid transcript, are transported into the oocyte at its anterior end, and localized to this end as soon as entry is completed (Frigerio et al. 1986; Berleth et al. 1988) . In dicephalic egg chambers these sites of entry are sometimes present at both the anterior and posterior poles (Frey et al. 1984). Thus under these conditions the bicoid transcript might be localized at both poles. This would provide a sufficient condition for the formation of double cephalons--in the absence of localized posterior determinants at the normal posterior pole. In fact pole cells do not form in dicephalic embryos (Lohs-Schardin, 1982). From the experiments of Illmensee and Mahowald (1974), it is clear that posterior pole cytoplasm is functional when injected anteriorly. Thus it is probably not the case that the presence of anterior determinants at the normal oocyte posterior pole is preventing the action of these elements. Rather, it seems

more likely that they are simply not localized correctly, perhaps due to the lack of normal positional signals which are present at the oocyte posterior pole during its development. Where might this signal originate? A likely source is the mesodermally derived follicle cells.

In the early stages of oogenesis, following the four mitotic divisions that give rise to the 16 cell cluster of germline cells that become nurse cells (15) and oocyte (1), about 80 follicle cells come to surround this cluster. As this structure, called the eggchamber, progresses through the phases of oogenesis, the follicle cells differentiate in a number of different ways. On the basis of morphology alone, 10 different groups have been identified (Margaritis et al. 1980). Between stages 2 and 5 of oogenesis a series of mitotic divisions occur in the follicle cells, increasing their number to about 1200 (King and Koch, 1963). Later, during stages 7 to 13, a series of follicle cell movements occur. During stages 7 to 10 most of the follicle cells move over the oocyte surface. A group of 6 to 10 anterior follicle cells migrate as a cluster between the nurse cells to the anterior surface of the nurse cells where they become located adjacent to the oocyte nucleus. These cells (border cells) are thought to be required for formation of the micropyle, which forms the sperm entry channel. At the posterior pole a specialized group of follicle cells is present which form a structure known as the areopyle (Margaritis et al. 1980). Interestingly, molecular differences (antibody immunoreactivity and enhancer driven lacZ gene expression) between follicle cells located at the

posterior pole and the rest of the follicular epithelium have been detected as early as stage 2 of oogenesis, and these differences are maintained throughout the rest of oogenesis (Brower et al. 1981; Fasano and Kerridge, 1988; Jan lab, unpublished).

The observation of molecular differences between follicle cells located at the posterior pole and the rest of the follicle is interesting since it has recently been shown that the somatically derived follicle cells and germline interact during oogenesis. The germline-dependent mutations (mutations whose effect is seen only when germline cells are mutant) fs(1)K10 and gurken (Wieschaus et al. 1978; Schupbach, 1987) alter the morphology of the somatically derived egg shell, in addition to the embryo. Conversely, the somatic-cell-dependent mutation torpedo changes the dorsal part of the embryo, in addition to the egg shell (Schupbach, 1987). Whether the follicle cell specializations seen at the posterior pole are a cause or consequence of oocyte polarity is unknown. However, it is an intriguing possibility that these cells might be important for some aspects of granule localization. As described below in the results, one of the new mutations isolated, in which germ cells fail to form, shows a pattern of expression which includes these posterior cells, consistent with this possibility.

CHAPTER 1

IDENTIFICATION OF A COMPONENT OF DROSOPHILA POLAR GRANULES

SUMMARY

Information necessary for the formation of pole cells, precursors of the germline, is provided maternally and localized to the posterior pole of the Drosophila egg. The maternal origin and posterior localization of polar granules suggest that they may be associated with pole cell determinants. We have generated an antibody (Mab46F11) against polar granules (Hay et al. 1988b). In oocytes and early embryos, the Mab46F11 antigen is sharply localized to the posterior embryonic pole. In pole cells, it becomes associated with nuclear bodies within, and nuage around, the nucleus. Immunoreactivity remains associated with cells of the germ line throughout the lifecycle of both males and females. This antibody recognizes a 72kd protein and is useful both as a pole cell lineage marker and in biochemical studies of polar granules. During late embryogenesis this antibody also recognizes an antigen with a similar apparent molecular mass that is present in nuclei of most, if not all cells. Based on experiments described in Chapter 2 we think these are not the same protein.

MATERIALS AND METHODS

Stocks

Drosophila melanogaster were raised on standard cornmeal-yeast-agar medium at 25°C. Normal flies were of Oregon-R (OR) wild-type strain. Balanced stocks of vasa (vas^{PD23}) and pumillio^{680} were kindly provided by Drs T. Schupbach and E. Weischaus, Princeton University, and Drs C. Nusslein-Volhard and R. Lehmann, Max-Planck Institute, Tubingen, respectively.

Antibody production

Polyclonal antisera directed against horseradish peroxidase (HRP) stain the nervous system of Drosophila as well as that of other insects (Jan and Jan, 1982). In an attempt to generate antibodies directed against subpopulations of neurons, a hybridoma fusion and screen for monoclonal antibodies was carried out, in which HRP (Sigma type VI) was used as the immunogen. In the course of screening the clone supernatents on tissue sections, an IgM-secreting clone (designated Mab46F11) was isolated which recognizes cells of the Drosophila germ line. This antibody does not bind HRP and thus the relationship of the antigen recognized to the immunogen is unknown.

Antibody staining

Embryo collections were done according to Allis et al. (1977). Embryos were kept at 25°C and time points were taken at 1hr

intervals. Embryos were processed for fixation, devitellinization and antibody staining as described by Bodmer and Jan (1987). Mab46F11 culture supernatant was diluted fivefold in blocking buffer (0.1M-sodium phosphate, pH7.5, 0.3% Triton X-100, 0.3% sodium deoxycholate, 2% bovine serum albumin (Miles, fraction V)) prior to use.

For cryostat sections, embryos or ovaries were fixed as above for 5 min, covered with a drop of OCT compound (Lab Tek products), frozen onto a chuck at -20°C and sectioned at 12um with a Reichert-Jung 2800 Frigocut. Sections were placed on slides coated with 0.1% gelatin and fixed for 10 min in 2% formaldehyde, 0.1M-phosphate buffer, pH7.6 Slides were processed for antibody staining as above.

For wholemount staining of larval and adult gonads, the following procedure was adopted. A number of gonads were dissected in Schneiders Drosophila medium (Gibco) and placed at 4°C for up to 1 hr. Gonads were then placed on a glass slide coated with 0.1% gelatin and the bulk of the excess solution withdrawn. A second slide similarly coated was then gently lowered down onto the first slide, pressing the tissue between them. The two slides were then placed against a block of dry ice for 5 min. The slides were then split apart with a razor blade and transfered to pre-chilled acetone (-70°C) for 5 min, to pre-chilled methanol (-70°C) for 3 min, and then through an ethanol series (at room temperature) into 0.1M phosphate buffer, pH 7.6. Slides were processed for antibody staining as above.

Immuno-electron microscopy was carried out as follows. Embryos were dechorionated in bleach:water (1:1), rinsed extensively with H_2O and placed onto double-stick tape. They were then covered with fixative (2% glutaraldehyde, 4% paraformaldehyde, 15% picric acid, 0.1 M sodium cacodylate, pH 7.2-7.4). The vitelline membrane was ruptured with a glass microelectrode and embryos allowed to fix for 10 min. Embryos were then nudged out of their vitelline membrane with a pair of blunt forceps and fixation allowed to continue for another 30 min. Tissues were dissected under the fixative and incubated similarly. Samples were then rinsed three times with 0.1 M sodium cacodylate pH 7.2-7.4. Enblock staining was performed with 2% aqueous uranyl acetate for 2 hrs. A brief H_2O rinse was followed by dehydration in 35%, 75% and 95% ethanol. The tissue was infiltrated with Lowicryl K4M (Polyscience):ethanol in proportions 1:2, 1:1 and undiluted K4M for 2 hrs each, at room temperature. The initiator was benzoin ethyl ether. Polymerization was effected with a UV lamp (model UVG-11, Ultraviolet Products) at 356nm for 24-48 hrs. Immunostaining was carried out as follows. Sections were incubated in H_2O for 1 min, in blocking buffer (3% BSA, 2% normal goat senum, 0.1 M phosphate, pH 7.2, 0.3% Triton X100, 0.3% deoxycholic acid) for 5 min, and with Mab46F11, diluted 1:4 in blocking buffer, for 15 min. Sections were then rinsed in blocking buffer with 3 changes of 2 min each, and subsequently in the same buffer with secondary antibody (goat anti-mouse IgM conjugated with 5nm colloidal gold (Jansen)), for 10 min. Grids were then rinsed as above, then 3 times, for 1 min each, with H_2O. Sections were then post-stained with 2% aqueous

uranyl acetate for 5 min and rinsed thoroughly with H_2O. Sections were examined and photographed with a Phillips 400 transmission electron microscope.

Western blotting.

Protein extracts were prepared from various sources by homogenizing the tissue in a glass/glass homogenizer in approximately 10 volumes of extraction buffer (2.5% SDS, 0.1 M Tris.Cl, pH 6.8, 10mM ethylenediaminetetraacetic acid (EDTA)) to which the protease inhibitors (100*u*M benzamidine, 10mM sodium bisulfite, 2mM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethyleneglycol-bis-(Binoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 10uM leupeptin, 10uM pepstatin,100Ki.u./ml^{-1} aprotinin, were added just before use. Samples were then boiled for 5 min and spun at full speed in a microfuge (eppendorf) for 5 min to pellet insoluble material. The supernatants were aliquoted and stored at -80°C. For SDS-PAGE equal amounts of sample protein were precipitated with 15% trichloroacetic acid (TCA) at 4°C for 20 minutes, and then pelleted in a microfuge at full speed at 4°C. The supernatants were aspirated and the pellets resuspended with acetone at 4°C. The samples were repelleted as above and the supernatants aspirated. The acetone pellets were then processed for SDS-PAGE under reducing conditions according to Laemmli (1970), and run on 10% polyacrylamide gels with 4% stacking gels. Protein was transfered to nitrocellulose (Schleicher and Schuell, BA85) according to Towbin et al. (1979), using a Biorad trans-blot cell at 0.1 Ampere for 8-12 hrs. Nonspecific binding sites were blocked by

incubation of filters for 1 hr in blocking buffer (10mM Tris.Cl, pH 8.0, 150mM NaCl, 0.05% Tween-20 and 2% BSA), at 37°C. Filters were subsequently incubated at room temperature for 1 hr with blocking buffer and Mab46F11 (1:10 dilution of culture supernatant), 1 hr with affinity purified rabbit anti-mouse IgM antibody (Zymed 1:1000 dilution), and 1 hr with alkaline phosphatase conjugated goat anti-rabbit antibody (Promega Biotec, 1:7500). Three 15 min washes in blocking buffer followed each antibody incubation. Following a final wash, in blocking buffer without BSA, the alkaline phosphatase reaction product was developed according to the Promega Biotec protocol.

Embryo fractionation

To obtain a protein sample enriched for pole cells, a variation of the protocol of Allis et al. (1977) for the isolation of pole cells was adopted, in which Percol (pharmacia) was substituted for Renografin-60 as a centrifugation medium. A final crude cell pellet (Allis et al. 1977) was resuspended into 3-5 mls of Chan and Gehring buffer (Chan and Gehring, 1971) (55mM NaCI, 40mM KCl, 15mM MgS04, 5mM CaCl2, 20mM glucose, 50mM sucrose, 10mM Tricine, pH 7.0, 1 mg/ml BSA) minus calcium (C&G-Ca++), and loaded on a Percoll gradient constructed as described below. A 90% Percoll stock was created by diluting 1OX C&G-Ca++ 1:9 with 100% Percoll. This was diluted appropriately with 1 X C&G-Ca++ to generate 15%, 70% and 80% working Percoll solutions. A 35ml 15-70% linear Percoll gradient was created over a 5ml 80% bottom layer in 50 ml Falcon tubes. The gradient was centrifuged at 400xg

for 20 min in a Sorvall SS34 rotor with the brake off. 2 ml gradient fractions were collected from the top. These were diluted 1:1 with C&G minus BSA (C&G-BSA) and spun at 1 20xg for10 min to pellet the cells. Resuspension and pelleting were repeated twice to remove traces of Percoll. Cells were then resuspended in 500*u*l of C&G-BSA and counted using a hemocytometer. Cell rich fractions were saved. A 100*u*l aliquot of cells from each fraction was processed as described below for immunocytochemistry, and the rest of the sample processed for Western blotting.

For immunocytochemistry, cells isolated from Percoll gradients were allowed to attach to glass coverslips coated with 100*u*g/ml concanavilin A (Sigma type IV) and 1 mg/ml of poly-D-lysine hydrobromide (Sigma, approximate MW 345,000) for five min, fixed in 4% formaldehyde, 0.1 M phosphate buffer, pH 7.6, for 15 min, and then processed for antibody staining as above.

Pole cell counts.

Pole cell number and position in wholemount embryos were recorded at different developmental stages using a Nikon Optiphot microscope equipped with differential interference contrast optics. A Nikon camera lucida was used to outline each labelled cell. Counts were made from these drawings.

RESULTS

The antigen recognized is associated with polar granules.

Embryogenesis in Drosophila begins with a period of internal nuclear divisions; this is followed by migration of most of these nuclei to the egg periphery where blastoderm formation occurs. In the unfertilized egg and cleavage stage embryo, cortical cytoplasm at the posterior pole contains large numbers of polar granules, 0.2-0.5um, electron dense structures (Mahowald, 1962). At the end of the eighth nuclear division (beginning of stage 3; staging is that of Campos-Ortega and Hartenstein, 1985), several nuclei migrate into this cortex and become incorporated into three to five cytoplasmic buds that protrude from the posterior egg pole. Most polar granules become incorporated into these buds (Counce, 1963; Mahowald, 1968). Subsequently, during stages 3 and 4, these buds divide twice (Foe and Alberts, 1983), before 12-32 pole cells pinch off. These cells divide from 0-2 times prior to blastoderm formation (Technau and Campos-Ortega, 1986). Polar granules are segregated into these newly formed pole cells, which also contain large, electron dense nuclear structures that are distinct from nucleoli, and known as nuclear bodies (Mahowald et al., 1976). Later during embryogenesis, polar granules and nuclear bodies fragment and small bits of electron dense material (presumably derived from these structures) are found dispersed throughout the cytoplasm. Some of this electron dense material is associated with the cytoplasmic face of the nuclear envelope and is called nuage

(Mahowald, 1971a; Allis et al., 1979). Nuage is also seen in germline cells in larval and adult stages, and appears to be a germline-specific structure.

Staining with Mab46F11 parallels the localization of polar granules, nuclear bodies and nuage. In the unfertilized egg and cleavage stage embryo immunoreactivity is confined to a thin cortical cap at the embryonic posterior pole (figure 1.1a). Immunoreactivity subsequently becomes associated with the nuclei that migrate into the posterior pole cortex (figure 1.1 b). This localization may involve active sequestration by the nuclei or degradation in the surrounding regions, since cortical internuclear regions are relatively devoid of immunoreactivity. By the time pole cells form, immunoreactivity is almost always localized only to cells with a typical pole cell morphology (figure 1.1 c,d).

At the EM level immunocytochemical label is associated with polar granules and nuclear bodies of newly formed pole cells (figure 1.2). Later in embryogenesis immunoreactivity in the pole cells becomes scattered throughout the cytoplasm and associated with nuage (data not shown; nuage associated label is shown in figure 1.6a, in the adult ovary).

Although material similar to Drosophila polar granules and nuage has been observed in the germ line of a number of species (Beams and Kessel, 1974; Eddy, 1975), the antibody does not recognize these structures in Xenopus laevis, Caenorhabditis elegans, or various other Drosophila species, including the closely related species, D. simulans and D. mauritiana.

The fate of pole cells during embryogenesis.

In Drosophila roughly twice as many pole cells are initially produced as ultimately become associated with the gonads (Rabinowitz, 1941; Sonnenblick, 1941; Turner and Mahowald, 1976; Zalokar and Erk 1976; Underwood et al., 1980; Technau and Campos-Ortega, 1986). From experiments in which exogenously labelled pole cells are transplanted to unlabeled embryos it appears that this reduction involves both pole cells becoming lost and pole cell death during migration to the site of the gonad. There is little or no evidence suggesting pole cells contribute to tissues other than the germline (Underwood, 1980; Technau and Campos-Ortega, 1986). In order to determine the utility of Mab46F11 as a pole cell lineage marker, we have used this antibody to follow the fate of pole cells and their migrations during embryogenesis. For this purpose the number of Mab46F11 immunoreactive cells and their distribution between lost and appropriately migrating cell populations, through stage 14 of embryogenesis (by which time the presumptive gonads have formed), was tabulated and plotted as frequency histograms in figure 1.3.

Most of the Mab46F11 immunoreactive cells present at the posterior pole of the embryo by the beginning of gastrulation (stages 6-7) ($X = 41 \pm 0.7$; $\pm$ = standard error of the mean; $n = 100$ for each sample; figure 1.3a) have a typical pole cell morphology (figure 1.1 e,f) and are found outside the blastoderm. A variable number (from 0-8) are found lying interdigitated between neighboring, nonstaining, blastoderm cells or underneath the blastoderm layer, apposed to its inner face but clearly separated

from the yolk by the yolk membrane (figure 1.1 c, arrowhead; 1.1 d,e arrows). At the beginning of gastrulation, pole cells are carried dorsally and anteriorly above a plate of about 150 cells (figure 1.1 e,f) which forms a sack that encloses the pole cells and carries them inside the embryo during germ band elongation (figure 1.1 g,h). At the end of germ band elongation (stage 10), the pole cells leave this sack by passing through the walls of the posterior midgut primordium (figure 1.1i,j). Occasionally a cell appears trapped in the midgut, or between cells of the midgut epithelia; however the vast majority migrate through without apparent mishap. The average number of immunoreactive cells present during late stage 10 and early stage 11 is 43$\pm$0.5 (figure 1.3b).

During early germ band shortening (early stage 12), pole cells become displaced laterally (figure 1.1 k,l), and a number (5$\pm$0.4, figure 1.3c) of apparently lost cells are seen (figure 1.1l). The total number of pole cells has also decreased, to 37$\pm$0.7 (figure 1.3c'). By the end of germ band shortening (early stage 13) most pole cells are closely associated with each other and are contacting mesoderm at the level of the fifth to eighth abdominal segments(A5-A8) (figure 1.1 m,n). The total number of labelled cells has decreased further (from 37$\pm$0.7 to 29$\pm$0.4, figure 1.3c,d). This is reflected in a decrease in the number of appropriately migrating cells (from 32$\pm$0.5 to 26$\pm$0.4, figure 1.3c,d) as well as in the number of apparently lost pole cells (from 5$\pm$0.4 to 3$\pm$0.2, figure 1.3 c,d). No further reduction is seen through the middle of stage 14, when the appropriately migrating pole cells have condensed at the level of

A5 and become surrounded by mesoderm (figure 1.1 o,p; figure 1.3e,e').

The following general observations are relevant concerning pole cell fate. First, immunoreactive cells which appear lost are found scattered throughout the embryo up through hatching. Therefore it is not the case that pole cells that do not reach the gonad invariably die or become incorporated into other tissues, resulting in a loss of expression of the Mab46F11 antigen. Second, weakly staining cells which have a distorted, blebbing morphology and are in the process of degeneration are found in both lost and appropriately migrating cell populations from the end of germ band elongation onward (data not shown). These observations are consistent with the hypothesis that the loss of labelled cells seen during germ band shortening is due to pole cell death, occurring in both lost and appropriately migrating cell populations. By stage 14 we find an average of 14 pole cells per gonad. This number is higher than that reported by others during roughly stages 15-16, with 5-7 pole cells in gonads of some embryos and 9-13 in gonads of others (Sonnenblick, 1941; Technau and Campos-Ortega, 1986). This difference in pole cell number suggests that pole cell death continues to occur following initial gonad formation, during stage 14. Pole cell fate, as revealed by Mab46Fl1 staining, generally agrees with conclusions obtained from previous studies (Underwood et al., 1980; Technau and Campos-Ortega, 1986). However, compared to studies using cell transplantation, immunocytochemical staining with Mab46Fl1 provides a much simpler and more quantitative method of following pole cells in

wildtype and mutant embryos. To illustrate this point, we have carried out Mab46F11 staining of embryos from homozygous pumillio mothers (figure 1.4). The maternally supplied pumillio gene product (located at the embryonic posterior pole) is required for proper abdomen specification; mutant embryos lack variable numbers of abdominal segments. In these embryos the formation of pole cells and their subsequent migrations during gastrulation are normal (Fig. 1.4a,b). The behavior of these cells during germ band shortening is severely disturbed, however. Lost cells are found scattered throughtout the stage 13 mutant embryo (figure 1.4c,d), whereas in the wildtype condition they would be aligning themselves along the body wall at the level of A5-A7 (see Fig. 1.1 n). This defect in migration is likely due to an abnormal environment, since functional pole cells can be recovered when pumillio pole cells are transplanted into otherwise sterile host embryos (Lehmann and Nusslein-Volhard, 1987).

Continuity of the 46F11 antigen in the germ line following embryogenesis.

Adult female germ-line.

Synthesis of polar granule components, and assembly and transport of these elements to the future embryonic posterior pole occurs during oogenesis (Mahowald, 1962, 1971a). As a step towards understanding these processes, we have used light and EM immunocytochemistry to examine the cellular and subcellular localization of the Mab46F11 antigen throughout oogenesis.

Each of the two adult ovaries contains 10-20 parallel egg tubes or ovarioles within which developmental stages of oogenesis are arranged linearly. (See King, 1970, and Mahowald and Kambysellis, 1978 for reviews). In figure 1.5a a whole mount preparation of part of an ovariole stained with Mab46F11 is shown. At the most anterior end, in the germarium, perinuclear label is present in several large cells, probably stem cells. These cells divide unequally to produce, after each division, another stem cell and a daughter cystoblast. The cystoblast subsequently undergoes 4 mitotic divisions with incomplete cytokinesis to produce a cluster of 16 cells interconnected by intercellular bridges. These are the smaller labelled cells, located in the posterior germarium (figure 1.5a). One of these 16 cells enters prophase of meiosis 1 and will become the oocyte; the other fifteen become nurse cells. During vitellarium stages of oogenesis, in which the bulk of oocyte and nurse cell growth occurs, perinuclear and cytoplasmic label is found in the 15 nurse cells (figure 1.5a,b). Staining is not seen in the oocyte until stage 10 (figure 1.5b, see below). Label is also not apparent in the follicle cells or any other somatically derived tissues of the gonad. At the EM level, perinuclear label in germ line cells of the germarium (data not shown), and in nurse cells of the vitellarium (figure 1.6a), is found associated with electron dense clumps of material on the cytoplasmic face of the nuclear envelope. These are the nuage or fibrous bodies previously described in the Drosophila germ line (Counce, 1963; Mahowald, 1971a; Mahowald and Strassheim, 1970). Label in the nurse cell cytoplasm may reflect the site of synthesis of the antigen.

During stages 10-12 of vitellogenesls, the nurse cells rapidly decrease in size as they inject their contents into the growing oocyte through intercellular channels at the anterior end of the oocyte. Cytoplasmic mixing rapidly distributes this material throughout the ooplasm (Gutzeit and Koppa, 1982). Localization of polar granules to the posterior pole of the oocyte takes place during this period, beginning during stage 10 and continuing through stage 13 (Mahowald, 1962, 1971a). At the earliest stage when Mab46F11 immunoreactivity is detectable in the oocyte (around stage 10; figure 1.5b) it is located at the posterior tip of the oocyte, and is associated with polar granules (figure 1.6b). By stage 14 (the mature oocyte, figure 1.5c) the number and size of these granules has increased, and they are often found associated with mitochondria (figure 1.6c). Because both polar granules and nurse cell nuage label with Mab46F11, our data supports the hypothesis that polar granules may derive from the nuage (Mahowald, 1971a). Since materials from nurse cells enter the oocyte at its anterior end and are mixed rather thoroughly by the swirling movements in the oocyte, the rapid localization of the Mab46F11 antigen to the posterior tip of the oocyte suggests that specific targeting mechanisms are required for this localization.

Adult male germ line

Synthesis of the Mab46F11 antigen by nurse cells during oogenesis is consistent with a postulated role of the antigen as a component of polar granules, structures important in early germ line determination. Synthesis in the adult male germ line, however,

would suggest that this antigen plays additional roles in germ line function. We have carried out immunocytochemical staining with Mab46F11 to address this possibility.

Figure 1.5d shows the apical half of a wholemount testis stained with Mab46F11. Small cells near the apex are germ line stem cells and their offspring the cyst progenitor cells. Cyst progenitors divide mitotically four times to produce a cluster of 16 primary spermatocytes. (See Lindsley and Tokuyasu, 1980 for a detailed review of spermatogenesis). Perinuclear label is present in each of these cell types (figure 1.5d). One or more large dots of label are also seen in the cytoplasm or associated with the nuclear envelope (figure 1.5d, arrow). These are probably the dense cytoplasmic masses described by Kessel (1981) (see discussion). More distal to these stages, larger maturing spermatocytes are seen. Perinuclear and punctate staining are detectable until about the time spermatocytes enter meiosis. At the EM level, Mab46F11 label in germ line cells of the testis is primarily found over perinuclear, electron dense structures (data not shown), similar to those labelled in the nurse cells of the ovary (figure 1.6a). The significance of antigen localization to these structures will be returned to in the discussion.

Nuclear Mab46F11 immunoreactivity

During late embryogenesis (stage 16-onward) and during larval life, a general nuclear staining is apparent in essentially all cell types (figure 1.7d), in addition to the germ-line staining seen with Mab46F11. Similar staining is not seen with second antibody alone

or with several other mouse IgM monoclonals used (data not shown). Although it persists throughout larval stages, this staining becomes undetectable in adult somatic tissues (cf. somatic cells of ovary in figure 1.5).

The antigen recognized by Mab46F11 is a 72kd protein.

We have carried out Western blotting with Mab46F11 to identify the antigen recognized by this antibody. Each of the tissues that label immunocytochemically with Mab46F11 contains an immunoreactive 72kd protein (Fig. 1.7a). The intensity of this band on Western blot is roughly correlated with the abundance of the Mab46F11 antigen seen immunocytochemically. Thus, stage 2-4 embryos show a weak immunoreactive band. This band is much more prominent in the lanes containing total protein from adult ovaries or a fraction enriched (roughly 10 fold) for pole cells from stage 2-5 embryos. A similar band is also present in protein from adult testis.

If the 72kd protein is in fact a component of polar granules it should be absent in mutant embryos which are missing polar granules. One mutant which displays such a phenotype is the maternal-effect mutant vasa (Schupbach and Wieschaus, 1986). Embryos from homozygous mutant vasa mothers lack detectable polar granules and fail to make pole cells (Schupbach and Wieschaus, 1986). They also lack detectable Mab46F11 antigen immunocytochemically (see also chapter 3). Figure 1.7c shows that these embryos contain no detectable immunoreactive protein, even following prolonged development of the alkaline phosphatase

reaction product. The 45kd band in wild-type as well as mutant embryos is one of the yolk polypeptides, major constituents of the early embryo. This band only appears following prolonged exposure and is also present in control experiments using other mouse IgM monoclonal antibodies (data not shown). Thus it is likely the 72kd immunoreactive protein from early embryos is the antigen immunocytochemically associated with polar granules.

Previous studies by Waring et al. (1978) revealed a single major protein species of 95kd enriched for in pole cell enriched cell populations. A band of approximately 70kd also appears as a fairly major component of the various fractions (Waring et al., 1978). The relation between this band and the Mab46F11 antigen is not clear. However, of the various tissues and fractions we have examined, the 72-74X10^3Mr antigen does not comigrate with any major protein species detectable with staining by commasie blue. Neither have we detected any immunoreactivity associated with proteins of higher molecular weight, even when special attention was paid to avoid possible degradation.

Total protein from late (stage 16 and 17) wild type embryos (figure 1.7a) (and from homozygous vasa embryos derived from homozygous vasa mothers), has a large amount of an immunoreactive protein. This cannot be accounted for by an increase in the germ line associated antigen, due to gonad growth, but may be due to the nuclear staining of somatic tissues that is well developed by this time (figure 1.7d). Furthermore, the level of the nuclear staining at different stages of the life cycle correlates well with that of the immunoreactive 72kd band (figure 1.7b). This band is found at low levels in early embryos. A large increase is apparent by stage 16, when nuclear staining appears. During the second and third larval instars the intensity of the 72kd immunoreactive band decreases. In adults with gonads removed there is even less immunoreactive protein, which corresponds to the lack of detectable nuclear staining in adult somatic tissues. We have not looked at the EM level to be able to say with what structures this nuclear antigen is associated. The 72kd protein identified as a component of polar granules comigrates with the protein associated with somatic nuclei. From experiments described in Chapter 3 we think these are not encoded by the same gene, however.

DISCUSSION

Polar granules are localized to the posterior pole of mature oocytes and early embryos of Drosophila and may be associated with determinants of germline cells (Illmensee and Mahowald, 1974). We have isolated a monoclonal antibody, Mab46F11, that recognizes a component of polar granules. This antibody labels germ cells throughout the lifecycle of the fly. Using this antibody, we have made the following observations: (1) The rapid rate at which the Mab46F11 antigen is transported from anteriorly located nurse cells to a thin cortical cap at the posterior end of the oocyte suggests the presence of a specific and effective targeting machinery. (2) The association of this antigen with nuclei in the pole buds of cleavage stage embryo, and its clearing from the internuclear cortical regions, raises the possibility that it may be actively sequestered. Likely candidates for this sequestration may include cytoskeletal components that show distinctive patterns of distribution at these early stages (Warn et al., 1985; Karr and Alberts, 1986). (3) Germ line specific structures, including polar granules, nuclear bodies, nuage and dense cytoplasmic masses, are related biochemically because they all contain the Mab46F11 antigen. (4) The Mab46F11 antigen appears as a 72-74X10^3Mr protein on Westem blots. This study suggests that the Mab46F11 antibody may be used for further biochemical characterizations of the components of polar granules. In addition, this antibody serves

as a faithful marker for pole cells throughout embryogenesis. Discussions of some of these points are presented below.

The number and fate of pole cells

In Drosophila and other Dipterans many more pole cells are formed than are found in the gonads (Rabinowitz, 1941; Sonnenblick, 1941). This has suggested that pole cells may contribute to tissues other than the germ line (cf Poulson and Waterhouse, 1960). However, recent transplantation experiments using [^{3}H]thymidine or HRP labelled pole cells have found no evidence for pole cell incorporation into other tissues (Underwood, et al., 1980; Technau and Campos-Ortega, 1986), and suggest instead that unaccounted cells may have gotten lost and/or died. Consistent with these observations, we find that two major causes of the low number of pole cells found in the embryonic gonad are, 1) pole cells becoming lost, primarily during germ band shortening, and 2) pole cell death, occurring in both the lost and appropriately migrating populations, also primarily during germ band shortening.

In pole cell transplantation experiments, in which large numbers of labelled pole cells are transplanted into the posterior pole of an unlabelled blastoderm embryo, the final number of pole cells in the gonads is similar to that of an uninjected embryo (X = 9). This has suggested that there is regulation of the final number of pole cells in the gonad, with an upper limit of about 13 pole cells per gonad at about stage 16 (Technau and Campos-Ortega, 1986). Our finding of an average of 14 labelled cells per gonad during stage 14 suggests

that some of this regulation may occur following gonad formation, during stages 15-16.

This antibody should prove useful in examining aspects of postembyonic germ cell behavior since it recognizes premeiotic germ cells throughout the fly lifecycle. It should also greatly facilitate study of the fate of pole cells in embryos in which maintenance of the germ line, following pole cell formation, is affected (Engstrom, et al., 1982; Oliver, et al., 1987; Togashi, et al., 1986).

Association of the Mab46F11 antigen with germline specific structures

Polar granules, nuclear bodies, nuage, and dense cytoplasmic masses are germline-specific structures. The observations that these structures are all recognized by Mab46F11, and that tissues in which they are found show a common immunoreactive 72-74X10^3Mr protein on Western blotting, indicates they are biochemically related. The fact that similar structures are found in the definitive germ line of phylogenetically distant groups, including other dipterous insects (Mahowald and Boswell, 1983), nematodes (Strome and Wood, 1982), amphibians (Smith et al., 1983) and mammals (Eddy and Hahnel, 1983), suggests that they are important for some fundamental aspect of germ cell life. The function of all of these structures is unknown. It is perhaps relevant, however, to note that in Drosophila polar granules (in oocytes and early embryos) and dense cytoplasmic masses (in spermatocytes) have been demonstrated to contain RNA, and are

present at stages when mRNA storage for later use is likely to be important (Mahowald, 1971 b; Kessel, 1981). Thus, it seems plausible, though there is no direct evidence, that these structures store RNA necessary for the determination of germ line cells in the early embryo, and for spermatid formation in the testis, respectively.

If polar granules are directly involved in pole cell formation and germline determination, it should be possible to isolate mutants which lack polar granules and which fail to form pole cells. Such mutants, members of the grandchildless-knirps class of maternal effect mutations, have been isolated in Drosophila (tudor, Boswell and Mahowald,1985; vasa, valois, staufen, Schupbach and Wieschaus,1986; oskar, Lehmann and Nusslein-Volhard,1986; cappuccino and spire, Manseau and Schupbach, 1989). Embryos derived from females homozygous for mutations at these loci lack a germ cytoplasm that contains polar granules and they fail to form pole cells. In addition, however, embryos show deletions of a variable number of abdominal segments, and may exhibit head defects or incomplete blastoderm cellularization, depending on the allele. The absence of this germ cytoplasm appears closely associated with the loss of pole cells and not with deletions of abdominal segments since, in the cases of tudor and oskar, the abdominal segmentation and pole cell phenotypes are separable (Boswell and Mahowald, 1985; Lehmann and Nusslein-Volhard, 1986) Similarly, in mutants showing a bicaudal phenotype (a mirror image duplication of the posterior abdomen at the anterior end) the transformed anterior end lacks polar plasm and does not form pole cells. (Bull, 1966; Nusslein-

Volhard, 1977; Mohler and Wieschaus, 1985). The above grandchildless mutations thus seem most likely to be due to defects in shared elements necessary for the synthesis, packaging, transport or localization of abdomen and pole cell determinants. Indeed, Mab46F11 staining has revealed defects in these processes in the above mutants (Chapter 3). The most direct way of addressing whether polar granules are necessary and sufficient elements for pole cell formation is to isolate them and their constituents from oocytes or preblastoderm embryos (when polar plasm is competent for nuclear induction), for biochemical and genetic analysis. A step in this direction is the identification of a 72-74X10^3Mr protein as a likely component of polar granules, nuclear bodies and nuage. If, based on this association, Mab46F11 can be used to purify polar granules for further molecular characterization of their protein and RNA components, it may be possible to critically test polar granule function in the early embryo.

Figure 1.1. Distribution of the Mab46F11 antigen before pole cell formation, and at various stages of pole cell migration during embryogenesis.

Optical sections of wholemount embryos (anterior is to the left and dorsal is uppermost). In e-p, figures on the left are parasagittal sections and those on the right are horizontal sections of similarly staged embryos. a) Cleavage stage (stages 1 and 2) embryo. Label is present in a cortical cap at the posterior pole. b) Stage 3 embryo in the process of forming pole buds. Label becomes concentrated around nuclei and depleted in the cortical regions between nuclei. c) Middle of stage 5, during blastoderm formation. A cap of immunoreactive pole cells sits on top of a nonstaining blastoderm. Several cells in the blastoderm layer have taken up Mab46F11 immunoreactive material (arrow). One of these (arrowhead) has blastoderm cell morphology. d) Stage 6 embryo, following blastoderm formation. Pole cells which are beneath the blastoderm cell layer are apparent (arrows). e) Parasagittal view of a stage 7 embryo. Note the 3 labelled cells lying underneath the blastoderm layer (arrow). f) Horizontal view of embryo similar to that in e. g) Parasagittal view of a late stage 8-early stage 9 embryo. Pole cells are found in the posterior midgut pocket (pm). One pole cell is just outside the midgut. h) Horizontal view of late stage 9 embryo. Some pole cells are beginning to migrate through the midgut. i) Parasagittal view of a stage 10 embryo. Most pole cells have migrated through the dorsal half of the posterior midgut. j) Horizontal view of a stage 10 embryo with pole cells migrating

through the midgut. k) Early stage 12 embryo, the first half of germ band shortening. Pole cells are arranged bilaterally, in groups that run from roughly A5 to A8. 1) Dorsal view of embryo during the first half of stage 12. Pole cells have become arranged bilaterally. Lost cells are present slightly out of the plane of focus (arrows). m) Almost parasagittal view of an early stage 13 embryo, the end of germ band shortening. Pole cells are contacting each other and contacting lateral mesoderm at the level of A5 and A6. Labelled cells, displaced but close to the condensing gonad (arrows) and others lost in the yolk (arrowhead) are present. n) Horizontal view of a stage 13 embryo. Pole cells lie in contact with mesoderm at the level of segments A5-A7, roughly. Lost cells are apparent in the yolk and tail region of the embryo (arrows). o) Almost parasagittal view of a stage 14 embryo. Pole cells have become surrounded and encapsulated by mesoderm at the level of A5. p) Horizontal view through a stage 14 embryo. Pole cells are clustered at A5. A lost pole cell is present near the forming gonad (arrowhead). Other lost pole cells are present in the yolk (out of the focal plane, large arrow) and along the body wall of more posterior abdominal segments (small arrows). pm = posterior midgut. (bar=100*um*).

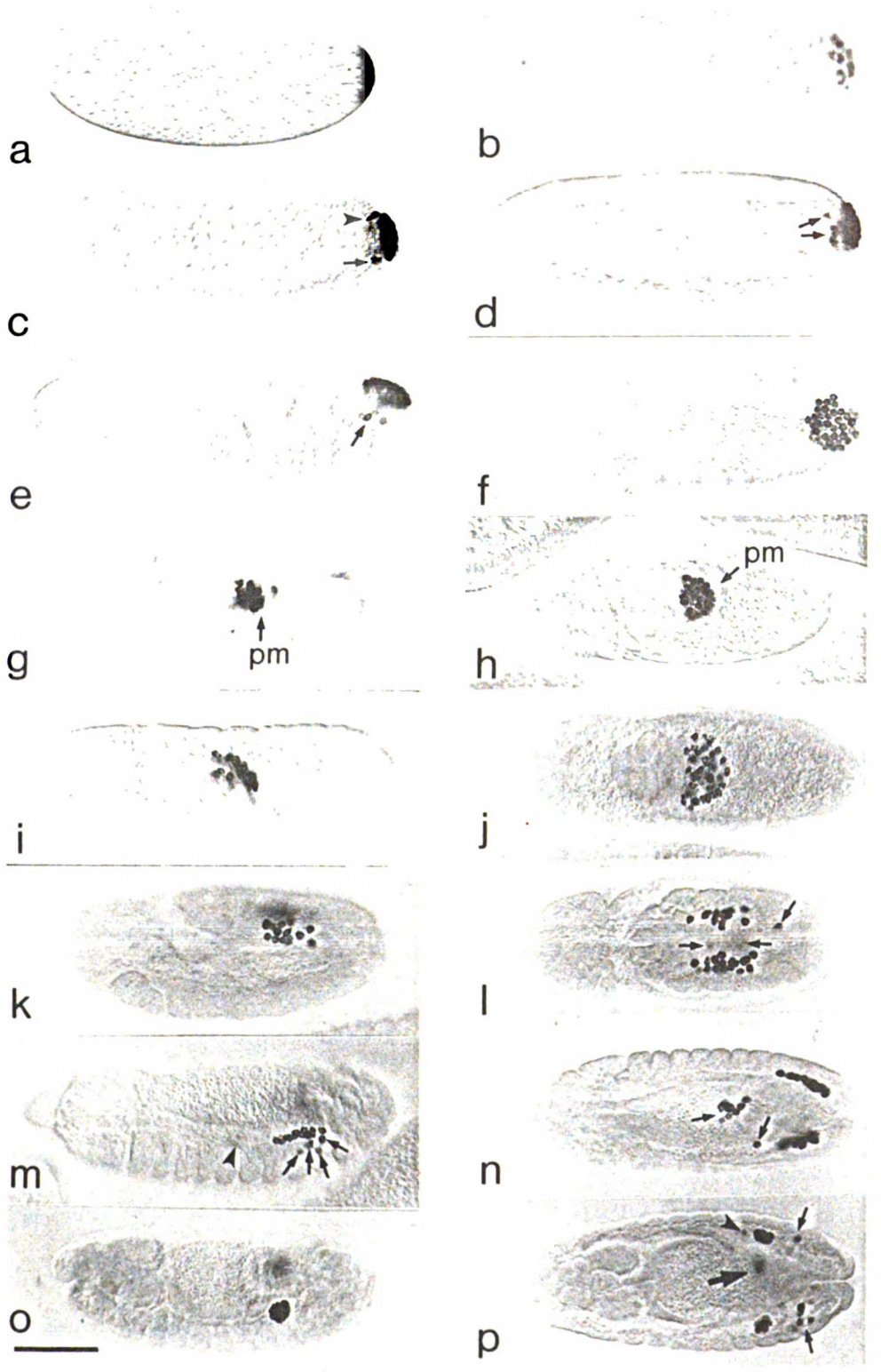
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Figure 1.2. Immunocytochemical staining of polar granules and nuclear bodies of a pole cell with Mab46F11.

A thin section through a pole cell labelled with Mab46F11 and a 5nm gold conjugated second antibody is shown at low magnification in the inset. At higher magnification the boxed portion of the cell is shown. Black dots of 5nm gold label are apparent over polar granules (pg) and nuclear bodies (nb) (arrows). (bar=100nm; inset, bar=1um).

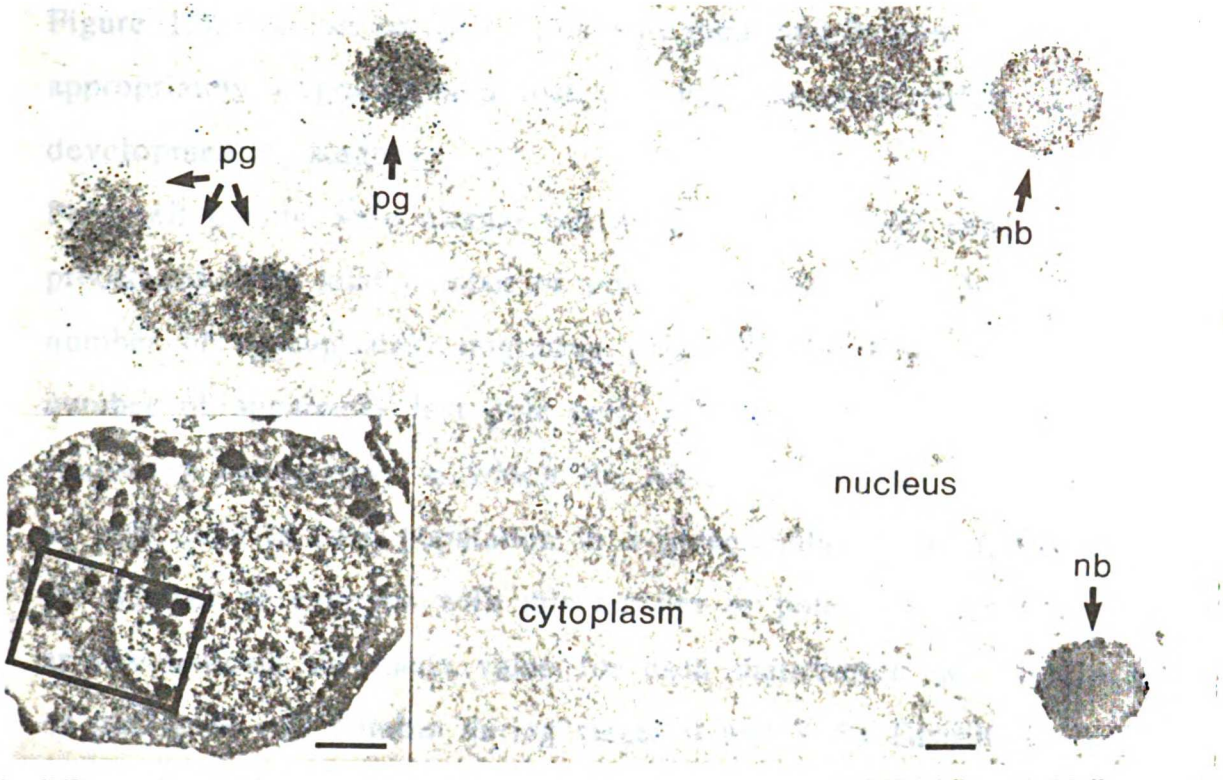

pg
pg
nb
nucleus
nb
cytoplasm

Figure 1.3. Number of pole cells and their distribution between appropriately migrating and lost cell populations at different developmental stages.

Pole cell counts were carried out as described in the experimental procedures. The total number of pole cells (solid black bars), the number of appropriately migrating pole cells (dashed bars), and the number of apparently lost pole cells (dotted bars) were plotted as frequency histograms, in which the abscissa indicates the number of pole cells in each population in a given embryo, and the ordinate the number of embryos with this number of pole cells. Xs and arrows indicate the mean value for each distribution. a) Distribution of total pole cell number during stages 6 and 7. b) Distribution of total pole cell number during late stage 10 and stage 11. c) Distribution of pole cells into appropriately migrating and lost cell populations during early stage 12, early germ band shortening. Lost cells at this stage are defined as those labelled cells which do not lie dorsolaterally against the body wall, or which are more than several cell diameters from the main body of pole cells. c') Distribution of total pole cell number during early stage 12, early germ band shortening. d) Distribution of pole cells into appropriately migrating and lost cell populations at the end of germ band shortening, early stage 13. Cells were scored as lost if they were more than one cell diameter from the main body of pole cells or in a different plane of focus from the main body of pole cells. d') Distribution of total pole cell number at the end of germ band shortening, early stage 13. e) Distribution of pole cells into

appropriately migrating and lost cell populations during stage 14. Pole cells not contained in the mesodermal sheath that surrounds the germline cells were scored as lost. e') Distribution of total pole cell number during stage 14.

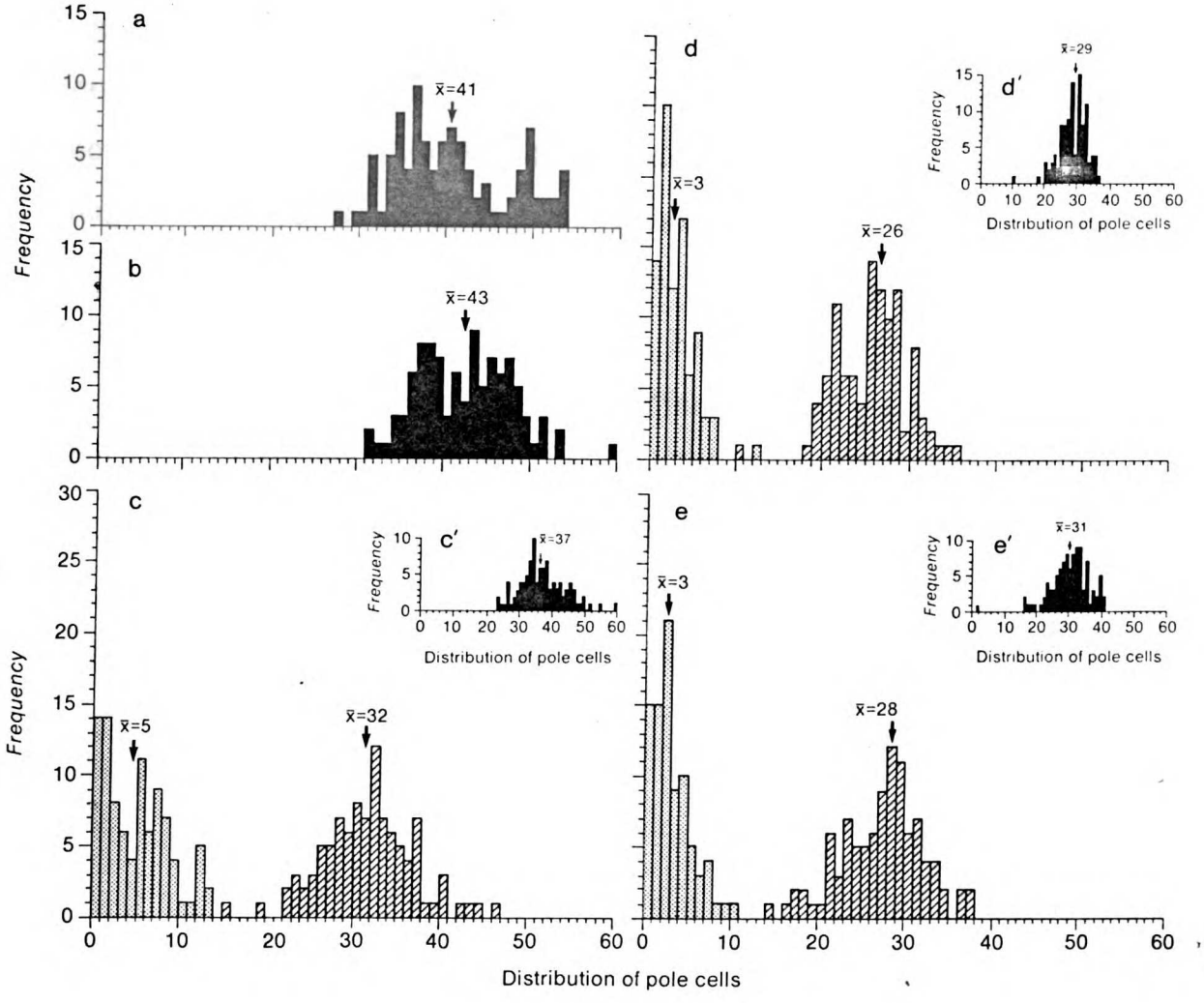

a
x̄=41
b
x̄=43
c
x̄=5
x̄=32
c′
x̄=37
d
x̄=3
x̄=26
d′
x̄=29
e
x̄=3
x̄=28
e′
x̄=31
Frequency
Distribution of pole cells

Figure 1.4. Stages in pole cell migration in pumillio embryos (embryos from females of genotype pum/pum). Staining of whole mount embryos with Mab46F11 was carried out as in Fig. 1.1 a. a) Stage 4 embryo showing Mab46F11 immunoreactive pole cells at the posterior embryonic pole. b) Stage 10 embryo showing pole cells migrating through the midgut. c) Horizontal view of stage 13 embryo showing lost pole cells. d) Different focal plane of embryo shown in c) showing other lost pole cells. (Magnification is the same as in figure 1.1).

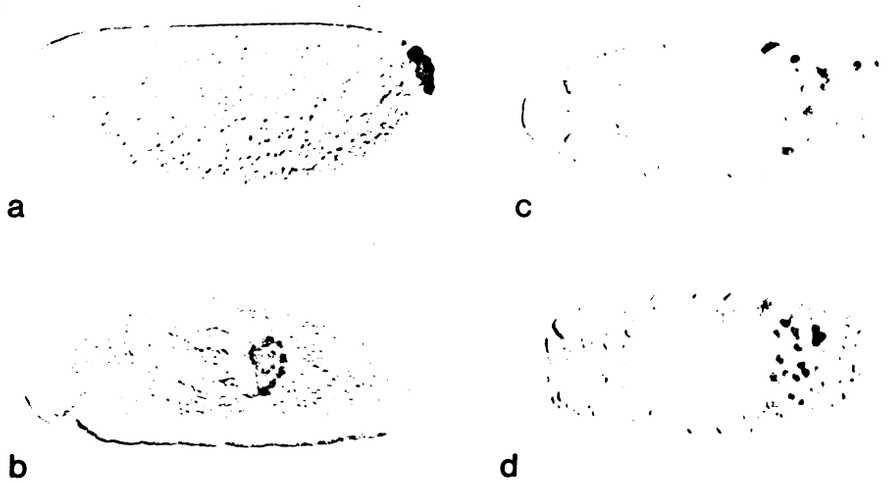
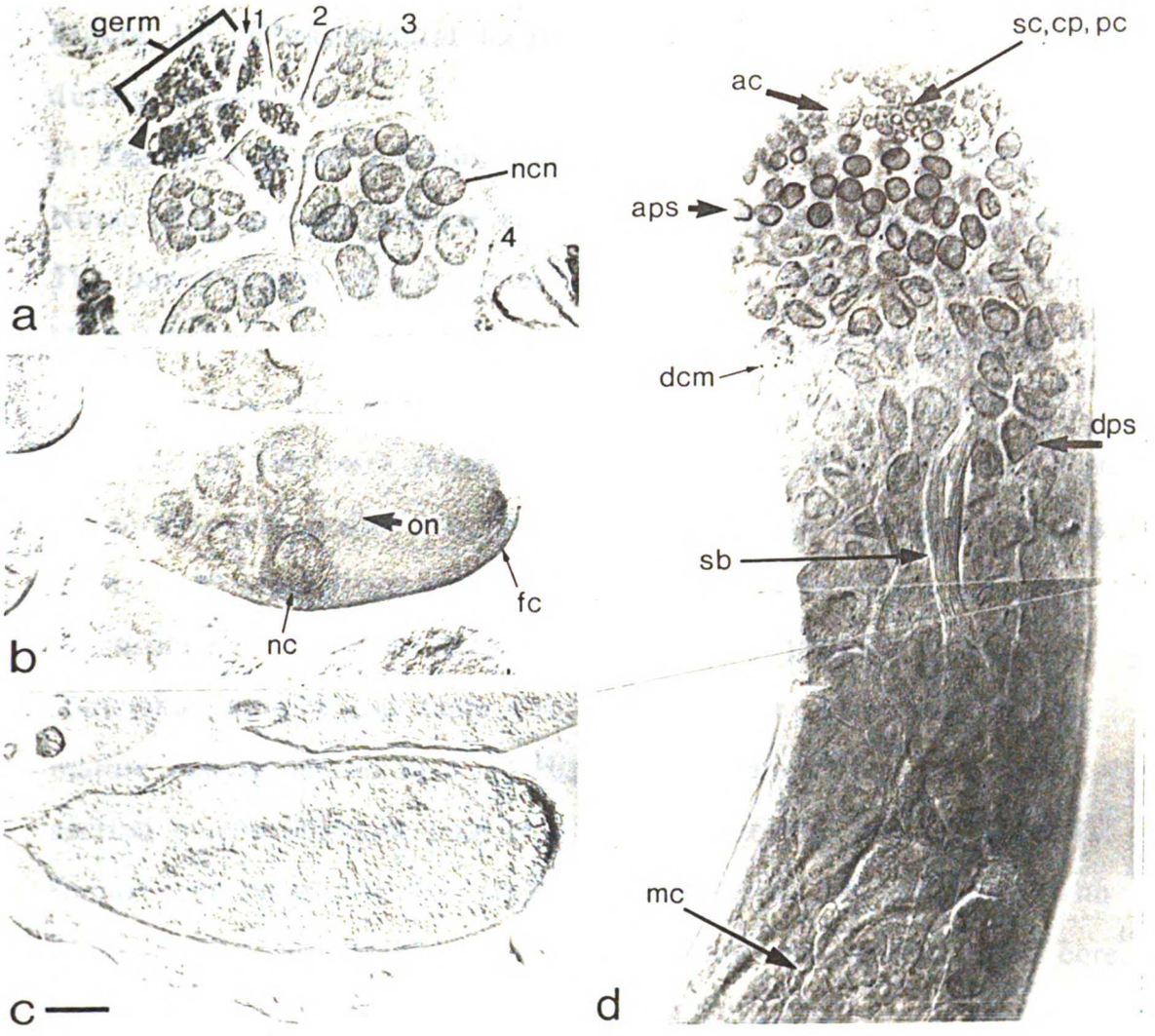

a
c
b
d

Figure 1.5. Distribution of Mab46F11 immunoreactivity during stages of oogenesis and in the adult testis.
a) Whole mount preparation of an ovariole showing early stages of oogenesis, including the germarium and early stages of the vitellarium. The germarium (germ) is in the upper left. Later vitellariam stages are found in a clockwise direction. A second length of ovariole, also showing germarium and vitellarium stages, lies directly against the inner radius of the ovariole to be described. In the anterior germarium large cells with perinuclear label are found (arrowhead). These are likely germ line stem cells. More posterior, to the right, are a number of smaller labelled cells. These are the clusters of 2, 4, 8, or 16 cystocyte cells that derive from the mitotic divisions of the primary cystocyte (cystoblast) in the anterior germarium. In the most posterior germarium, labelled cells are surrounded by nonstaining prefollicle cells (arrow). In the vitellarium stage egg chambers, labelled 1-4, localized label is confined to the nuclear envelope of nurse cells (ncn). This is particularly clear in the egg chamber labelled 4, in which 15 labelled nuclear envelopes can be seen. Diffuse label is present in the nurse cell cytoplasm. The nonstaining oocyte nucleus is not visible. b) Wholemount stage 10 egg chamber labelled with Mab46F11. The posterior pole of the oocyte is to the right. Nurse cells (nc) show perinuclear and diffuse cytoplasmic label. In the oocyte, localized label is restricted to a thin cortical band at the posterior pole. Note that label is not associated with the follicle cells (fc) immediately apposing the posterior pole of the oocyte. The

oocyte nucleus (on) is visible in the anterior oocyte, but not stained. c) Cryostat section through a mature stage 14 oocyte. Label is localized to a dense cortical band at the oocyte posterior pole. d) Wholemount preparation of the tip of an adult testis stained with Mab46F11. The nonstaining apical cell cluster (ac) sits near the apical tip of the testis. The small cells with perinuclear label that surround it are probably stem cells (sc), cyst progenitor cells (cp), proliferating cysts (pc), and apical primary spermatocytes (aps). In these cells large cytoplasmic or perinuclear dots of label are also usually observed. These are probably dense cytoplasmic masses (dcm) (see text). In later stage, distal primary spermatocytes (dps), label becomes less distinct around the nucleus. By the time spermatocytes have begun meiosis (mc = meiotic cell), label is not detectable in germ line cells. Part of a nonstaining mature sperm bundle (sb) is visible in this optical plane. (5a-d, bar= 200*um*).


germ
1
2
3
4
ncn
a
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fc
nc
b
c
sc,cp, pc
ac
aps
dcm
dps
sb
mc
d

Figure 1.6. Ultrastructural localization of the Mab46F11 antigen during oogenesis.

a) The inset shows a section through a stage 3 or 4 egg chamber. Nurse cells and the follicle cells that overlay them are apparent. The boxed region of a nurse cell is shown at higher magnification. Here label can be seen over electron dense clumps of material (nuage) along the cytoplasmic face of the nuclear envelope (arrows). b) The inset shows the posterior pole of a stage 10 oocyte. Numerous yolk bodies are present in the oocyte cytoplasm. In the upper right are follicle cells. The boxed region is shown at higher magnification. Label is associated with polar granules (arrows), somewhat smaller and more irregular in shape than those in the mature oocyte, shown in c. c) High magnification view of a thin section of the posterior pole of a stage 14, mature oocyte. Label is found over polar granules (pg), many of which are associated with mitochondria (m). Many polar granules show an electron lucid core. (6a-c, bar=100nm; insets, bar = 200um).

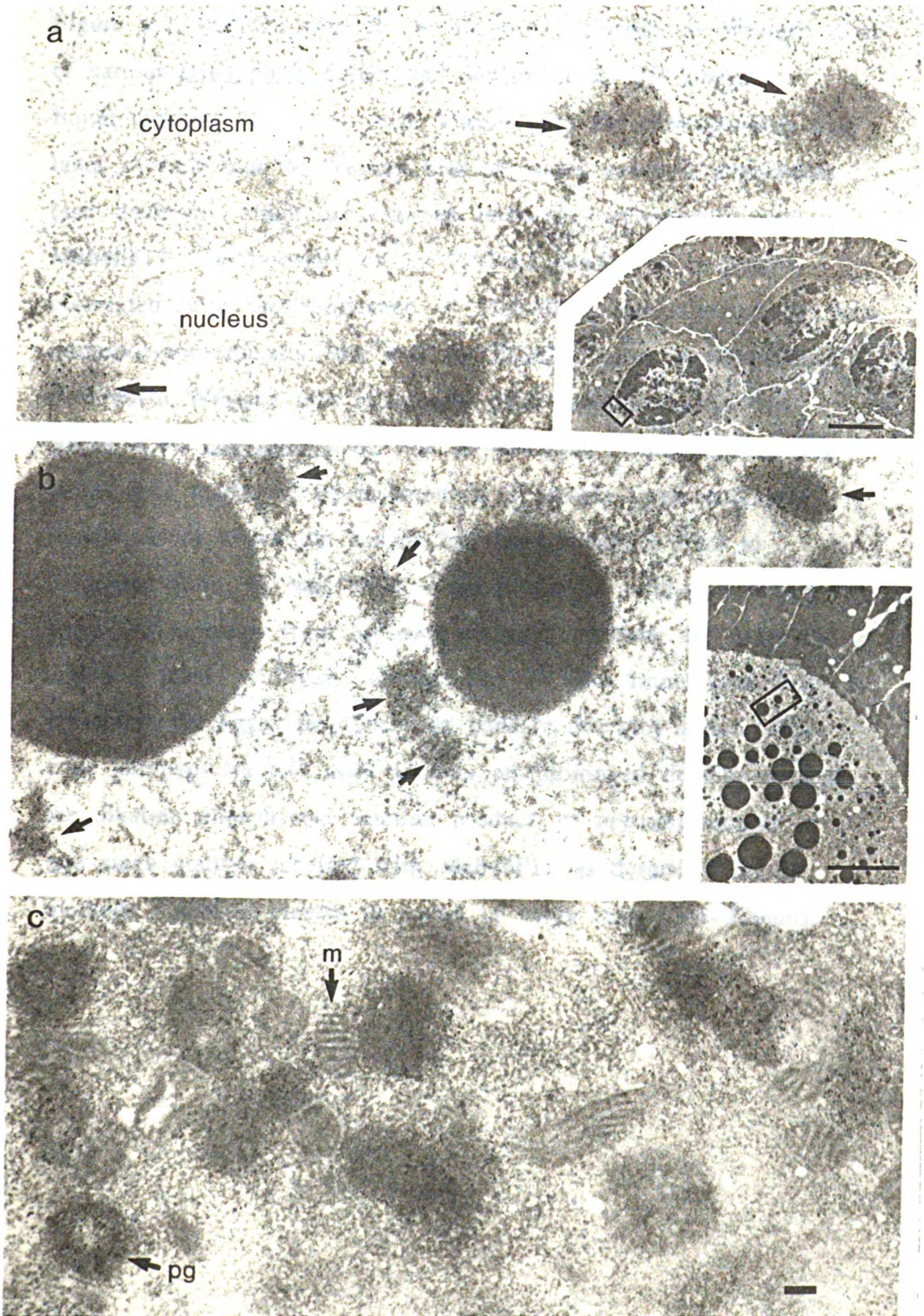
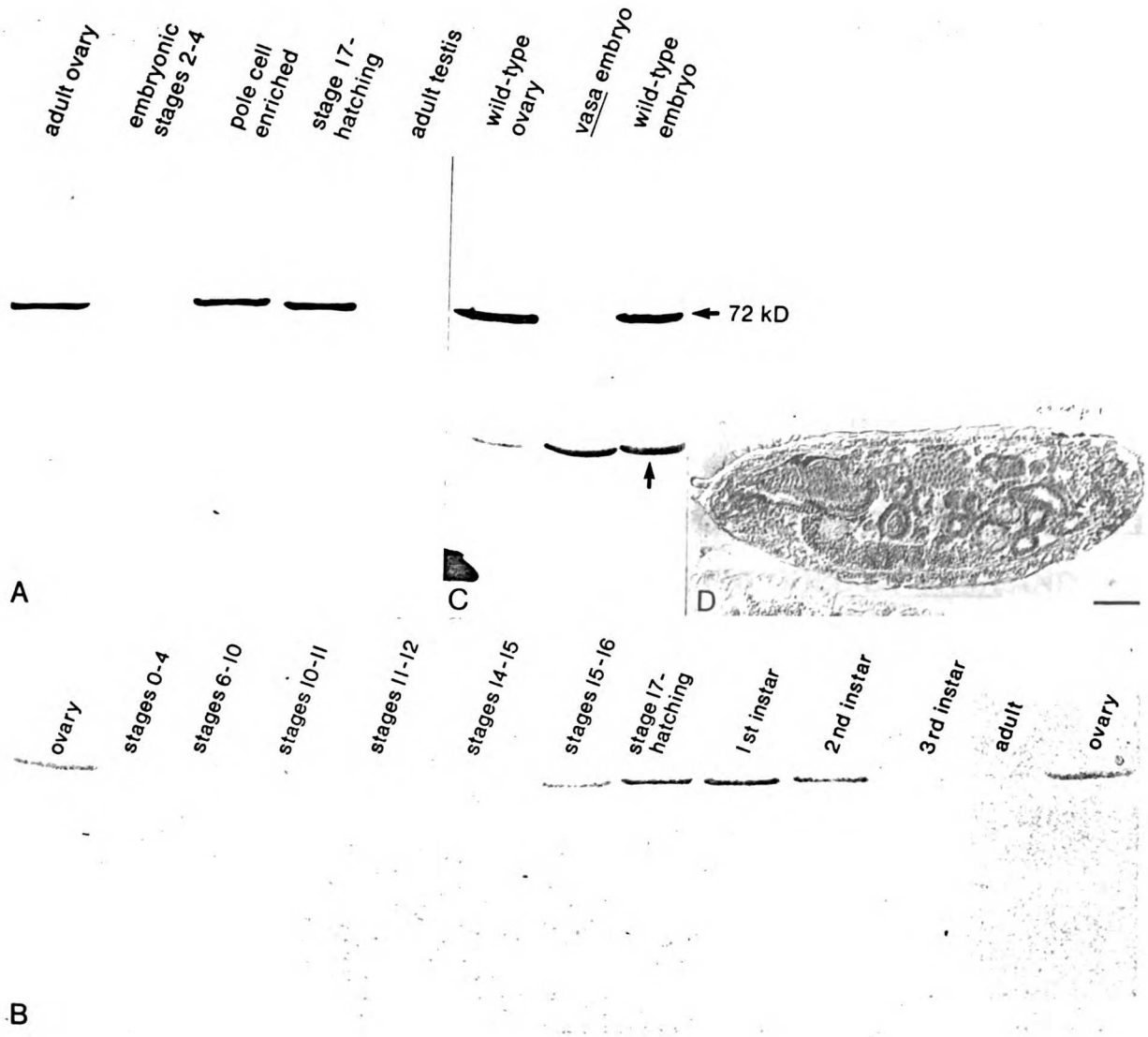

a
cytoplasm
nucleus
b
c
m
pg

Figure 1.7. Identification of the Mab46F11 antigen on Western blots of various Drosophila tissues and developmental life stages.
Equal amounts of protein were loaded onto the corresponding gel lanes of each blot. a) Western blot of tissues and cell populations (see methods) which are reactive immunocytochemically with Mab46F11. The intensity of the 72-74kd band seen is roughly correlated with the abundance of Mab46F11 immunoreactivity seen immunocytochemically (see text for details). b) A developmental timecourse Western blot containing total protein from various stages of the Drosophila lifecycle, as described in the methods. The adults lane contained equal amounts of protein from male and females from which the gonads had been removed prior to homogenization. c) Mab46F11 Western blot of wild-type ovary, stage 0-3 embryos from homozygous vasa mothers, and stage 0-3 embryos from wild-type mothers. No 72-74kd immunoreactivity is present in the vasa lane. The 45kd band in this blot is one of the yolk polypeptides and only appears on prolonged development of the alkaline phosphatase reaction product. d) cryostat section of a late stage embryo labelled with Mab46F11 as described in the methods. Label is present in nuclei of many cells. (bar=200um).


adult ovary
embryonic stages 2-4
pole cell enriched
stage 17-hatching
adult testis
wild-type ovary
vasa embryo
wild-type embryo
72 kD
A
C
D
ovary
stages 0-4
stages 6-10
stages 10-11
stages 11-12
stages 14-15
stages 15-16
stage 17-hatching
1st instar
2nd instar
3rd instar
adult
ovary
B

CHAPTER 2

THE PROTEIN COMPONENT OF DROSOPHILA POLAR GRANULES RECOGNIZED BY MONOCLONAL ANTIBODY MAB46F11 IS ENCODED BY VASA AND HAS EXTENSIVE SEQUENCE SIMILARITY TO ATP-DEPENDENT HELICASES

SUMMARY

Determinants of pole cells, which are precursors of the germ line, are provided maternally and localized to the posterior pole of the Drosophila egg, as are polar granules. It has been hypothesized that certain RNA molecules associated with polar granules may be necessary for pole cell determination (Mahowald, 1968). Using a monoclonal antibody (Mab46Fll) against polar granules, we have cloned the gene for one of their components. This gene turns out to be vasa, which is required maternally for the formation of polar granules and germ cells. This polar granule component shows significant sequence similarity to eIF-4A, a translation initiation factor which binds to mRNA, and other helicases.

MATERIALS AND METHODS

Stocks

vasa^{PD23} and deficiency stock Df(2L)75c were provided by Drs. Trudi Schupbach and Eric Wieschaus, Princeton University. Deficiency stocks Df(2L)A72 and Df(2L)64j were obtained from Dr. Michael Ashburner and the Bowling Green stock center.

Immunocytochemistry

Mab46F11 is a monoclonal antibody that recognizes polar granules and other germ line specific structures. Immunocytochemical techniques were as described in Hay et al. 1988a.

cDNA isolation and sequencing

RNA was isolated from hand dissected ovaries using the hot phenol method (Schwarz et al. 1988). Poly A+ RNA was isolated by two cycles of adsorption to and elution from oligo(dT)-cellulose (Aviv and Leder, 1972). This RNA was used to construct a Lambda Zap expression library (Stratagene). cDNAs for the Mab46F11 antigen were isolated from the expression library using standard techniques for plating, IPTG induction and transfer to nitrocellulose filter (Huynh, et al. 1985; Stratagene protocol). Monoclonal antibody Mab46F11 was diluted 1:10 in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20 (TBST) and incubated for one hour with the filters. Three 15 min. washes at room temperature in TBST were followed by a one hour incubation with rabbit anti-mouse IgM (Zymed)

diluted 1:1000 with TBST. Washes as above were followed by incubation with goat anti-rabbit antibody conjugated to alkaline phosphatase (Promega Biotech) diluted 1:7500 in TBST. Development of the alkaline phosphatase reaction product followed the Promega Biotec protocol.

cDNAs were subcloned from Lambda Zap using a plasmid rescue procedure (Bluescript manual, Stratagene). cDNAs from the Lambda Zap ovarian library were used as hybridization probes to screen a lambda gtll library made from ovarian poly A+ enriched RNA (Dr. Laura Kalfayan; University of North Carolina, Chapel Hill) and a genomic Lambda Dash library (Stratagene) using standard techniques (Maniatis et al. 1982). cDNA and genomic DNA were subcloned in Bluescript (Stratagene).

Sequencing of single-stranded DNA was carried out using the Sequenase sequencing kit (U.S. Biochemical) and oligonucleotides corresponding to previously determined sequences. Sequences were determined for both strands of cDNAs and one strand of the genomic DNA.

Chromosome in situ hybridization

In situ hybridizations to polytene chromosomes were performed as described in Papazian et al. 1987.

Northern blot analysis

RNA extraction was either by the hot-phenol method (Schwarz et al., 1988) or by the guanidinium method (Chomczynski and Sacchi, 1987). RNA was electrophoresed on formaldehyde-agarose gels,

blotted onto Nytran membranes (Schleicher and Schuell), and hybridized as described in Vaessin et al. 1987.

Tissue in situ hybridization

Ovaries and embryos were fixed, embedded in paraffin and sectioned according to Vaessin et al. 1987. Testes were dissected and processed through the methanol fixation step described in Hay et al., 1988. They were then post fixed in 4% paraformaldehyde in PBS (130 mM NaCl, 10 mM sodium phosphate, pH 6.8) for 15 minutes and processed for hybridization as with the other tissue. Probes for hybridization were ^{35}S-labeled antisense RNA derived from cDNA sequences using either the T3 or T7 promotors of Bluescript. Probes were reduced to a mean length of 100-200 base pairs using base hydrolysis (Cox et al. 1984). Hybridizations, washes and signal detection were as described in Vaessin et al. 1987.

RESULTS

Isolation of cDNA for the polar granule antigen and localization of the transcripts

Monoclonal antibody Mab46F11 binds to polar granules at the posterior pole of late stage oocytes and early embryos, as well as to other germ line specific structures throughout the fly life cycle (Hay et al. 1988a). To clone the gene for this antigen, we screened an ovarian cDNA (lambda zap) expression library with Mab46F11. Two classes of positive cDNA clones were isolated; clones within each class hybridize to one another and are probably derived from a single gene. Because cDNAs in the first class hybridize to transcripts expressed exclusively in germ cells (see below), whereas cDNAs in the second class hybridize to RNA throughout the embryo (not shown), we have only pursued clones in the first class and have used these cDNAs as hybridization probes to isolate full length cDNA clones from an ovarian gtll library kindly provided by Dr. Laura Kalfayan. We have previously found a nuclear antigen which is present in somatic cells of late embryos and is stained weakly by Mab46F11 (Hay et al. 1988a), but have not determined whether this nuclear antigen is related to the cDNAs of the second class.

cDNA from the first class (hereafter referred to simply as the cDNA) hybridizes to transcripts that show essentially the same distribution, notwithstanding one exception, as the strong Mab46F11 staining in embryos, ovaries and testes (Fig. 2.1,2.2). In the embryo,

strong Mab46F11 staining is first localized to the posterior pole and subsequently associated with pole cells (Hay et al. 1988a). As shown in Fig. 2.1, high levels of hybridizing transcripts are found uniformly distributed throughout the preblastoderm embryo. By blastoderm formation hybridizing transcript is essentially absent. Hybridization signal does not reappear until roughly stage 12 or early stage 13, the end of germ band shortening (Staging is that of Campos-Ortega and Hartenstein, 1985). When it does reappear, label is found over pole cells, which are arranged in bilateral clusters located in the posterior third of the embryo (Fig. 2.1C). At this and later stages of embryogenesis, pole cells are the only cells that contain hybridizing transcripts and show strong staining with the Mab46F11 antibody. This temporal distribution of the transcript suggests that zygotic transcription of this gene occurs primarily, if not exclusively, in pole cells (Fig. 2.1).

In the adult female the Mab46F11 antigen is found in germline stem cells, nurse cells (sister cells of the oocyte), as well as at the oocyte posterior pole. In the adult male the antigen is present in germ cells in the testes at early stages of spermatogenesis (Hay et al. 1988a). With the exception of late stage oocytes, which show uniform distribution of the hybridizing transcript, as in early embryos, transcript hybridizing to the cDNA is found exclusively in cells of the germ line lineage, paralleling the localization of the Mab46F11 antigen (Fig. 2.2).

Although the Mab46F11 antigen is found sharply localized to the posterior pole of late stage oocytes and early embryos, the transcript that encodes this antigen appears to be uniformly distributed in the

cytoplasm of these cells (Fig. 2.1). This difference may be accounted for by the following scenario. The Mab46Fl1 antigen is likely to be synthesized in the nurse cells and then transported into the oocyte where it is targeted to the oocyte posterior pole (Mahowald, 1962; Hay et al. 1988a). Transcript for the Mab46Fl1 antigen, however, probably flows into the oocyte with the bulk of nurse cell cytoplasm during the second half of oogenesis (Mahowald and Kambysellis, 1978), and does not appear to be targeted to any specific location.

Consistent with the observed distribution of transcripts for the Mab46Fll antigen, Northern analysis shows the presence of a single major transcript of about 2.2 kb, which is abundant in ovaries (not shown), female flies, and early embryos (Fig. 2.3). Much lower levels of expression can be detected in late embryos (Fig. 2.3) and in larvae (not shown).

vasa is the gene for the Mab46Fll antigen

Since the Mab46Fl1 antigen is a component of the polar granules, a prime candidate for its structural gene might be one of the grandchildless-knirps class of maternal-effect gap genes, because mutations of these genes cause females to give rise to embryos without polar granules or pole cells (Boswell and Mahowald, 1985; Schupbach and Wieschaus, 1986; Lehmann and Nusslein-Volhard, 1986; Nusslein-Volhard, Frohnhoefer and Lehmann, 1987). Of the mutations in this class that we have tested (tudor, valois, oskar, staufen, vasa), only the vasa mutation ($vasa^{PD23}$) removed the Mab46Fl1 antigen. Early embryos from $vasa^{PD23}$ mutant mothers lack the 72-74X10^3Mr protein (Hay et al. 1988a), as well as the Mab46Fll

staining (Fig. 4). In *vasa*^{PD23} adult females, immunoreactivity is limited to the germarium of the ovary. Substantial levels of the antigen, however, are still present in the adult male germ line of this *vasa* allele (data not shown). Since *vasa*^{PD23} is a hypomorphic allele (R. Lehmann, personal communication), these observations are compatible with the possibility that *vasa* codes for the Mab46F11 antigen.

The cDNAs isolated with Mab46F11 hybridize to the 35BC region on the left arm of the second chromosome (Fig. 2.5), the same region that contains *vasa*. Further, deletions that remove the *vasa* gene, such as Df(2L)75c (35A1,2; 35D4-7) and Df(2L)A72 (35B2-3; 35B7-8) (Schupbach and Wieschaus, 1986; Lasko and Ashburner, 1988), remove the hybridization signal whereas nearby deletions that leave *vasa* intact, such as Df(2L)64j, do not remove the signal on the polytene chromosome (data not shown). These results strongly suggest, but do not prove, that *vasa* is the gene coding for the Mab46F11 antigen. The *vasa* gene has just been cloned in a chromosome walk (Lasko and Ashburner, 1988). Sequence comparisons reveal that the *vasa* gene product is essentially identical and that its gene has the same organization as the gene we have isolated (see below). Therefore, *vasa*, which is required for the formation of polar granules (Schupbach and Wieschaus, 1986; Nusslein-Volhard, Frohnhoefer and Lehmann, 1987), is most likely the gene coding for the 72-74X10^{3}Mr Mab46F11 antigen component of the polar granules (Hay et al. 1988a).

The organization of the gene for the Mab46F11 antigen, hereafter referred to as *vasa*, is shown in the inset to Fig. 2.6. The coding

sequence for the Mab46Fl1 antigen has been deduced from sequencing a cDNA that apparently contains the entire coding sequence, several overlapping incomplete cDNAs from a second library, and corresponding genomic DNA. The first methionine codon in the open reading frame is flanked by nucleotide sequences that agree with the consensus sequence for a ribosome initiation site (Cavener, 1987) and the stop codon is followed by a consensus polyadenylation signal and a poly A tail (Fig. 2.6). Conceptual translation yields a protein of 648 amino acids (Fig. 2.6) so that the primary translation product has a molecular weight of $71X10^3$Mr. This predicted molecular weight is very similar to the size ($72-74X10^3$Mr) of the Mab46Fll antigen as measured from SDS gels (Hay et al. 1988a), in agreement with the assignment of the vasa gene product as the Mab46Fll antigen.

The predicted vasa protein sequence

We have noticed four regions of interest in the predicted vasa protein. A large internal domain of vasa has sequence similarity with eukaryotic translation initiation factor 4A (eIF-4A) (Nielsen, McMaster and Trachsel, 1985; Nielsen and Trachsel, 1988), a nuclear antigen, p68, whose appearance is associated with cell proliferation (Ford, Anton and Lane, 1988) (Fig. 2.7), and a murine testis specific protein, PL10 (Leroy et al. 1989). This sequence similarity has been noticed independently by Lasko and Ashburner (1988). Near its amino terminus the vasa protein contains a five fold tandem repeat of the heptad sequence F(or S)RGGE(or Q)GG (Fig. 2.8). In the carboxy terminal half vasa contains a domain that is a candidate for a helix-

turn-helix structure, which has the general pattern of hydrophobicity and conserved residue types suggested by Pabo and Sauer (1984) to be involved in interactions with double stranded DNA. Finally, the carboxy terminus of vasa contains multiple negatively charged residues (Fig. 2.6), as in several single-stranded nucleic acid binding proteins.

Sequence similarity with eIF-4A and other helicases

The vasa product shows 27% amino acid identity with eIF-4A, 31% identity with human nuclear antigen p68, and 45% homology with a murine testis-specific protein, PL10 (Fig. 2.7). The function of the latter two proteins is unknown, though it has recently been demonstrated that p68 does function as an ATP-dependent RNA helicase (Hirling et al. 1989). The activity of eIF-4A, on the other hand, has been well characterized. eIF-4A is a component of a high molecular weight protein complex (called eIF-4F) involved in 5' cap recognition and the binding of mRNA to ribosomes. It has been suggested that eIF-4A functions to bind and unwind mRNA in an ATP-dependent manner; i.e. that eIF-4A is an ATP dependent RNA helicase (Ray et al. 1985; Lawson et al. 1986; Abramson et al. 1987; Rhoads, 1988).

Sequence motifs thought to be required for ATP-binding (Walker et al. 1982) are present in eIF-4A and p68 (Ford, Anton and Lane, 1988) and vasa (motifs I, II in Fig. 2.7). In addition to these two motifs, five others have been found in a large number of ATP-dependent helicases (motifs Ia, III, IV, V and VI) (Hodgman, 1988a,b; Lane, 1988). Six of these seven motifs can be readily identified in vasa (Fig 7). The extensive sequence similarity between

vasa, eIF-4A and p68, and the presence of these motifs strongly suggests that the vasa product is also an ATP-dependent nucleic acid-binding protein.

The F(or S)RGGE(or Q)GG heptad repeats

The amino terminal region of vasa is glycine-rich (61 glycines in 200 residues, or 30%) and very hydrophilic (76, or 38%, charged residues). In particular, a five-fold tandem repeat of the heptad F (or S)RGGE(or Q)GG is found (Fig. 8). Glycine-rich sequences are found in a number of likely RNA-binding proteins, including proteins in the nucleolus (Lischwe et al. 1985; Jong et al. 1987; Lapeyre, Bourbon, and Amalric, 1987) and heterogeneous nuclear ribonucleoproteins (hnRNP) (Cobianchi, et al. 1986; Kumar, William and Szer, 1986; Cruz-Alvarez and Pellicer, 1987). Direct association of these proteins with RNA has been demonstrated (Thomas et al. 1981; Bugler et al. 1987; Schenkel et al. 1988) or implicated (Jong et al. 1987; Parker and Steitz, 1987). The glycine-rich regions in these proteins often contain multiple aromatic residues and arginines (Lischwe et al. 1985; Cobianchi et al. 1986; Kumar, William and Szer, 1986; Cruz-Alvarez and Pellicer, 1987; Jong et al. 1987; Lapeyre et al. 1987). The clustering of aromatic residues and basic residues in these proteins, as well as in proteins with the RNP consensus sequence (Swanson et al. 1987), has been suggested to be indicative of single stranded nucleic acid binding domains, as demonstrated for E. coli single-stranded binding proteins, bacteriophage T4 gene 32 protein, and bacteriophage fd (M13) gene 5 protein (O'Connor and Coleman, 1983; Chase and Williams, 1986).

Putative helix-turn-helix region

Pascal et al. (1989) have carried out secondary structure predictions for vasa, PL10, and eIF-4A and find that a carboxy terminal region of each of these proteins contains a candidate for a helix-turn-helix structure. This region, amino acids 530-550 of vasa, contains 4 amino acids at positions which Pabo and Sauer have found to be conserved in a large number of potentially helix-turn-helix DNA binding proteins. The general pattern of hydrophobicity is also consistent with the possibility that this region might be involved in double stranded DNA binding (Pabo and Sauer, 1984).

What is the significance of this motif in proteins which are known to function as RNA helicases (eIF-4A and p68)? One possibility, which remains to be demonstrated, is that double stranded RNA forms a structure on which these proteins act, using a helix-turn-helix binding domain. It should be pointed out, however, that RNA cannot fit into a B-form structure equivalent to that of B-form double stranded DNA, and thus the relevance of the helix-turn-hel;ix binding motif of DNA binding proteins, in known RNA helicases is unclear.

The acidic carboxy terminus of vasa

The carboxy terminus of the vasa product is negatively charged; six of the last twelve residues are glutamate or aspartate residues (Fig. 6). This feature is interesting in light of the sequence similarities between vasa and known helicases, since similar acidic carboxy termini have been found in other RNA or single-stranded DNA

binding proteins (Chase and Williams, 1986; Swanson et al. 1987; Williams et al. 1987).

DISCUSSION

We have cloned the gene for a polar granule component which is recognized by Mab46F11 (Hay et al. 1988). This gene was subsequently identified as *vasa* because its sequence is essentially identical to that of *vasa* (Lasko and Ashburner, 1988). Since the *vasa*^{PD23} mutation removes polar granules (Schupbach and Wieschaus, 1986) as well as the *vasa* protein in oocytes and early embryos, most likely the *vasa* protein is either a major component of polar granules or a component important for their integrity. The failure of these mutant embryos to form pole cells, then, is consistent with the hypothesis that polar granules are involved in the formation of pole cells and the determination of the germ cell lineage (Mahowald, 1968).

Sequence of the vasa protein suggests that it may be a RNA-binding helicase

The *vasa* sequence contains four features that are interesting in light of the association between polar granules and RNA (Mahowald, 1971), and of the implication of maternal RNA in pole cell determination (Okada and Kobayashi, 1987).

First, *vasa* has extensive sequence similarity with the translation initiation factor eIF-4A and contains six of the seven motifs identified in a number of helicases. This suggests that the *vasa* protein may be able to associate with, and to unwind, RNA. Secondly,

the amino terminal region of vasa contains a five fold tandem repeat of F(or S)RGGE(or Q)GG. It has been pointed out that from purely physical considerations, the extent of a heptad repeat in any non-alpha-helical structure is unlikely to exceed two or three heptads (Cohen and Parry, 1986). The presence of five tandem heptads in vasa would therefore suggest an alpha-helical structure, though the presence of multiple glycine residues may weaken this prediction. These heptads and the flanking sequences are arranged in such a way that in an alpha-helix of 12 turns there would be six arginines along one face, next to four phenylalanines and two serines (Fig. 8B). Bearing in mind the involvement of basic residues and aromatic residues in the interaction with single-stranded nucleic acids (O'Connor and Coleman, 1983; Chase and Williams, 1986), we are tempted to consider the possibility that a single-stranded nucleic acid (e.g. RNA) interacts with the structure of heptad repeats so that the phosphate backbone has electrostatic interaction with arginines and the nucleotide bases interact with the phenylalanines by stacking or with the serines by forming hydrogen bonds. This model further raises the intriguing question as to whether the postulated association with RNA has any specificity for primary sequences or secondary structures. Sequence specific binding domains have been found at the amino terminal region, separate from the putative helicase domain, of the SV40 large tumor antigen and of the transcription termination factor rho (Brennan, Dombroski and Platt, 1987; Gish and Botchan, 1987; Dombroski and Platt, 1988; Goetz, 1988). Such a model for the heptad repeats should be regarded with great caution, however, since no examples of a single alpha-helical

structure interacting with nucleic acids have been shown. Besides, other possible functions for the heptad repeats exist, such as formation of coiled-coil structure, though the heptad sequences in vasa do not agree very well with the preferred arrangement of heptads found in coiled coils (Cohen and Parry, 1986). Thirdly, the carboxy terminal region contains sequences consistent with the possibility of forming a helix-turn-helix structure similar to that present in a number of DNA binding proteins. There is no evidence, however, that such structures can interact with RNA in a similar way. If, however, a helix-turn-helix structure is important for some aspect of unwinding double stranded RNA or DNA regions it would be predicted that mutations in this region, specifically at the conserved residues would have a strong affect on helicase activity. Finally, the highly acidic carboxy terminus is reminiscent of the carboxy termini of several single-stranded nucleic acid binding proteins. Some of these carboxy termini appear to be involved in the interaction between the nucleic acid-binding protein and other proteins (e.g. between the T4 gene 32 product and other proteins involved in DNA replication, or between the high mobility group non-histone proteins and histones); such interactions may alter the nucleic acid-binding affinity (Burke, Alberts and Hosoda, 1980; Reeck, Isackson and Teller, 1982).

Possible relation between polar granules and pole cell determinants

Polar granules are found to be associated with RNA during late oogenesis and early (pre-pole cell formation) stages of embryogenesis (Mahowald, 1971b). Following fertilization polysomes

are found associated with the granules. This has led to the hypothesis that the polar granules function to localize and store mRNA which is then used following fertilization for some aspects of germ cell determination (Mahowald, 1971b). Several observations are consistent with a postulated role for mRNA in pole cell formation and germ cell determination. UV irradiation can prevent pole cell formation, and this effect is photoreversible at longer wavelengths, a characteristic of UV-induced damage to nucleic acids. This UV-induced inability to form pole cells can be rescued with RNA from cleavage stage embryos (Okada and Kobayashi, 1987). These cells die, however, upon reaching the embryonic gonad, indicating that the effects of UV irradiation are probably more complex than simply damage to RNA. Also, there is greatly increased translation in the posterior pole cytoplasm during early embryogenesis relative to the somatic regions of the egg, though essentially no transcription is occuring at the posterior pole (Zalokar, 1976). There is no direct evidence, however, that mRNA associated with polar granules is affected by UV irradiation, responsible for the increased translation seen at the posterior embryo pole, or necessary for pole cell formation.

Since only posterior cytoplasm (the pole plasm) from late stage oocytes or early embryos is capable of inducing the formation of pole cells (Illmensee and Mahowald, 1974; Illmensee, Mahowald and Loomis, 1976), either the relevant maternal RNA is restricted to the posterior pole or it has wider distribution but is only functional in the posterior pole. In the former case, the posterior localization

of these maternal RNA species may be attributed to polar granules, e.g. binding of specific mRNA by the vasa protein (see below, however). Translation of such mRNA in the embryo may follow either its release from polar granules or interactions between polar granule components and other cellular constituents. If the relevant maternal RNA is not restricted to the posterior pole, one could imagine that vasa, or other polar granule components, has to bind to these species of maternal RNA to allow for their translation (Kozak, 1983;1988). It remains to be determined whether one or a combination of mechanisms such as those described above, or others, are important in pole cell determination. These points are expanded on below.

Possible functions of vasa in RNA localization and/or translational regulation.

RNA localization

Examples of RNA localization as well as translational regulation during development exist in the literature. There are several examples of developmentally important RNAs being localized to regions of the oocyte and egg. In the amphibian Xenopus the veg 1 transcript becomes localized to the vegetal hemisphere cortex during oogenesis (Melton, 1987). In Drosophila the transcripts for several proteins important in early development are localized before, or following fertilization. The bicoid transcript becomes localized to the anterior oocyte pole during oogenesis as the nurse cells (the site of transcript synthesis) dump their contents into the oocyte (Frigero et

al. 1986; Berleth et al. 1988). Following fertilization the cyclin B transcript becomes localized to the posterior embryo pole around nuclear cycle 8 (Whitfield et al. 1989). It is unclear, however, whether the cyclin message is actively localized prior to nuclei entering the posterior pole or whether the localization seen is due to de novo synthesis by the migrating pole cell nuclei. Unpublished data (Ruth Lehmann) indicates that the nanos transcript is also localized to the embryo posterior pole. It is not known, however, whether this transcript becomes localized during late oogenesis or early embryogenesis.

The vasa product is required for nanos and cyclin B mRNA localization to the posterior pole (Ruth Lehmann, unpublished; Bruce Hay, Tom Jongens, unpublished). There is no direct evidence, however, that the vasa product functions to bind specific RNAs. There are now several well documented cases in which it is clear that specific proteins can bind to specific mRNAs (Crawford and Richter, 1987; Bugler et al. 1987; Query et al. 1989; Kearney and Nomura, 1987; Gregory et al. 1988). In several cases in which the sequence of the RNA-binding protein is known (nucleolin, Bugler et al. 1987; 70k U1 snRNP protein, Query et al. 1989) the protein contains one or more copies of an RNA-binding consensus motif (Dreyfuss et al. 1988; Query et al. 1989). This motif encompasses about 90 amino acid residues, including a highly conserved octamer (Adam et al. 1986). In the case of the 70kd U1snRNP protein this region has been shown to be largely sufficient to confer sequence-specific RNA binding (Query et al. 1989). vasa, as well as p68 and eIF-4A lack this RNP consensus motif and eIF-4A and p68 bind RNA in a sequence

nonspecific manner. For DNA helicases also, there are none known with sequence-specific binding properties. Thus, simply based on homology, vasa falls into a class of RNA binding proteins that suggests its function may be related to some aspect of RNA function other than the actual localization process.

Translational regulation

One possibility is that vasa functions to regulate translation of transcripts at the posterior pole. In a number of systems translational regulation of mRNA has been demonstrated to occur. Translational control is a common regulatory mechanism during oogenesis and early embryogenesis (Davidson, 1986; Richter, 1987; Rosenthal and Wilt, 1987). There is a general inhibition of translation in unfertilized versus fertilized eggs (reviewed in Davidson, 1986). In addition there are examples of both selective inhibition (Ford et al. 1977; Braun et al. 1989; Driever and Nusslein-Volhard, 1988a) and selective activation (Rosenthal et al. 1980) of translation. To pick a relevant example in Drosophila, for example, the transcript for the bicoid product is synthesized in nurse cells during oogenesis and localized to the anterior pole of the oocyte. It is not translated, however, until after egg activation (Driever and Nusslein-Volhard, 1988a).

Translational regulation is also important during the process of spermatogenesis. Transcripts may be stored for as long as one month before translation (Iatrou and Dixon, 1977). In Drosophila there is no postmeiotic transcription (Olivieri and Olivieri, 1965), and in the mouse transcription ceases at about stage 9 (Kierszenbaum and Tres,

1975). However, in both these systems many proteins required for spermatid assembly are not synthesized until many days later.

In oogenesis and early embryogenesis the two mechanisms that are thought to play a role in translational inhibition are limitations of specific translation factors (Richter and Smith, 1981; Audet et al. 1987; Collin and Hille, 1986) and masking of the mRNAs by proteins (Rosenthal et al. 1980). The rest of this discussion will focus on the former class of possibilities, given the vasa product's homology with translation initiation factors.

One possibility is that vasa functions as a specialized initiation factor which is required for the translation of a specific class of messages. In this capacity it might, for example, function in a fashion analogous to that of eIF-4A, to unwind double stranded regions of RNA. Activation of this activity following fertilization would unwind secondary structure in RNAs, providing a permissive environment for translation initiation. Is there a reason, though, to postulate the existence of "special factors" which would facilitate the translation of specific messages?

There is a large body of work which demonstrates that different RNAs have different translatibilities. In a number of proteins these differences can be accounted for at the level of the initiation steps (cf. Lawson et al. 1988; Rhoads, 1988). Experimental evidence suggests that at least some of the basis for mRNA discrimination has to do with mRNA secondary structure. mRNAs with a large amount of secondary structure at their 5' ends are found to translate less efficiently than those which do not. Regulation of translation initiation by RNA secondary structure appears to occur at two

different steps: the steric accessability of the m^7G cap found at the 5' end of all eukaryotic and most viral mRNAs, and the amount of secondary structure formed by sequences 3' to the cap site.

The group of peptides responsible for carrying out the initial reactions of translation initiation are eIF-4E (also known as the cap binding protein), eIF-4A, eIF4B, and eIF-4F, a multiprotein complex consisting of eIF-4A, -4E, and a 220kDa polypeptide termed p220. In in-vitro assays the eIF-4F complex is probably most important for the differential translation observed with different transcripts. This is based on the observation that it is the only translation initiation component which can relieve competition between different mRNAs (Panniers et al. 1985; Ray et al. 1983; Sarkar et al. 1984). eIF-4E (probably as a component of eIF-4F) is the first factor to interact with an initiating RNA. It binds in an ATP-independent manner to the m^7G cap present at the 5' end of mRNAs (Sonnenberg et al. 1978). It serves as a nucleating site for the subsequent binding of other initiation factors. Evidence that the accessibility of the cap structure is a primary determinant of translation efficiency comes from studies of viral RNAs. Alfalfa mosaic virus mRNAs (AlMV) are decapped at very different rates by tobacco acid pyrophosphatase, and these rates correspond to the translational efficiencies of these mRNAs (Godefroy-Colburn et al. 1985). Since the sequence and structures formed by the 5' ends of these mRNAs are all well characterized they were able to deduce that the different rates of capping are likely due to the steric accessibility of the cap.

Other evidence, notably from Sonnenberg's lab (Edery et al. 1984; Sonenberg et al. 1982), has suggested that secondary structure distal

to the cap itself is also important in modulating the translation efficiency of the AlMV mRNA. Factors that are required to overcome this secondary structure induced block are probably components of the eIF-4F complex, the most directly important of which is probably eIF-4A. The primary findings of this group are 1), that AlMV mRNA 4, which is translated much more efficiently than the other AlMV mRNAs, lacks secondary structure at its 5' end (Gehrke et al. 1983; Godfroy-Colburn et al. 1985), and 2), that when inosine-substituted mRNAs (which are relatively unstructured), are translated in extracts from polio virus infected cells (in which cap binding protein has been inactivated and thus translation is cap-independent), they are translated much more efficiently than their native counterparts (Sonnenberg et al. 1982). On the basis of these results they have suggested that RNA secondary structure functionally downstream of the cap-dependent step, plays an important role in regulating translation initiation, by virtue of the fact that RNAs with more secondary structure will require higher concentrations of initiation factors for optimal translation initiation than those which have little secondary structure. More recent results have distinguished between an early ATP-independent, and a later ATP-dependent, step in regulating the rates of translation (Lawson et al. 1988). The ATP-independent step reflects the initial requirement for eIF-4E binding. The later ATP-dependent step probably reflects a requirement for eIF-4A, probably as a component of the eIF-4F complex. Interestingly, this latter step shows a salt-dependency which is consistent with the formation of secondary structure as being an important step in regulating translation efficiency.

To elaborate on the possibility that vasa might be functioning in some manner analogous to eIF-4A, one possibility, already mentioned above, is that vasa is required for unwinding secondary structure in the 5' end of specific transcripts, following fertilization, placing them in a permissive condition for optimal translation initiation. A second possibility related to the above is that vasa might, by virtue of its homology to eIF-4A, function primarily to repress translation of specific messages at the posterior pole prior to fertilization by binding to sites where eIF-4A would bind. ATP-dependent unwinding in this case would function to release these transcripts for translation. Alternatively, a third possibility is that vasa functions primarily to bind and localize specific transcripts to the posterior pole where they subsequently become released, again perhaps in an ATP-dependent manner for translation following fertilization.

Posterior localization of the vasa protein

Although the vasa protein is sharply localized to the posterior pole as soon as it becomes detectable in the oocyte (Hay et al., 1988a), the vasa transcript is uniformly distributed in late stage oocytes and early embryos (Fig. 2.1). Therefore, the targeting machinery must operate at the level of the protein but not of the vasa mRNA. The vasa protein may be either directly interacting with the targeting machinery or associated with other proteins that are targeted to the posterior pole, possibly other components of polar granules. Indeed, our preliminary studies of maternal effect mutations of the

grandchildless-knirps class (tudor, valois, oskar, staufen; chapter 3), as well as the grandchildless mutations described in chapter 4 indicate that they may be involved in the establishment of posterior localization or its maintenance.

FIGURES

Figure 2.1. Tissue in situ hybridization of embryos with cDNA isolated with monoclonal antibody Mab46F11. In each panel anterior is to the left. (A) Parasaggital section through a preblastoderm embryo showing high levels of hybridization uniformly distributed throughout the embryo. (B) Parasaggital section through an embryo at the beginning of blastoderm formation. Hybridization intensity has decreased uniformly throughout the embryo to background levels. (C) Late stage 12, early stage 13 embryo. The only labelled cells are the pole cells (arrows). This is about the earliest stage that we can detect the hybridization signal in pole cells during embryogenesis. (D) Semi-parasaggital section through a first instar larva. Hybridization is restricted to the pole cells (arrow).

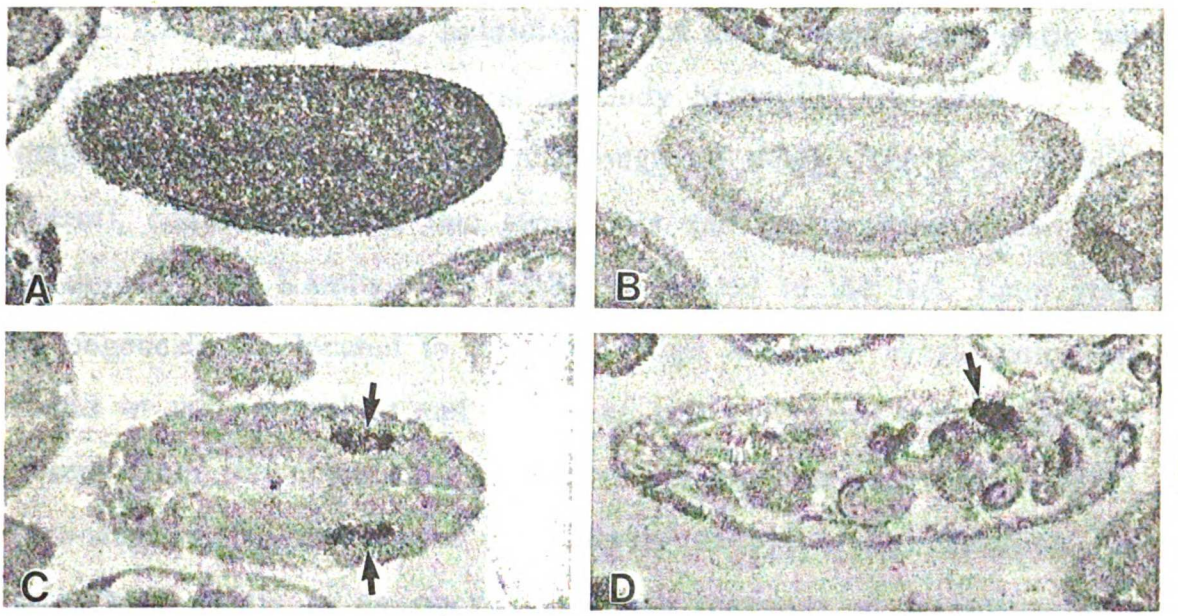
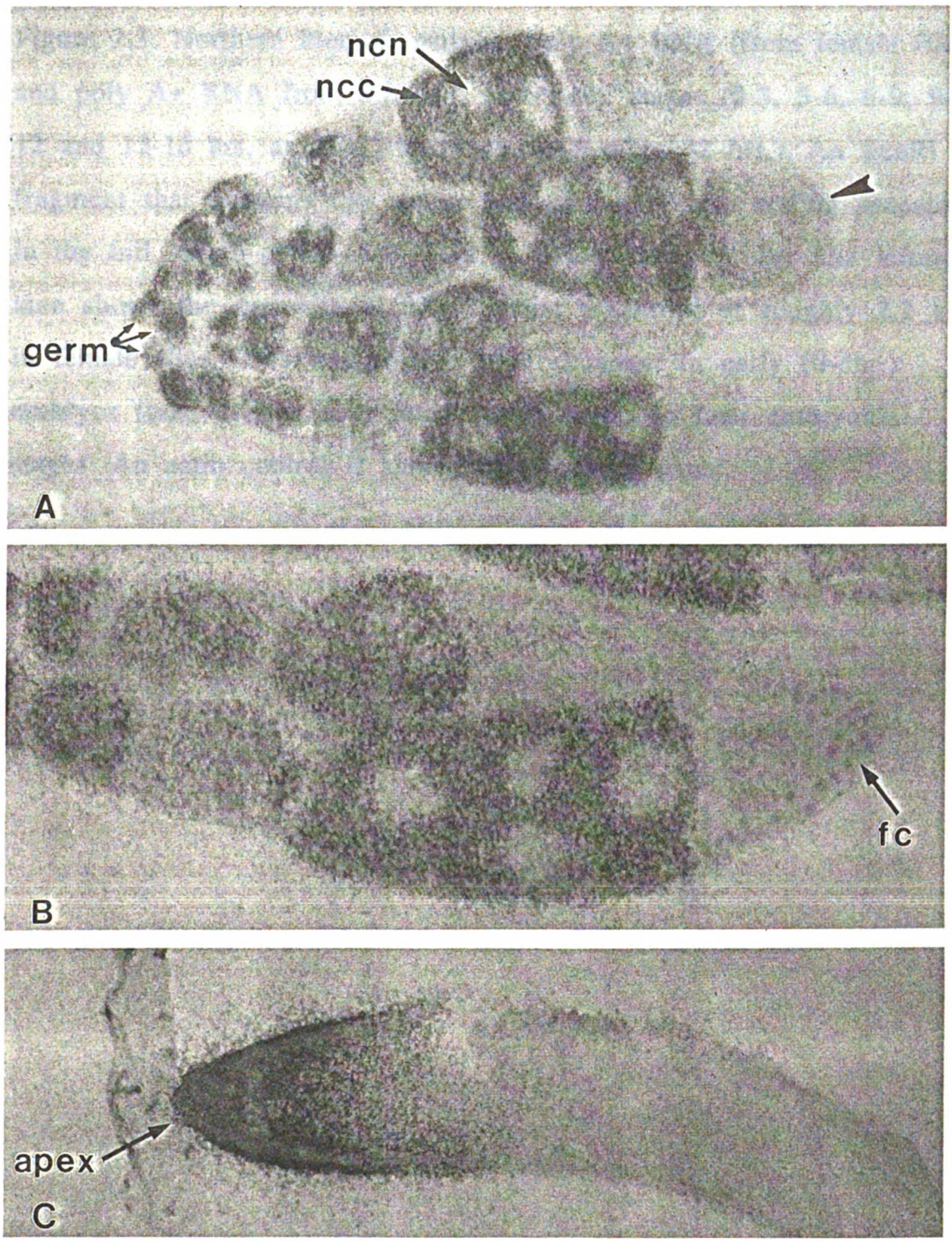

A
B
C
D

Figure 2.2. Tissue in situ hybridization of adult ovaries and testis with cDNA isolated with monoclonal antibody Mab46Fll. (A) Low magnification view of a section through an ovary. The germarium (germ), containing germ line stem cells and proliferating cysts (Mahowald and Kambysellis, 1978), is located to the left. Later stages of oogenesis are located to the right. High levels of hybridization are found in the germarium and the nurse cell cytoplasm (ncc). Signal is absent from the nurse cell nucleus (ncn), however. At these stages (pre-stage 9,10), the oocyte is relatively devoid of hybridization. The arrowhead points to an oocyte in which transport of transcript from nurse cells to the oocyte is beginning. Transcript accumulates throughout the oocyte beginning around stage 9 such that by stage 14, the mature oocyte, large amounts of transcript are found throughout the oocyte, similar to what is seen in the cleavage stage egg shown in figure lA. (B) Higher magnification view of a glancing section through a single egg chamber showing the lack of label associated with the somatic follicle cells (fc). (C) Whole mount squash of an adult testis showing hybridization limited to the apical tip. This is the region in which germ line stem cells and early stage spermatocytes reside.


ncn
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apex
C

Figure 2.3. Northern blot of maternal poly A+ RNA (from female flies) and poly A+ RNA from different embryonic stages (0-3, 3-6, 6-9, 9-12 and 12-16 hrs. at 250C; Embryogenesis takes 22 hrs.). An EcoRl fragment that contains the amino half of the protein coding seuence in the full length cDNA was used as hybridization probe. The leftmost lane shows the presence of an abundant transcript of roughly 2.2 kb in female flies. This transcript is also abundant in early (0-3hr.) embryos (lane 2) but is at much lower levels in later embryonic stages. An actin control is shown in the lower panel.

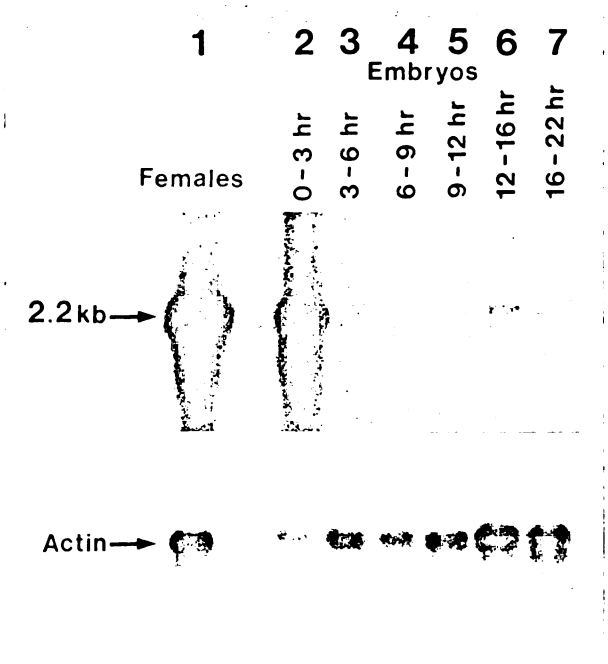

1
2
3
4
5
6
7
Embryos
Females
0-3 hr
3-6 hr
6-9 hr
9-12 hr
12-16 hr
16-22 hr
2.2kb
Actin

Figure 2.4. Monoclonal antibody Mab46Fll staining of wholemount wildtype embryos and embryos derived from homozygous <u>vasa</u>^{PD23} mothers. (A) Wildtype cleavage stage embryo showing Mab46Fll antigen localized to the polar plasm. (B) Wildtype embryo following pole cell formation. The Mab46Fl1 antigen has been incorporated into pole cells. (C) Cleavage stage <u>vasa</u>^{PD23} embryo showing absence of localized Mab46Fl1 immunoreactivity. Also note that the background level of antigen throughout the embryo is decreased with respect to wildtype. (D) <u>vasa</u>^{PD23} embryo at a similar stage to embryo in panel B. Pole cells are absent and Mab46Fl1 immunoreactivity is undetectable.

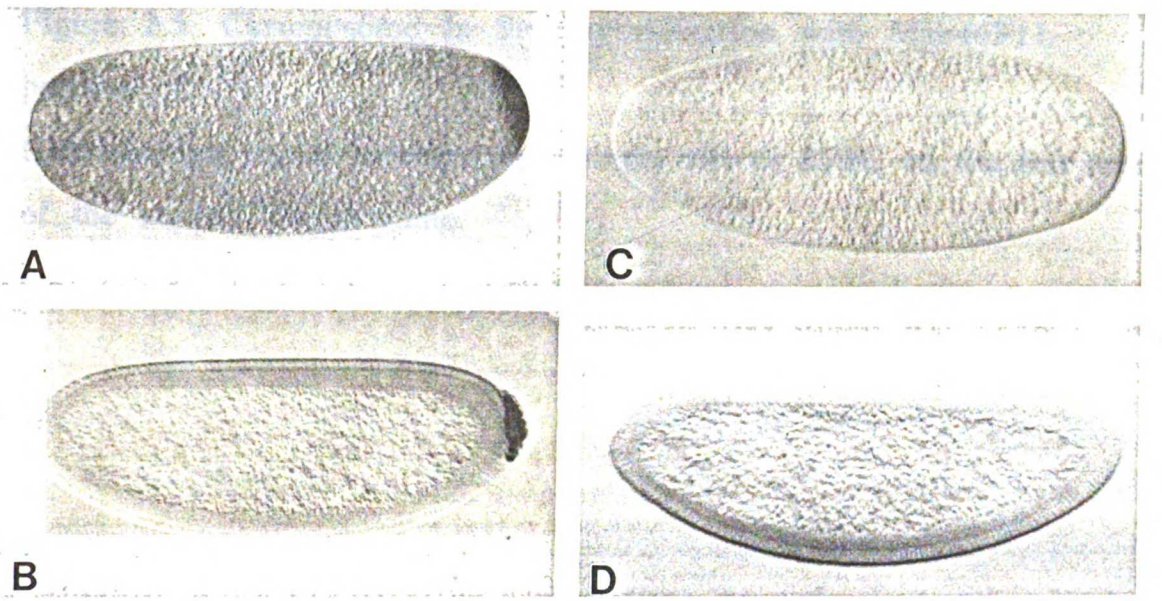

A
C
B
D

Figure 2.5. Chromosome In Situ hybridization with Mab46F11-positive cDNA.

Hybridization signal localizes to a single site at 35BC on the left arm of the second chromosome.

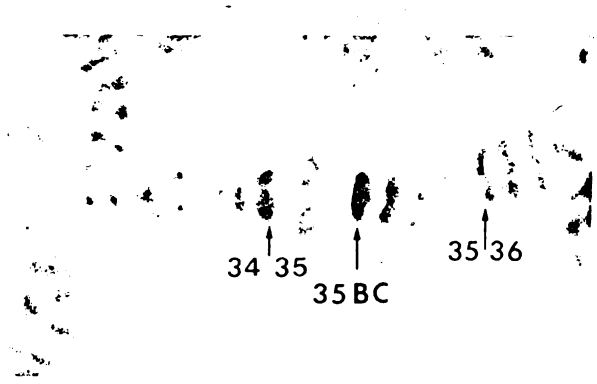

34
35
35BC
35
36

Figure 2.6. Genomic organization and coding sequence of the vasa gene. The inset shows the genomic organization of the vasa gene. The coding region is divided into seven exons. These are separated by five small introns of 53, 58, 57, 63, and 70 base pairs, respectively, and a large intron of about 3.5 kb. The positions of EcoRl sites used in subcloning genomic fragments are indicated (E), as are the initiation codon (ATG), termination codon (UGA) and the position of the poly A splice site (Poly A).

The sequence of the vasa cDNA as well as the locations for the six introns (arrowhead) are shown. cDNAs were sequenced on both strands. The one cDNA isolated from the Kalfayan library contains the entire sequence shown in figure 6. Several incomplete cDNAs, coresponding to the amino terminal half of the Kalfayan clone down to the end of exon 4, were isolated from the Lambda ZAP library. The size of the largest Kalfayan clone, 2229 bp, is similar to the size of the single transcript identified by Northern analysis, indicating this clone encodes a nearly full lenght or full length cDNA. The genomic clone spans the 3 EcoRl fragments shown in the figure 6 inset. All of these were sequenced with two exceptions. Only the ends of the 3.5 kb intron were sequenced and genomic sequence corresponding to the 30 bp 5' to the polyadenylation site of the cDNA was not sequenced. We also note that 12 out of 60 bp from base 2583 to base 2638 are different between the genomic and cDNA clones. The cDNA sequence is shown. All but the last 30 nucleotides of the cDNA sequence has been confirmed by sequencing genomic DNA (on one strand). Boxed regions refer to features of the sequence discussed in

the text and figure 7 and 8. These are the five heptad repeats, the six helicase motifs (labelled with Roman numerals I,Ia,II, III,IV,V) and the acidic tail (with asterisks underneath the charged residues). The consensus polyadenylation sequence in the 3' end of the sequence is underlined.

We have noted a number of minor differences between our sequence and that of Lasko and Ashburner (1988), including sequences of the introns. Amino acid changes are also noted: #252, Y for F in Lasko and Ashburner (1988); #569, R for C; #581, H for D. A tandem repeat of 39 nucleotides (#539 to 577) is present in Lasko and Ashburner (1988) as well as in one, but not in the other, of our cDNA clones. Because only one copy is found in our genomic DNA sequence, only one copy is shown in this figure. Numbers of the first nucleotide and amino acid in each line are indicated on the left.

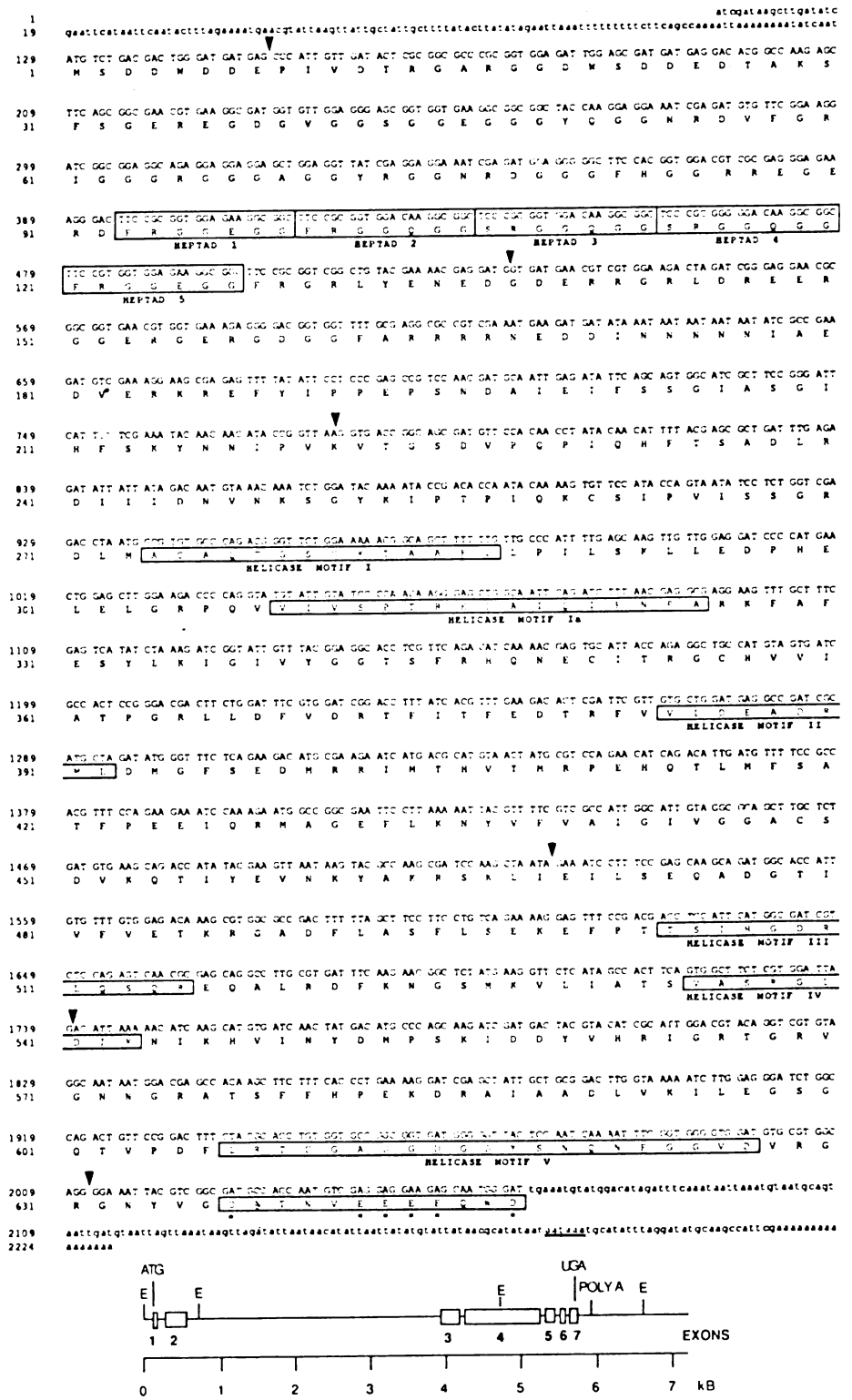

HEPTAD 1
HEPTAD 2
HEPTAD 3
HEPTAD 4
HEPTAD 5
HELICASE MOTIF I
HELICASE MOTIF Ia
HELICASE MOTIF II
HELICASE MOTIF III
HELICASE MOTIF IV
HELICASE MOTIF V
ATG
UGA
POLYA
E
EXONS
1 2
3
4
5 6 7
0
1
2
3
4
5
6
7
kB

Figure 2.7. Homology of vasa coding sequence with murine eIF-4AI and human p68, and identification of domains shared by a large number of helicases. Alignments have been performed visually between EIF-4AI, p68 and vasa. Boxed regions indicate identity with the vasa sequence. Vertical lines and Roman numerals show our alignment of six domains within the vasa sequence identified as common to a large number of helicases (Hodgman, 1988a,b; Lane, 1988). Letters below the protein sequence alignments indicate the Hodgman consensus sequences (in single letter codes for amino acids) for these domains of helicases (1988a,b).

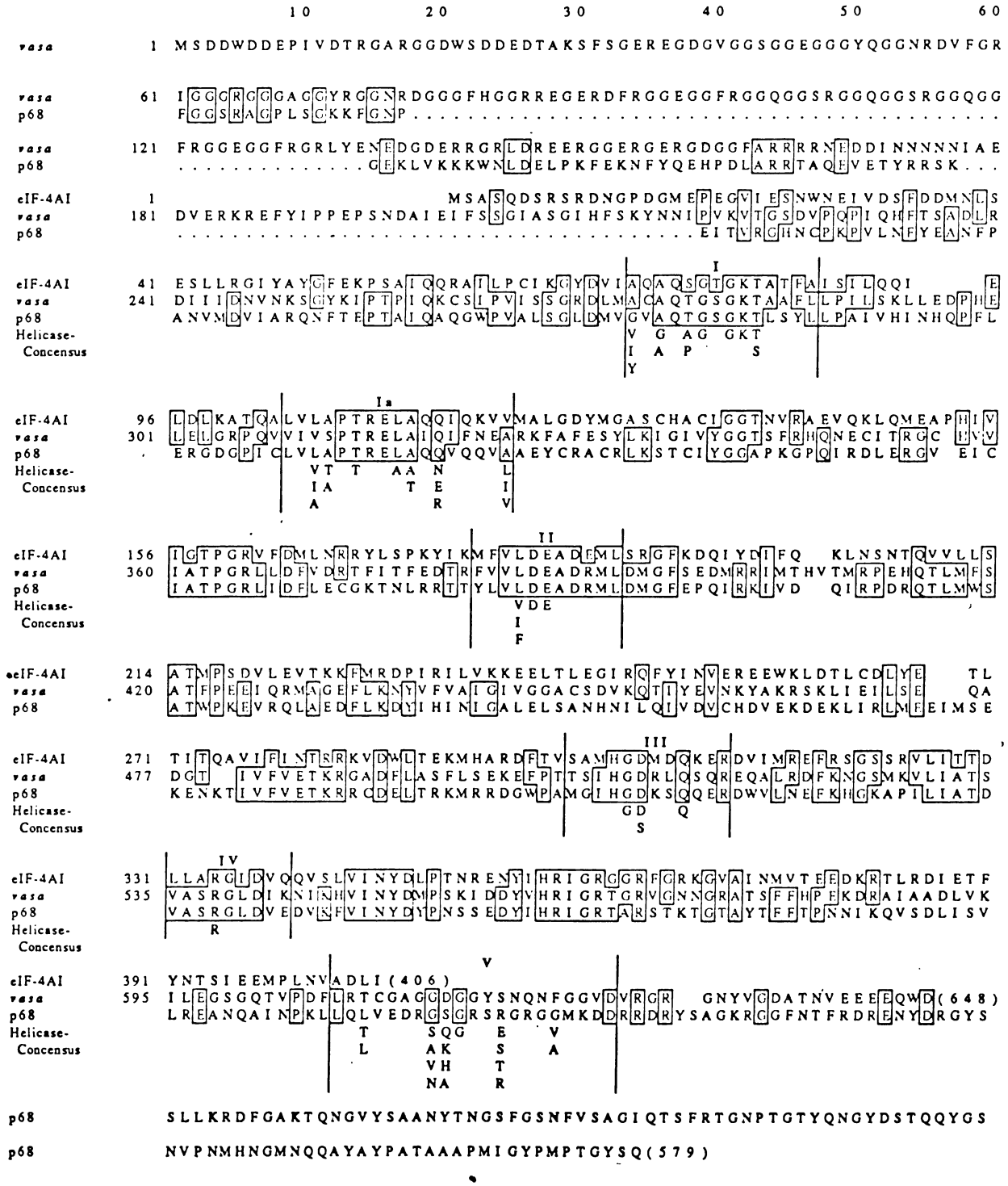
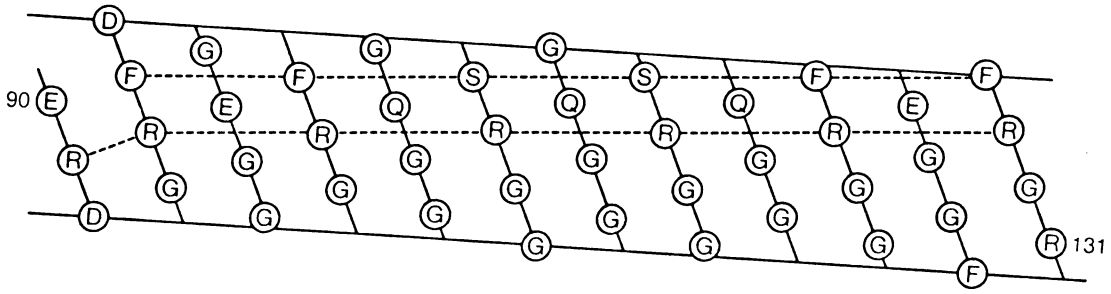

10 20 30 40 50 60
vasa 1 MSDDWDDEPIVDTRGARGGDWSDDEDTAKSFSGEREGDGVGGSGGEGGGYQGGNRDVFGR
vasa 61 IGGGRGGGAGGYRGGNRDGGGFHGGRREGERDFRGGEGGFRGGQGGSRGGQGGSRGGQGG
p68 FGGSRAGPLSGKKFGNP
vasa 121 FRGGEGGFRGRLYENEDGDERRGRLDREERGGERGERGDGGFARRRRNEDDINNNNNIAE
p68 GEKLVKKKWNLDELPKFEKNFYQEHPDLARRTAQEVETYRRSK
eIF-4AI 1 MSASQDSRSRDNGPDGMEPEGVIESNWNEIVDSFDDMNLS
vasa 181 DVERKREFYIPPEPSNDAIEIFSSGIASGIHFSKYNNIPVKVTGSDVPQPIQHFTSADLR
p68 EITVRGHNCPKPVLNFYEANFP
eIF-4AI 41 ESLLRGIYAYGFEKPSAIQQRAILPCIKGYDVIAQAQSGTGKTATFAISILQQIE
vasa 241 DIIIDNVNKSGYKIPTPIQKCSIPVISSGRDLMACAQTGSGKTAAFLLPILSKLLEDPHE
p68 ANVMDVIARQNFTEPTAIQAQGWPVALSGLDMVGVAQTGSGKTLSYLLPAIVHINHQPFL
Helicase-Concensus V G AG GKT I A P S Y
I
eIF-4AI 96 LDLKATQALVLAPTRELAQQIQKVVMALGDYMGASCHACIGGTNVRAEVQKLQMEAPHIV
vasa 301 LELGRPQVVIVSPTRELAIQIFNEARKFAFESYLKIGIVYGGTSFRHQNECITRGCHVV
p68 ERGDGPICLVLAPTRELAQQVQQVAAEYCRACRLKSTCIYGGAPKGPQIRDLERGVEIC
Helicase-Concensus VT T AA N L IA T E I A R V
Ia
eIF-4AI 156 IGTPGRVFDMLNRRYLSPKYIKMFVLDEADEMLSRGFKDQIYDIFQKLNSNTQVVLLS
vasa 360 IATPGRLLDFVDRTFITFEDTRFVVLDEADRMLDMGFSEDMRRIMTHVTMRPEHQTLMFS
p68 IATPGRLIDFLECGKTNLRRTTYLVLDEADRMLDMGFEPQIRKIVDQIRPDRQTLMWS
Helicase-Concensus VDE I F
II
eIF-4AI 214 ATMPSDVLEVTKKFMRDPIRILVKKEELTLEGIRQFYINVEREEWKLDTLCDLYETL
vasa 420 ATFPEEIQRMAGEFLKNYVFVAIGIVGGACSDVKQTIYEVNKYAKRSKLIEILSEQA
p68 ATWPKEVRQLAEDFLKDYIHINIGALELSANHNILQIVDVCHDVEKDEKLIRLMEEIMSE
eIF-4AI 271 TITQAVIFINTSRKVDWLTEKMHARDFTVSAMHGDMDQKERDVIMREFRSGSSRVLITTD
vasa 477 DGTIVFVETKRGADFLASFLSEKEFPTTSIHGDRLQSQREQALRDFKNGSMKVLIATS
p68 KENKTIVFVETKRRCDELTRKMRRDGWPAMGIHGDKSQQERDWVLNEFKHGKAPILIATD
Helicase-Concensus GD Q S
III
eIF-4AI 331 LLARGIDVQQVSLVINYDLPTNRENYIHRIGRGGRFGRKGVAINMVTEEDKRTLRDIETF
vasa 535 VASRGLDIKNINHVINYDMPSKIDDYVHRIGRTGRVGNNGRATSFFHPEKDRAIAADLVK
p68 VASRGLDVEDVSFVINYDYPNSSEDYIHRIGRTARSTKTGTAYTFFTPNNIKQVSDLISV
Helicase-Concensus R
IV
eIF-4AI 391 YNTSIEEMPLNVADLI(406)
vasa 595 ILEGSGQTVPDFLRTCGAGGDGGYSNQNFGGVDVRGRGNYVGDATNVEEEEQWD(648)
p68 LREANQAINPKLLQLVEDRGSGRSRGRGGMKDDRRDRYSAGKRGGFNTFRDRENYDRGYS
Helicase-Concensus T SQG E V L AK S A VH T NA R
V
p68 SLLKRDFGAKTQNGVYSAANYTNGSFGSNFVSAGIQTSFRTGNPTGTYQNGYDSTQQYGS
p68 NVPNMHNGMNQQAYAYPATAAAPMIGYPMPTGYSQ(579)

Figure 2.8. Fivefold tandem heptad repeats in the vasa sequence. (A) The fivefold heptad repeat F(or S)RGGE(or Q)GG in vasa. Numbers in parantheses indicate the positions of the first and the last amino acids of the repeats. (B) Helical-net analysis of residues 90 through 131, including the heptad repeats. Dotted lines mark the row of arginines and the adjacent row of phenylalanines and serines.

A.

(93) F R G G E G G
F R G G Q G G
S R G G Q G G
S R G G Q G G
F R G G E G G (127)

B.



CHAPTER 3

LOCALIZATION OF VASA, A COMPONENT OF DROSOPHILA POLAR GRANULES, IN MATERNAL-EFFECT MUTANTS THAT ALTER ANTEROPOSTERIOR POLARITY

SUMMARY

Cytoplasm at the posterior pole of the early Drosophila embryo, known as polar plasm, serves as a source of information necessary for germ cell determination and for specification of the more anterior abdominal region. Likely candidates for elements important in one or both of these processes are polar granules, cytoplasmic organelles concentrated in the cortical cytoplasm of the posterior embryo pole. Females homozygous for the maternal-effect mutations, tudor, oskar, staufen, vasa, and valois give rise to embryos which lack localized polar granules, fail to form the germ cell lineage and have abdominal segment deletions. Using antibodies against a polar granule component, the vasa product, we find that vasa synthesis or localization is affected by these mutations. In vasa mutants, synthesis of vasa is absent or severely restricted. In oskar and staufen mutant females, vasa synthesis appears normal, but the vasa product is not localized. In tudor and valois mutant females, vasa is localized to the posterior pole of oocytes, but this localization is lost following egg activation. In addition, we examined embryos from Bicaudal-D mothers, in which abdominal determinants are incorrectly localized to the anterior pole. This ectopic localization appears to require vasa function, yet Bicaudal-D embryos do not show any ectopic vasa localization.

MATERIALS AND METHODS

Stocks

Drosophila melanogaster were raised on standard cornmeal-yeast-agar medium at 25°C. Normal flies were of Oregon-R (OR) wild-type strain. Balanced stocks of vasa (vas^{PD23} and vas^{DL}), tudor (tud^{WC8}), a deficiency which uncovers the tudor locus (Df(2L) PL3), staufen, $stau^{HL54}$, a deficiency which uncovers the staufen locus (Df(2L) PC4), valois, (val^{RB}) and a deficiency that uncovers the valois locus (Df(2L)TW2), were provided by Trudi Schupbach, Princeton University, Princeton, NJ. Other stocks of tudor (tud^{2}, tud^{3}, tud^{4}) were provided by Robert Boswell, University of Colorado, Boulder, CO. Stocks of the two deficiencies Df(2L)A267 and Df(2L)TE116-GW18, which in heterozygous combination delete part of the vasa locus, staufen ($stau^{D3}$), oskar (osk^{166}, osk^{336}, osk^{301}), a deficiency which uncovers the oskar locus, stocks of valois (val^{PE}), and stocks of Bicaudal-D ($Bic\text{-}D^{7134}$, $Bic\text{-}D^{IIIE}$) were provided by Ruth Lehmann, Whitehead Institute, Cambridge, MA.

Fly crosses

For most experiments homozygous females were collected from balanced stocks and mated with OR males. For the creation of a deletion of a part of the locus, virgin heterozygous Df(2L)A267/Cyo females were crossed to Df(2L)TE116-GW18 males and non-Cyo progeny sleected. Females carrying a mutation on one chromosome and a deficiency for the

chromosome and a deficiency for the region on the other were created by crossing heterozygous deficiency virgin females to homozygous males of the appropriate mutant strain. Progeny females lacking the appropriate balancer chromosome were selected for further study.

Production of polyclonal anti-vasa rabbit serum.

A trpE-vasa fusion protein was used as the immunogen. Site directed mutagenesis was carried out to create an Nde1 restriction site at the initial methionine of a full length vasa cDNA cloned into bluescript SK- (Stratagene) as described (Kunkel, et al., 1987). A pair of oligonucleotides was synthesized, phosphorylated and annealed using standard techniques (Maniatis et al., 1982) such that BamH1 and Nde1 sites were created at opposite ends of the oligonucleotide. A gel purified Nde1-Xba1 fragment containing the entire vasa coding region was mixed with these oligonucleotides and ligated into the vector PATH1, cut with BamH1 and Xba1. This vector contains the trpE coding sequence (Dieckmann and Tzagaloff, 1985). Bacteria containing either a trpE-vasa construct or trpE alone were grown and induced according to Driever et al., 1988. Fusion protein was isolated according to Rio et al. (1986), dissolved in sample buffer (Laemmli, 1974) and subjected to preparative SDS-PAGE. Protein bands were visualized by incubating the gel with 0.25 M KCL in H_20. The trpE-vasa fusion protein was cut out and chopped into small bits. These were then suspended in complete Freund's adjuvant (incomplete for booster injections). Rabbits (Berkeley Antibody Company, Berkeley, CA) were injected at multiple subcutaneous and intramuscular sites,

boosted after 4 weeks, and bled at biweekly intervals thereafter. Two rabbits produced high titers against the vasa protein. To purify antibodies specific for the vasa portion of the fusion protein, the following procedure was carried out. Bacterial extract from cells expressing either the trpE-vasa protein or trpE alone were dialyzed into coupling buffer (0.1 M HEPES (pH 7.5), 10% glycerin, 5 mM EDTA). Precipitated material was sedimented at 10000 x g, 10 min. The supernatents were used to prepare affinity columns (Affigel 10/15, 1:1 ratio; Biorad) according to the manafacturer's protocol. Following coupling, these columns were washed exhaustively with 0.5M NaCl, 10mM TrisCl, pH 7.5, and then with elution buffer containing 0.2 M glycine, 0.115M HCl, 0.5% Tween-20, pH 3.5. The column was then equilibrated with antibody binding buffer (50mM TrisCl, pH 7.5, 5mM EDTA). Rabbit serum was dialyzed into antibody binding buffer and then incubated overnight at 4°C batchwise with column material containing bacterial extract induced to express trpE alone. The unbound material was incubated batchwise for four hours at room temperature with affigel containing extract from the trpE-vasa containing bacteria. This affigel was then poured into a column and washed with 50 column volumes of antibody binding buffer. Bound protein was eluted with 3 column volumes of elution buffer. Elution volumes were immediately neutralized with 1 M Tris base and brought to a final concentration of 1% BSA, 0.02% sodium azide.

Immunocytochemistry

Staining of stages of oogenesis and embryogenesis with monoclonal antibody Mab46F11, which recognizes the product of the

vasa locus, were carried out as described in Hay et al. 1988a. For immunocytochemistry using the polyclonal antisera, tissue was incubated for 1 hr at room temperature with the affinity purified anti-vasa antibodies at a dilution of 1:1000 in antibody buffer (0.1 M sodium phosphate, pH7.5, 0.3% Triton X-100, 0.3% sodium deoxycholate, 2% bovine serum albumin (Miles, fraction V)). 3, 15 min. washes in antibody binding buffer were followed by incubation of the tissue for 1 hr at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Biorad, EIA grade) at a dilution of 1:1000. Following three 15 min. washes as above the diaminobenzidine reaction product was developed as described in Hay et al., 1988a. DNA was visualized by incubating embryos with 0.5 ug/ml 4',-6 diamidine-2-phenylindole dihydrochloride (DAPI, Boehringer Mannheim Biochemicals) in 0.1M TrisCl for 10 min. at room temperature and then washing for 10 minutes in Tris buffer alone. The embryos were then mounted in 80% glycerol, 0.1 M TrisCl, pH 9.0, 5% (wt/vol) n-propyl gallate.

Western blotting

For Western blotting one hr collections of embryos were dechorionated in bleach:water (1:1), rinsed extensively with water and staged under a dissecting microscope. Cleavage stage embryos were then processed as described in Hay et al. (1988a) for Western blotting using the monoclonal antibody Mab46F11.

RESULTS

Embryos derived from females homozygous for strong alleles of the five maternal-effect gap loci, vasa, staufen, oskar, valois and tudor, all have abdominal deletions, fail to form germ cells and lack polar granules at the posterior embryo pole. To examine the effects of these mutations on granule localization, we used monoclonal and polyclonal antibodies against the vasa product, a component of polar granules, and carried out Western (immunoblot) analysis and immunocytochemical staining of oocytes and embryos derived from homozygous mutant mothers. Null, as well as weaker alleles of vasa were first analyzed to verify the specificity of the antibodies, before the studies of the other mutations. In order to access the severity of these mutations, the phenotypes generated by homozygous mutant females are compared to those arising from females carrying the same mutation on one chromosome and a deletion of the locus on the other chromosome. In wild-type embryos, vasa immunoreactivity, as seen with monoclonal antibody Mab46F11, is concentrated in the form of polar granules at the posterior pole (Hay et al., 1988a,b). Lower levels of immunoreactivity are also found throughout the embryo during cleavage stages (Hay et al., 1988b). Vasa immunoreactivity, as seen with the polyclonal antiserum, shows a similar pattern during early embryogenesis (Fig. 3.1A). In embryos from homozygous $vasa^{PD}$ females (a hypomorphic allele), the immunoreactivity seen with monoclonal or polyclonal antibodies,

localized to the posterior pole and present at lower levels throughout the embryo, is absent (see Hay et al 1988b Fig.4,) (Fig 3.1B). Thus, both antibodies appear specific for the vasa product.. The specificity of these antibodies for the vasa product is further demonstrated by Western analysis of proteins extracted from vasa cleavage stage embryos (Hay et al., 1988a Fig. 7) (Fig. 3.2). In ovaries of homozygous $vasa^{PD}$ females vasa immunoreactivity is restricted to early stages, primarily the germarium, in which the geometry of the oocyte-nurse cell complex is set up. Occasionally, low levels of vasa immunoreactivity are also found in early stages of the vitellarium, in which the bulk of oocyte and nurse cell growth occurs (figure 3.1D; compare with wild type in figure 3.1C). In a female null for vasa function (see experimental methods for details) oogenesis is usually not completed. In ovaries of many females, egg chambers do not develop beyond the early stages of the vitellarium (Fig. 3.1E). However, in other ovaries egg chambers at later stages of oogenesis are present (up through stage 14) (figure 3.1F). None of these show any vasa immunoreactivity with either the monoclonal or polyclonal antibody. These observations show that our anti-vasa antibodies recognize only the vasa product during oogenesis and early embryogenesis.

The oskar and staufen mutations affect vasa localization during oogenesis. Ovaries from strong (strength being defined relative to the amount of abdominal segment deletion seen) alleles of oskar, (osk^{336}) and staufen, ($stau^{D3}$), or these mutations in trans to deficiencies uncovering the respective loci, show a normal pattern of vasa expression in the nurse cells (Fig. 3.3A, B). Immunoreactivity appears

in the oocyte around stage 10, but never becomes localized to the posterior pole of stage 14 oocytes (Fig. 3.3C, D). Localization is also not seen in early cleavage stage embryos (Fig. 3.3E, F). Normal levels of vasa product are present in early embryos of oskar^{166} and staufen^{D3} mothers (Fig. 3.2). This may derive from vasa synthesized by nurse cells and transported into the oocyte, or it may come from translation of vasa from the large amount of maternally derived vasa RNA present in the early embryo (Hay et al. 1988b). On the basis of Western blotting we cannot distinguish between these two posibilities, and thus cannot address whether these mutations directly affect the localization of vasa to the posterior pole or perhaps the stability of just that population of vasa which ends up at the posterior pole. From the fact that the absolute levels of vasa are not affected in these mutations, and in the absence of any information suggesting the existence of populations of vasa which differ in their stability, we favour the hypothesis that these mutations directly affect vasa localization. This phenotype is likely due to a reduction or loss of function, because a similar pattern of expression is seen immunocytochemically in oskar^{336} and staufen^{D3} females which carry the mutation on one chromosome and a deficiency for the region on the other chromosome (data not shown).

A correlation between vasa localization and mutant phenotype is evident in a temperature-sensitive oskar mutant, osk^{301}. At 18°C, the permissive temperature, many embryos show normal abdominal segmentation and hatch, though pole cells do not form. At 29°C they have strong abdominal deletions, in addition to the absence of pole cells (Lehmann and Nusslein-Volhard, 1986). The degree of vasa

localization in these embryos is correlated with the severity of the abdominal defect. At 18°C some vasa product is found localized to the posterior pole of stage 10 oocytes, and following oviposition weak localized staining is sometimes seen in cleavage stage embryos (figure 3.3G, H). Occasionally structures which resemble polar buds are formed, but these do not develop into pole cells and by blastoderm formation localized vasa immunoreactivity is undetectable. At the nonpermissive temperature oskar^{301} resembles oskar^{336} with respect to its vasa localization phenotype. (figure 3.3I, J).

The valois and tudor mutations tested affect vasa localization during early embryogenesis. Oocytes of homozygous valois^{PE}, valois^{RB71}, or valois^{PE} or valois^{RB71}/deficiency females have vasa localized to their posterior pole at stage 10 through stage 14, as in the wild type (Fig. 3.4A, B). However, following oviposition localized vasa immunoreactivity rapidly disappears, such that by the time of cellular blastoderm formation no localized immunoreactivity is present (Fig. 3.4C, D). Wildtype amounts of vasa immunoreactivity are present, however, by Western blotting (Fig. 3.2). When mutant embryos are also stained with DAPI to visualize their DNA, embryos with localized vasa show no more than a single nucleus, whereas embryos which have proceeded through some cleavage divisions show no signs of localized vasa. This indicates that vasa localization is lost very rapidly following fertilization, long before pole cell formation would have occured.

Embryos from homozygous tudor^{WC8} mothers have a very variable segmentation defect, with some embryos showing normal

segmentation and others having abdominal deletions of varying size (Schupbach and Wieschaus, 1986). Females carrying tudor^{WC8} on one chromosome and a deficiency for the region on the other consistently give rise to embryos with a stronger phenotype, indicating that tudor^{WC8} (as with other published tudor alleles) is probably a hypomorphic allele (Schupbach and Wieschaus, 1986). In both the homozygous mutant and transheterozygous situation, normal vasa localization and accumulation at the posterior pole is observed during the second half of oogenesis (Fig. 3.4E, F). Following oviposition early cleavage stage embryos can be seen to have localized vasa immunoreactivity (Fig. 3.4G), whereas in later stage cleavage embryos it becomes progressively weaker (Fig. 3.4H). In some embryos pole cell buds are observed to form (Fig. 3.4I), but these buds disappear by blastoderm formation (Fig. 3.4J, K). Localized vasa immunoreactivity is always absent by blastoderm formation (Fig. 3.4K), as is the general background seen during cleavage stages in wildtype and mutant embryos.

Tudor^{WC8} embryos have some small electron dense structures associated with mitochondria. These structures may be some form of polar granule. In a phenotypically weaker allele, such as tudor^{2}, more of these structures can be found and a more normal abdomen develops in the embryo (Boswell and Mahowald, 1985). Correspondingly, in these embryos localized vasa immunoreactivity is more intense and frequent, though this immunoreactivity still disappears prior to blastoderm formation (data not shown).

Vasa localization in Bicaudal-D embryos.

In some of the embryos from mothers carrying the dominant bicaudal mutation Bicaudal-D anterior structures are reduced or absent, and in extreme cases a posterior abdomen is duplicated in mirror image symmetry to the normal abdomen, at the anterior end (Mohler and Wieschaus, 1986). At the transformed anterior end polar granules are not apparent and pole cells do not form. Cytoplasmic transplantation experiments suggest that in Bicaudal-D, but not in wildtype embryos, a source of abdomen inducing activity is localized at the anterior of the egg (Lehmann and Nusslein-Volhard, 1986). Interestingly, in Bicaudal-D females that are also heterozygous for vasa the bicaudal phenotype is suppressed (Mohler and Wieschaus, 1986). The vasa-dependent suppression of the bicaudal phenotype may indicate that vasa is required for the ectopic localization of an abdomen inducing activity and that the reduced level of vasa from heterozygous vasa females results in localization of insufficient amounts of this activity. On this hypothesis one might expect to see localization of vasa to the anterior pole as well as the posterior. This hypothesis has not been born out; under egg laying conditions in which Bicaudal-D females produce large numbers of embryos with a bicaudal phenotype, we see no localization of the vasa product to the anterior pole of oocytes, in-vitro activated eggs or early cleavage stage embryos (data not shown).

DISCUSSION

Polar granules are concentrated at the posterior pole of late stage oocytes early embryos, and may be involved in the posterior localization of germ cel determinants and activities that specify abdominal segmentation. We have s previously that the vasa protein is an essential component of the polar granule (Hay et al., 1988a,b). In an attempt to study the mechanism and function of its localization, we have followed the fate of the vasa product in six maternal effect mutants, vasa, staufen, oskar, valois, tudor, and Bicaudal-D, because these mutations alter localization of cytoplasmic determinants required for development of the germ line and for embryonic abdomen formation.

Functions of vasa

The phenotype of vasa is complex. A hypomorphic allele, $vasa^{PD}$, shows vasa immunoreactivity restricted to the germarium, and fails to localize (or perhaps even assemble) polar granules. In contrast, null mutants are usually blocked during early oogenesis, at roughly the stages in which vasa protein seems to be restricted in $vasa^{PD}$. These results suggest a requirement for vasa at two distinct phases of oogenesis. Early during oogenesis vasa is necessary for the development of the oocyte and nurse cells following their formation, whereas in the second half of oogenesis vasa is required for the localization of cytoplasmic determinants required for germ line formation and abdomen specification. During embryogenesis there

may be additional requirements for vasa. At the stage of the somatic blastoderm formation, $vasa^{PD}$ and $vasa^{DL}$ embryos show frequent cellularization defects (data not shown). This may reflect an essential function of the low level of vasa seen throughout the wildtype embryo, as revealed in immunocytochemical experiments. Furthermore, although the Bicaudal-D mutation causes a mirror-image duplication of abdominal structures in a manner that depends on the dose of the vasa gene, vasa is not detectably localized to the anterior pole in embryos from Bicaudal-D females. Thus it seems possible that the vasa protein present throughout the somatic regions of the cleavage stage embryo provides an activity necessary for the function of the anteriorly localized abdominal determinants, or perhaps an environment that is permissive for ectopic localization in a Bicaudal-D background. Decreasing the level of vasa in heterozygotes reduces the amount of available anterior vasa below a critical threshold and thus prevents the activation or localization of the abdominal determinants. Whether the bicaudal phenotype would be augmented by extra copies of the vasa gene is unknown.

A similar phenotype to that seen with vasa/Bicaudal-D double mutants is also seen with double mutants of Bicaudal-D with tudor (Mohler and Wieschaus, 1986), valois, oskar (Lehmann and Nusslein-Volhard, 1986; Lehmann, unpublished) and cappuccino and spire (Manseau and Schupbach, 1989). If the products of these genes are also distributed throughout the oocyte and early embryo, perhaps as mini-polar granules, and are required for some aspect(s) of the spatial or temporal function of the posterior determinants, the block in anterior function of posterior determinants could be similarly

explained. Interestingly, staufen/Bicaudal-D double mutants show an increased frequency of the bicaudal phenotype compared to Bicaudal-D alone (Mohler and Wieschaus, 1986). Homozygous staufen embryos have head defects (Schupbach and Wieschaus, 1986) and decreased bicoid protein levels at the anterior end (Driever and Nusslein-Volhard, 1988), in addition to the posterior defects. Transplanting wildtype posterior pole cytoplasm to the anterior pole of a wildtype embryo results in a similar head phenotype (Frohnhofer et al. 1986). One possibility consistent with these observations is that staufen is required for transporting posterior activity to the posterior pole. In embryos from homozygous staufen mothers some of this activity remains at the anterior pole, resulting in the head defect phenotype, while in Bicaudal-D/staufen embryos it becomes more concentrated at the anterior pole because of the opposing action of an independent Bicaudal-D dependent localizing process.

Localization of vasa

Following fertilization or egg activation polar granules undergo rapid cha their associations with other structures and in their morphology. During oog polar granules are associated with mitochondria and appear to contain large amounts of RNA. Following either egg activation or fertilization they lose thi association with mitochondria, fragment and become associated with polyso Mahowald, 1962, 1968, 1971b). These observations raise the possibility that different (cytoskeletal?) mediators of localization may be used during oogen and early embryogenesis.

One approach to identify elements important for posterior localization of granules is to study mutations that affect localization. This process is affecte

localization. This process is affected at an early stage by oskar and staufen mutations. What appear to be strong alleles of staufen and oskar, based on their abdominal deletion phenotype, synthesize roughly normal amounts of vasa. In the oocytes of homozygous females, however, vasa is not localized. Thus both oskar and staufen gene products are required for some aspect of the initial vasa localization event. Further evidence for this hypothesis derives from studies of $oskar^{301}$, the temperature sensitive allele. The temperature sensitive period for the oskar-dependent development of the abdominal deletion phenotype corresponds to the last half of oogenesis (stages 10-14), the same time period during which polar granules become localized to the posterior oocyte pole (Lehman and Nusslein-Volhard, 1986). Indeed, some vasa is localized at permissive, but not at nonpermissive temperatures during oogenesis. Thus the strength of the oskar phenotype is related to the amount of vasa, and probably polar granules that become localized to the posterior pole. The oskar activity in $oskar^{301}$ mutants is not normal even at the permissive temperature, because pole cells never form in these embryos and the vasa protein that is localized to the posterior pole during oogenesis does not remain localized following oviposition. The latter observation suggests that oskar dependent activity (which may be either quantitatively or qualitatively insufficient in the mutant) is also required during cleavage stages of embryogenesis to maintain vasa localization.

The phenotype of tudor is more difficult to interpret, since $tudor^{WC8}$, a strong allele of tudor, is almost certainly hypomorphic. Boswell and Mahowald (1985) have shown that the number and size

of structures tentatively identified as polar granules, based on their electron-dense appearance and association with mitochondria, decreases as the severity of the abdominal deletion phenotype increases in a series of tudor mutants. Polar granules are not apparent in tudor^{WC8}/Df(2L)PL3 heterozygotes yet we see good vasa localization during oogenesis and during early cleavage stages following fertilization. However, consistent with this phenotypic series, we find that in tudor 2, a weaker tudor allele, the vasa localized immunocytochemically typically is more abundant than in tudor^{WC8}/Df(2L)PL3 heterozygotes. It is not clear from this phenotypic series, however, whether vasa would be localized in oocytes from mothers totally deficient in tudor function. One possibility is that the strong phenotype (if viable) is a complete lack of localization. Alternatively, or perhaps in addition to this aspect of tudor function, tudor may play some other role in polar granule function following fertilization. This seems likely because, though vasa localization is quite good in early cleavage stage tudor embryos, and vasa immunoreactivity remains somewhat localized until about the time the pole cells would normally be forming, pole cells do not form. The fact that vasa is well localized but that localized polar granules are not visible with electron microscopy suggests that the tudor product might be required for protein-protein interactions necessary for assembly of a functional granule structure. The observation that the abdominal segment deletion phenotype is variable in tudor^{WC8}, whereas pole cells never form may simply reflect a higher threshold requirement for tudor-depentent functions for pole cell formation in the mutant situation, or it may indicate a

qualitative difference in the mutant's ability to carry out functions required for abdomen and pole cell development.

What appears to be a strong allele of valois (valois^{PE}), based on the consistency of both the abdominal deletion and vasa localization phenotypes, either as a homozygote or in heterozygous combination with a deficiency for the region, shows good localization during oogenesis, but this disappears very rapidly following fertilization. This may suggest that the maternal valois product is required for some aspect of stabilization of vasa localization. Interestingly, many valois embryos do not cellularize, or cellularize incompletely, even though normal amounts of vasa protein can be detected on Western blots. This phenotype is consistent with the possibility that valois might be important for some aspects of cellular function (the cytoskeleton perhaps) in the embryo which are required for both somatic cellularization and polar granule localization. As with tudor, though, we cannot rule out a role for valois also during the initial localization process.

In summary, we have used vasa immunoreactivity as a marker for polar granule localization. In stages where polar granules have been examined or searched for with electron microscopy, their lack of localization is correlated (with the possible exception of tudor) with the absence of localized vasa immunoreactivity. All four loci, staufen, oskar, tudor and valois, appear to be involved in the posterior localization and/or its maintainence. Strong alleles from staufen, oskar and valois show a similar localization phenotype as homozygotes and as flies carrying one mutant allele and a deficiency of the locus. These phenotypes suggest that both staufen and oskar affect early

steps of vasa localization in oocytes. There is also an indication that oskar dependent activity is required for this localization later during embryogenesis. The valois locus, on the other hand, may be mainly involved in the maintainence of polar granule localization in early embryos. Although tudor alleles also affects localization in embryos, this affect may be indirect, the consequence of a loss of tudor-dependent functions required following fertilization for proper granule function. The alleles tested, however, are hypomorphic and may not have revealed all aspects of tudor function.

Figure 3.1. Immunoreactivity in wildtype and vasa mutant ovaries as visualized using polyclonal rabbit anti-vasa antibodies. Younger stages of oogenesis, and the anterior of any given egg chamber are located to the left. (A) Cleavage stage wildtype embryo. Label is concentrated at the posterior embryo pole. A general background level of staining is present throughout the somatic regions of the embryo. (B) $vasa^{PD}$ cleavage stage embryo. No staining is present, at the posterior pole or throughout the rest of the embryo. (C) Wildtype ovary. (D) Homozygous $vasa^{PD}$ ovary. Label is primarily present in the germarium and early vitellarium stages (indicated by the arrows). (E) A vasa null ovary which does not show any late stage oocytes. Label is undetectable. (F) A vasa null ovary which does show a later stage oocyte (approximately stage 10). Label is not present.

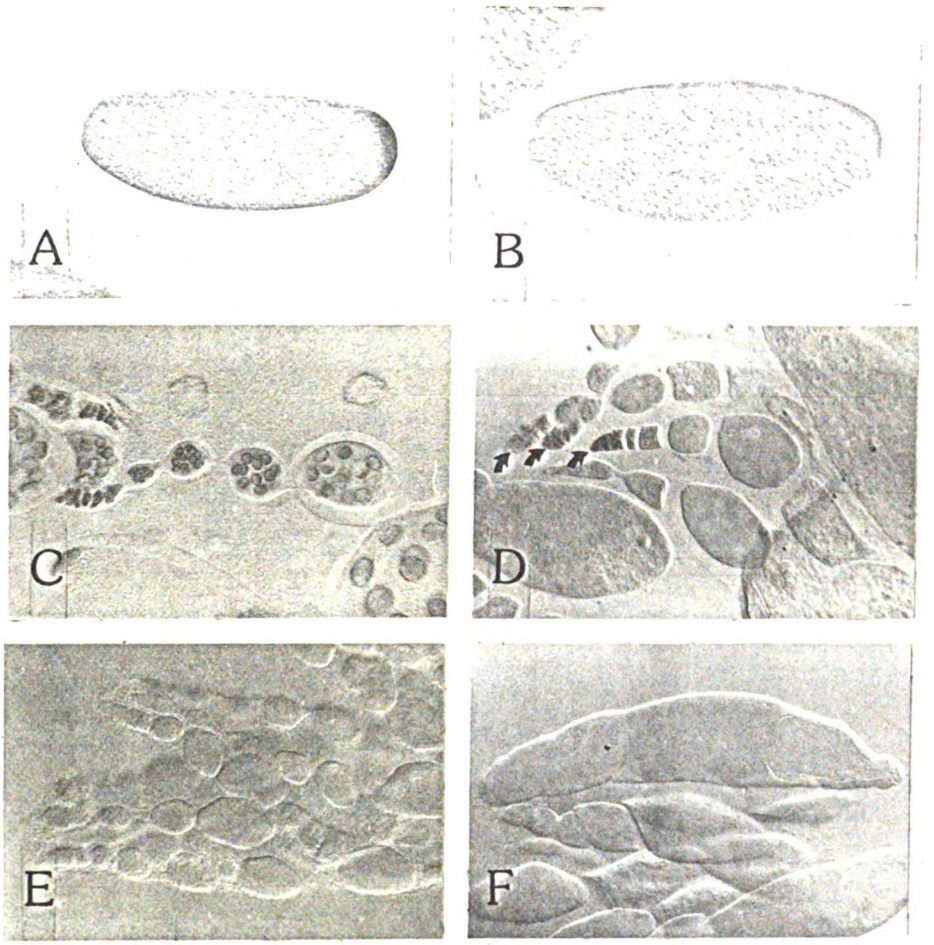

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Figure 3.2. Western blot of cleavage stage embryos from wildtype and homozygous mutant mothers for the mutations vasa, tudor, oskar, staufen, and valois. A 72X103Mr band is absent only in the vasa embryo lane.

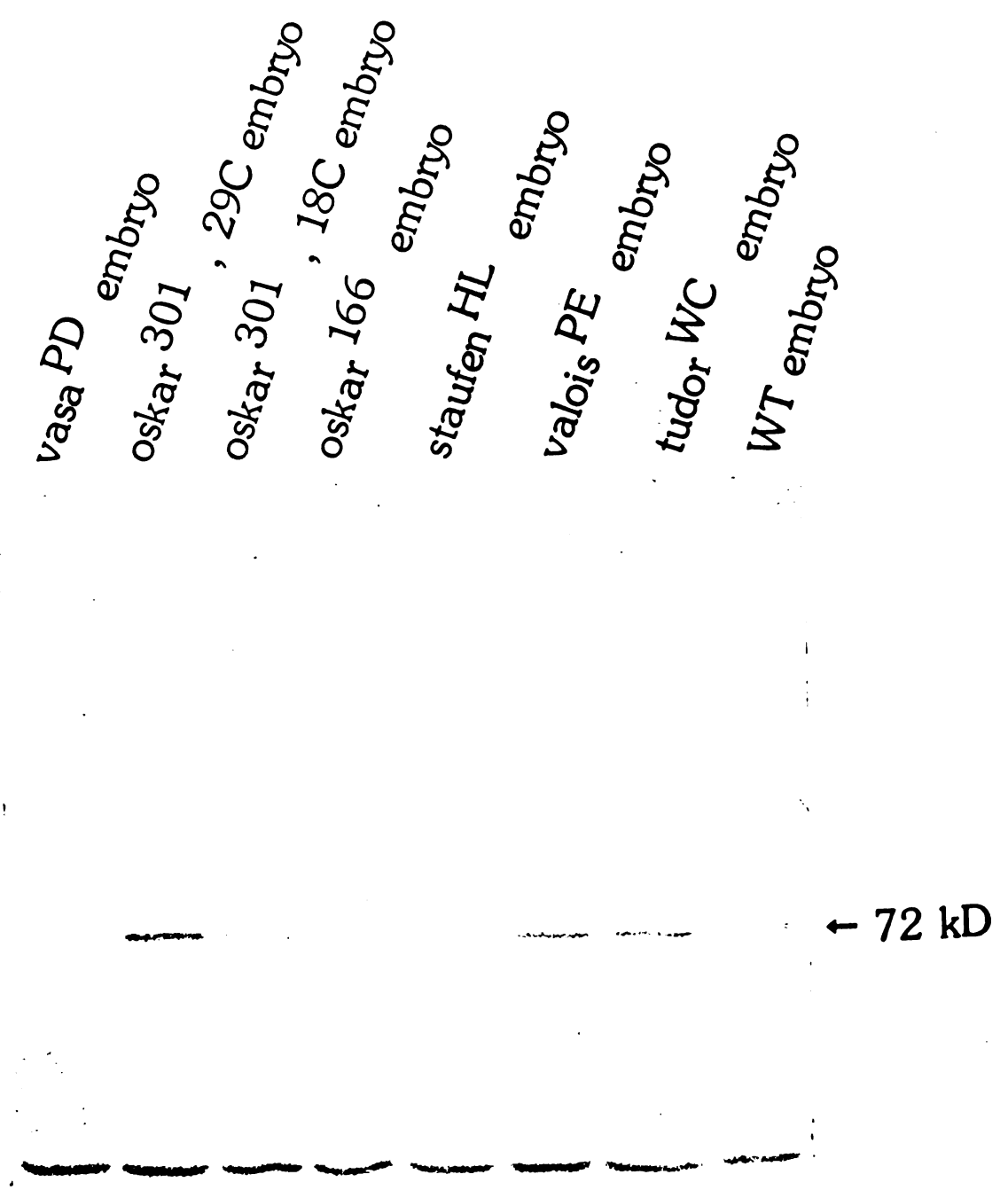
vasa PD embryo
oskar 301, 29C embryo
oskar 301, 18C embryo
oskar 166 embryo
staufen HL embryo
valois PE embryo
tudor WC embryo
WT embryo
← 72 kD

Figure 3.3. vasa localization in stages of oogenesis and early embryos from homozygous oskar and staufen mothers. All antibody stainings were carried out with polyclonal anti-vasa antibodies. (A) Stage 10 $oskar^{336}$ egg chamber. Label is present in nurse cells and in the oocyte but is not localized to the posterior oocyte pole. (B) Stage 10 $staufen^{D3}$ egg chamber. Label is present in nurse cells and oocyte but not localized to the posterior oocyte pole. (C) Cryostat section of an $oskar^{336}$ stage 14 oocyte. Posterior label is not present. (D) Cryostat section of a $staufen^{D3}$ stage 14 oocyte. Posterior label is not present. (E) $oskar^{336}$ early cleavage stage embryo. Localized label is not present. (F) $staufen^{D3}$ early cleavage stage embryo. Localized label is not present. (G) stage 10 oocyte from $oskar^{301}$ female raised at 18°C. Some localized label is present at the posterior oocyte pole. (H) Early cleavage stage embryo from $oskar^{301}$ female raised at 18°C. A small amount of label is localized to the posterior embryo pole. (I) Stage 10 oocyte from $oskar^{301}$ female raised at 29°C. Label is not detectably localized to the posterior oocyte pole. (J) Early cleavage stage embryo from $oskar^{301}$ female raised at 29°C. Label is not detectably localized to the embryo posterior pole.

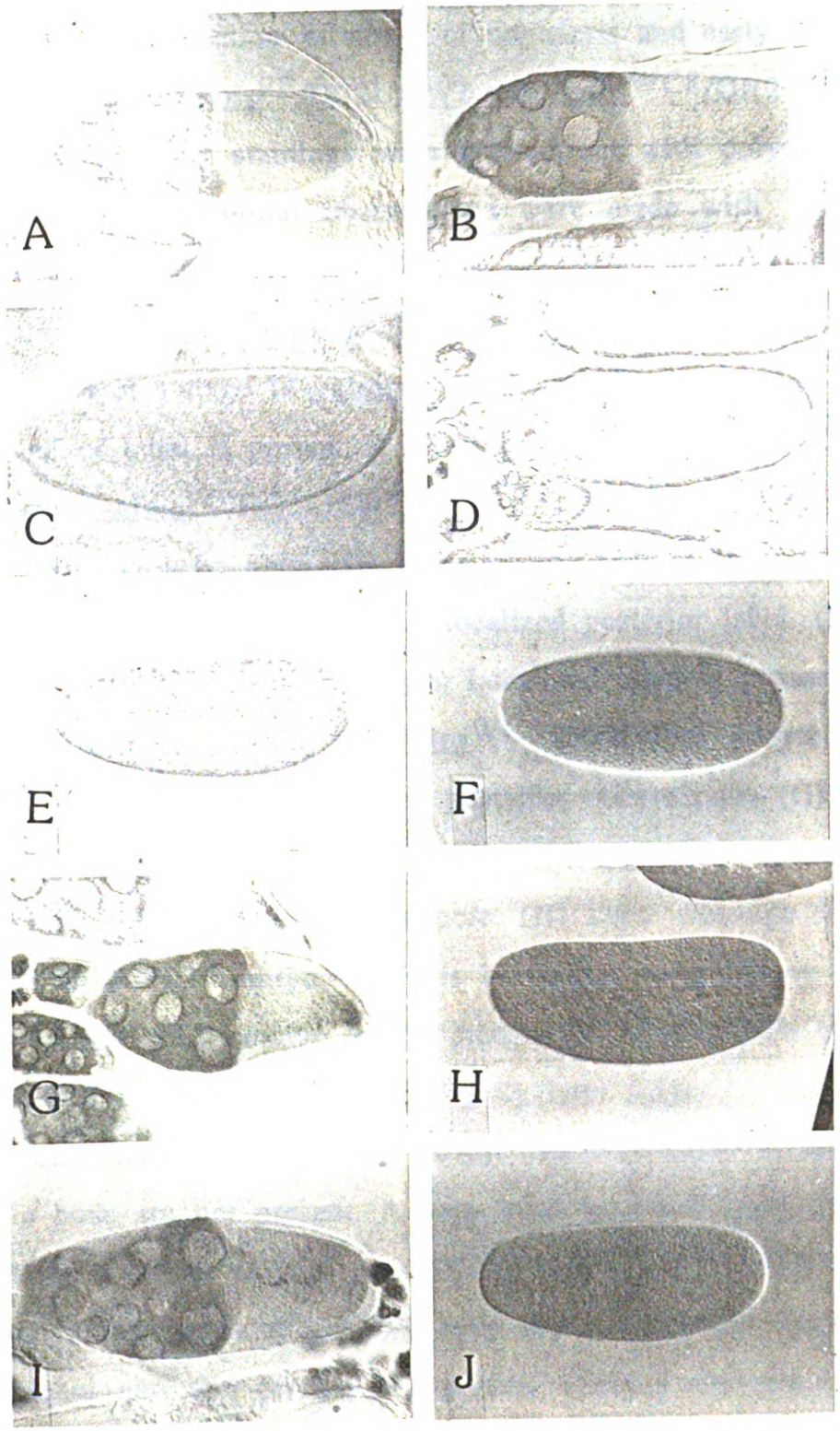
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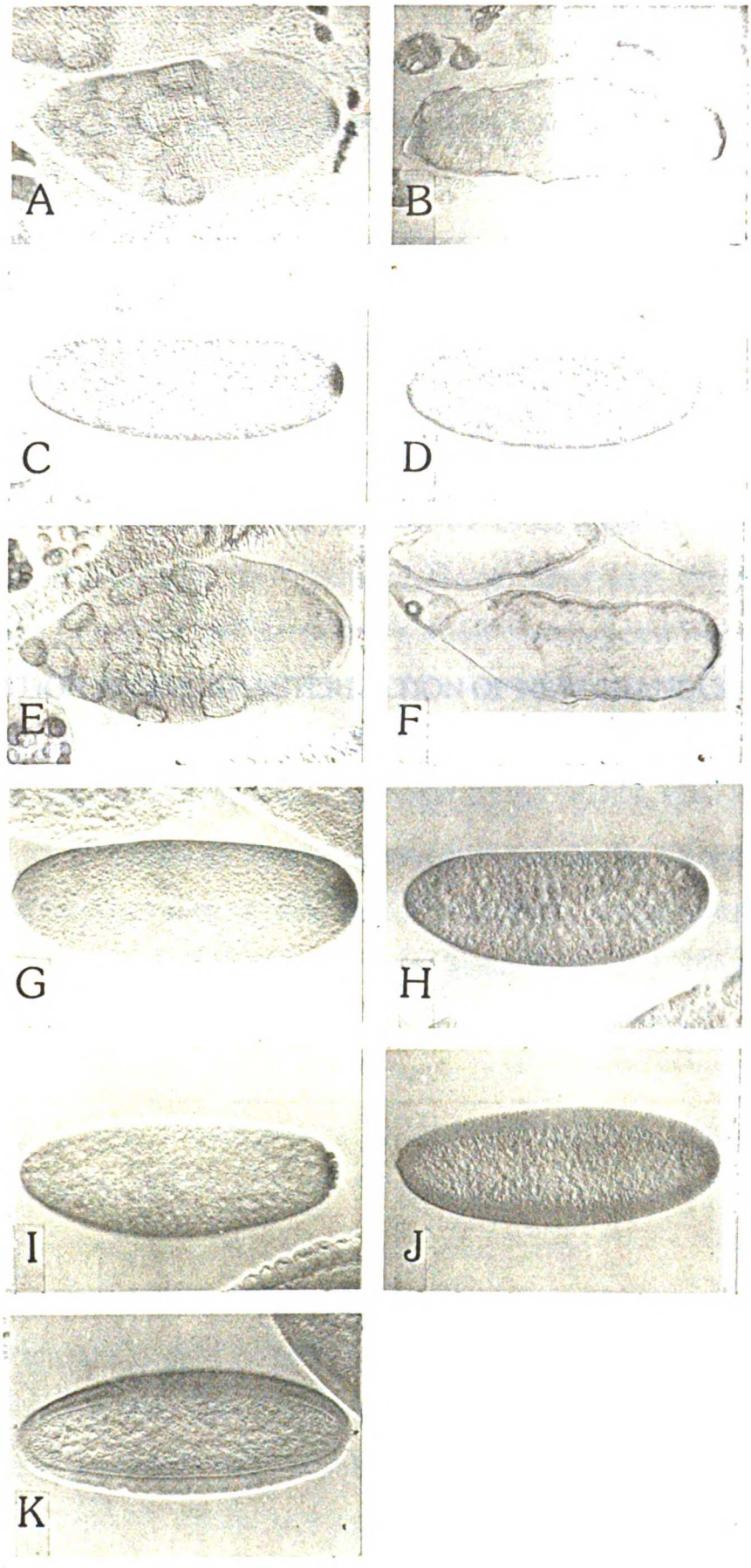
Figure 3.4. vasa localization in stages of oogenesis and early embryogenesis from valois^{PE}/Df(2L)TW2 and tudor^{WC8}/Df(2R)PL3 mothers. All antibody stainings were carried out with polyclonal anti-vasa antibodies. Similar observations were made with monoclonal antibody Mab46F11. (A) Stage 10 valois^{PE}/Df(2L)TW2 oocyte. Some label is localized to the oocyte posterior pole. (B) Cryostat section of a stage 14 valois^{PE}/Df(2L)TW2 oocyte. A dense cortical band of label is present at the oocyte posterior pole. (C) Early cleavage stage valois^{PE}/Df(2L)TW2 embryo which shows some label at the posterior embryo pole. (D) Later cleavage stage valois^{PE}/Df(2L)TW2 embryo with no localized posterior label. (E) Stage 10 tudor^{WC8}/Df(2R)PL3 oocyte. Localized label is present at the posterior oocyte pole. (F) Stage 14 tudor^{WC8}/Df(2L)PL3 oocyte. A dense band of label is present at the posterior oocyte pole. (G) Early cleavage stage tudor^{WC8}/Df(2R)PL3 embryo. Large amounts of label are localized to the oocyte posterior pole. (H) Later cleavage stage tudor^{WC8}/Df(2R)PL3 embryo. Label is becoming more diffuse. (I) tudor ^{WC8}/Df(2R)PL3 embryo in the process of forming pole buds. Some localized label is present. (J) Slightly later stage tudor^{WC8}/Df(2R)PL3 embryo than in (I). The blastoderm is about to form. Pole buds are not present. A very faint localized label is present at the posterior embryo pole. (K) tudor^{WC8}/Df(2R)PL3 embryo beginning gastrulation. Pole cells are not present. Localized posterior label and the general background staining are absent.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CHAPTER 4

ISOLATION AND CHARACTERIZATION OF NEW GRANDCHILDLESS MUTATIONS

SUMMARY

Recently in the Jan lab a P-element vector was constructed and used to generate lines of flies with single autosomal P-element insertions (Bier et al. 1989). I screened through these lines looking for mutations in which the ability of homozygous females to give rise to progeny that have a functional germline is impaired. Both female sterile (in which the progeny die during embryogenesis) and grandchildless mutants (in which viable progeny are obtained whose only defect is in the ability to form a functional germline) were obtained. One female sterile mutation was isolated in which pole cells fail to form in offspring of homozygous females. Two grandchildless mutations were found in which at least some viable, but sterile progeny were obtained from homozygous mothers. Complementation tests with known mutants that affect pole cell formation show that all these mutants represent new loci. Some characterization of these lines is presented.

MATERIALS AND METHODS

Stocks

Drosophila melanogaster were raised on standard cornmeal-yeast-agar medium at 25°C. Normal flies were of Oregon-R (OR) wild-type strain. Balanced stocks of vasa (vas^{PD23} and vas^{DL}), tudor (tud^{WC8}), a deficiency which uncovers the tudor locus (Df(2L) PL3), staufen, $stau^{HL54}$, a deficiency which uncovers the staufen locus (Df(2L) PC4), valois, (val^{RB}) and a deficiency that uncovers the valois locus (Df(2L)TW2), were provided by Trudi Schupbach, Princeton University, Princeton, NJ. Other stocks of tudor (tud^{2}, tud^{3}, tud^{4}) were provided by Robert Boswell, University of Colorado, Boulder, CO. Stocks of the two deficiencies Df(2L)A267 and Df(2L)TE116-GW18, which in heterozygous combination delete part of the vasa locus, staufen ($stau^{D3}$), oskar (osk^{166}, osk^{336}, osk^{301}), a deficiency which uncovers the oskar locus, stocks of valois (val^{PE}), and stocks of Bicaudal-D ($Bic\text{-}D^{7134}$, $Bic\text{-}D^{IIIE}$) were provided by Ruth Lehmann, Whitehead Institute, Cambridge, MA. Df(2L)GpdhA (25D7-E1; 26A8-9), which uncovers the E3 2nd 24 grandchildless mutant site of insertion was provided by the Bowling Green stock center.

Scheme to set up transformed lines with autosomal insertions and screen these for female sterility and grandchildlessness

Approximately 4000 lines in which P-element jumps to autosomes had b induced to take place were set up as balanced stocks in vials by Bier et al. (From this balanced stock homozygous males and females were selected and

homozygous males and females were selected and placed into fresh vials. I then scored these vials for the presence of eggs that hatch. Those vials which contained no eggs, or no eggs that hatched, were scored as female sterile. The majority of vials, in which viable progeny were obtained, were treated as follows. Prior to eclosion of progeny from non-female sterile lines the parents were removed. Following eclosion of the progeny they were transfered to a new vial and the vial checked a week later for the presence of eggs. Those vials which contained no eggs were scored as grandchildless. The absence of gonads in both males and females from this generation was confirmed by dissection.

Complementation testing of the mutants.

Heterozygous virgin females of a balanced mutant stock were mated with heterozygous males of a second mutant stock and progeny lacking the balancer chromosome were scored for for female sterility or, in the next generation, grandchildlessness.

Immunocytochemistry and X-gal staining.

Immunocytochemistry using Mab46F11 to localize the vasa product in sections of ovaries, wholemount embryos and testis, of mutants was carried out as in Hay et al. 1988a).

For staining for lacZ expression in embryos, embryos were collected overnight, washed into sample wells with 0.7% NaCl, 0.1% Triton X-100 (NaCl/Triton). They were then dechorionated with bleach, washed extensively with NaCl/Triton, fixed for 30 min. in 5% formaldehyde in 0.1M sodium phosphate, pH 7.2 mixed with an

equal volume of heptane, rinsed in NaCl/Triton, then incubated in an X-gal solution (Hiromi et al. 1985) overnight at room temperature in the dark with gentle shaking. The samples were placed in water, 0.1M sodium phosphate or NaCl/Triton for examination with a stereomicroscope. For staining for lacZ expression in ovaries and testes gonads were dissected into 0.7M NaCl and then transfered to an X-gal solution (Laski and Rubin, 1989) and processed as above.

Molecular techniques

Plasmid rescue from minipreps of fly DNA were performed according to Pirotta (1986). In situ hybridization to polytene chromosomes was done using the P-lacW vector as a probe as described in Papazian et al. 1988). Tissue in situ hybridizations were carried out according to Bier et al. in preparation) on wholemount ovaries, testes and embryos.

RESULTS

As a part of a P-element mutagenesis screen carried out by Bier et al. (1989) I was able to screen homozygous females from P-element containing lines for female sterility and grandchildlessness. 57 female-sterile lines were isolated. Progeny from homozygous females of two of these lines (fs 1 and fs 4) fail to form pole cells. The embryos also show abdominal segment deletions characteristic of members of the maternal-effect-gap mutants described in chapter 3 (V. Siegal, unpublished). The progeny of the major class of females, those that lay viable eggs, were then scored for sterility--the grandchildless phenotype. In this way four lines were isolated that showed a grandchildless phenotype; the progeny of homozygous females gave rise to viable, but sterile offspring. One of these lines was subsequently lost, though genomic DNA was isolated from flies containing the P-element. This line was subsequently shown to have a P-element at the vasa locus (previously characterized as a component of polar granules), and will not be discussed further. The other three grandchildless lines all contained P-elements on the second chromosome.

The chromosomal location of the P-elements in the three second chromosome grandchildless lines and the two female sterile lines fs 1 and fs 4 was obtained by using the P-lacW vector as a probe, as described in Hay et al (1988b). The sites of P-element insertion are given in table 1. Only vasa (35BC) is the site of any known mutation which affects pole cell formation.

The three grandchildless mutants complement each other as well as five other known mutations that affect pole cell formation and polar granule localization (tudor, oskar, valois, vasa, staufen) with the exception that A5 2nd 10 does not complement A2 2nd 27. These mutations also complement the second chromosome female-sterile mutations fs 1 and fs 4, which do not complement each other (V. Siegal, unpublished). The phenotypes of A5 2nd 10 and A2 2nd 27 are similar (described below). Thus it is likely that these are different alleles of the same locus. The similar phenotypes, and the same chromosomal localization make it likely that fs 1 and fs 4 are also allelic. Thus, it seems likely that three new loci that affect function of the germline have been isolated in this screen. E3 2nd 24 is uncovered by deficiency Df(2L) GpdhA (25D7-E1;26A8-9) (Kotarski et al. 1983), where the P-element maps. In trans to the deficiency chromosome the phenotype of the P-element line becomes more severe (described below). Thus it is likely that the P-element insertion is the cause of the mutant phenotype. Deficiencies or translocations are not available for the other 2nd chromosome P-element insertion sites. Thus it is not clear at this time whether the genes affected are at the site of P-element insertion.

A preliminary phenotypic characterization of the second chromosome grandchildless lines is presented below.

E3 2nd 24

The phenotype of the mutant E3 2nd 24 is complex. Ovaries from homozygous females are usually smaller than those from comparable wildtype controls (Fig. 4.1A). Many oocytes do not develop to

maturity. Eggs which are laid are often smaller than wildtype, have droopy and small chorionic appendages, and are often collapsed. Only about 5-10% of the fertalized eggs hatch. (Fig. 4.1B). In females in which the P-element chromosome is in trans to the deficiency Df(2L)GpdhA, which brackets both sides of the P-element insertion, the ovaries are smaller yet and only rarely give rise to laid eggs. These eggs never hatch. Usually they are not fertilized. Cuticle preparations of embryos from homozygous or transheterozygous females do not show any consistent abdominal segment deletions. In addition, transheterozygous males are sterile. Dissection of adult males shows that the gonads either fail to develop or are absent. When during the development of the germline this defect arises is unknown.

One use of P-element vectors is to search for cis-acting sequences which confer tissue-sepcific expression of a B-galactosidase (lacZ) fusion gene driven by the weak promoter of the P-element transposase (O'Kane and Gehring, 1987; Ghysen and O'Kane, 1989; Bier et al. 1989). In several cases involving P-element insertions near known genes the lacZ expression pattern approximates that of the pattern of gene expression (elav,Robinow and White, 1988; cyclin A, Lehner and O'Farrell, 1989, H. Vaessin and E. Giniger, in preparation; yasa, B. Hay, unpublished; rhomboid, Bier et al. in preparation). Since the P-element lines in this study all show a mutant phenotype we thought it reasonable that these elements would be near the genes being affected and thus the lacZ pattern might tell us something about the site of action of the genes being affected.

During embryogenesis E3 2nd 24 does not show a lacZ expression pattern. During oogenesis, however, a rather intriguing pattern of expression is seen. lacZ staining is present in some nurse cells throughout oogenesis. It is also expressed in two small subsets of follicle cells; one group of cells, known as the border cells, are located at the anterior pole. A second group of follicle cells is located at the posterior oocyte pole. (Fig. 4.1C). The border cells at the anterior pole are thought to form the micropyle, which forms the sperm entry channel. Those located at the posterior pole form a structure known as the aeropyle, function unknown. During spermatogenesis lacZ expression is seen in cells throughout the male testis, including what appear to be both germline and somatic cells. Ovaries from homozygous females show much more lacZ expression, both in cells that are expressing in the heterozygous condition, and in other follicle and nurse cells (Fig. 1D). In figure 4.1D it is also apparent that there are very few normal looking egg chambers. Most are arrested in early stages of oogenesis.

Anti-vasa antibody staining in E3 2nd 24 shows a variable phenotype. During oogenesis vasa is present in nurse cells but this immunoreactivity rarely becomes localized to the posterior pole (data not shown). During embryogenesis pole cells are seen to form in a few, but not in most embryos (Fig. 4.2A). In those embryos which do form pole cells, at least some of these migrate appropriately to the site of the embryonic gonad (Fig. 4.2B, C). Along the way, however, many pole cells can be seen that have an abnormal morphology and many immunoreactive anucleate blebs are present (Fig. 4.2B). Since all embryos from homozygous females that

hatch give rise to sterile adults, pole cells that are formed must die or become incorporated into other tissues at some point. When this occurs is unknown.

A5 2nd 10 and A2 2nd 27

The phenotypes of the mutants A5 2nd 10 and A2 2nd 27 are very simil and will be discussed together. Ovaries and testis from homozygous mutant females and males appear morphologically normal. As noted above, deficien are not available for these loci and thus we do not know whether the pheno of either line is close to the null phenotype, or whether the phenotype is ass with either P-element.

There is no lacZ expression pattern above background during embryogen oogenesis in either of these mutants. Staining of the two mutants with anti-v antibodies shows a variable phenotype. vasa is present in nurse cells and localized to the posterior oocyte pole during the second half of oogenesis. Following fertilization vasa immunoreactivity remains localized to the posterior pole during cleavage divisions (Figure 4.1B), as in the wildtype situation (Fig. 4.1A). In some embryos of both lines pole cells form, though there are usually many fewer (less then 10) than in the wildtype case (X = 40; chapter 1) (Fig. 4.3C, D)). Observation of these pole cells during early embryogenesis, at blastoderm formation, shows they are already somewhat morphologically different from wildtype pole cells (Fig. 4.3E, F), being somewhat smaller than wildtype pole cells, and more lightly labeled with anti-vasa antibodies.. As with the line E3 2nd 24, pole cells that do form do not contribute to the germline since the mutant is 100%

contribute to the germline since the mutant is 100% penetrant for the grandchildless phenotype. By stage 14 of embryogenesis, when the pole cells have become surrounded by mesoderm in the embryonic gonad, clear differences in the morphology of these cells as compared to wildtype pole cells can be seen (Fig. 4.3G-J). The mutant pole cells are comparatively weakly labeled with anti-vasa antibodies; they are tightly associated with each other, whereas pole cells are typically not in extensive contact with each other, and they are small and often somewhat elongate, whereas pole cells are large and round. When exactly these cells lose their potential to contribute to the germline is unknown. Interestingly, essentially all fertilized embryos hatch and give rise to viable larvae. Cuticle preparations from embryos that did not hatch do not show any of the abdominal segment defects associated with members of the maternal-effect-gap mutants described in chapter 3.

DISCUSSION

Using P-element mutagenesis we have identified three new loci which affect the initial formation of pole cells. A discussion of some aspects of the two grandchildless mutations on the second chromosome is presented below.

The mutant E3 2nd 24 has a variable phenotype in which the ovaries are small, some egg chambers fail to develop to maturity and eggs that are laid show various defects. In trans to a deficiency that deletes both sides of the P-element insertion site, the defects observed during oogenesis become more pronounced and male germ line development becomes arrested or terminated at an early stage. This suggests that the grandchildless phenotype is a hypomorphic condition. Embryos that lack a functional polar plasm but develop normally otherwise probably represent one end of a spectrum of phenotypes in which a failure to complete oogenesis (or in the case of males, spermatogenesis) is the null phenotype. This mutant may well be a case of a locus in which the posterior pole determinant system is the region of the embryo most sensitive to temporal and spatial expression of a gene function which is required for other aspects of normal development.

The relationship of the lacZ expression pattern of E3 2nd 24 to the phenotype observed is not straightforward. The large amount of lacZ expression seen in the testis is consistent with a strong mutant phenotype in the male germline. The strong phenotype seen during

oogenesis may be somewhat suprising in light of the limited lacZ expression pattern seen, largely limited to follicle cells located at the anterior and posterior oocyte pole, with some expression in a few nurse cells. At this point we have no obvious explanation for this apparent discrepency except to note that the lacZ expression pattern seen in the homozygous female is quite different from that in the heterozygote, suggesting that perhaps the P-element insertion in this line is, in the absence of a wildtype chromosome, somehow giving rise to an abberent quantitative and spatial pattern of nearby gene expression. That this may be the case is indicated by the observation that when the P-element chromosome is placed in trans to a deficiency for the region, the lacZ staining pattern is like more that of the homozygous mutant than the heterozygote in trans to a wildtype chromosome. Clearly, a genetic and molecular analysis of this region will be required to figure out what the effect of the P-element insertion is, and what the phenotype is of animals which are null for the gene(s) affected.

The identification of a locus (E3 2nd 24) that affects pole cell formation whose expression pattern may include the posterior follicle cells is intriguing from the standpoint of the question as to how anteroposterior polarity becomes set up in the oocyte. Perhaps the posterior follicle cells play a role in this process. Gap junctions are present between follicle cells and nurse cells, as well as the oocyte, providing a potential pathway for signaling to occur (Mahowald and Kambysellis, 1976). Other cell surface receptors which could be involved in intercellular interactions are also likely to be present. The recent demonstration that the Drosophila epidermal

growth factor receptor is required in follicle cells for proper oocyte development provides one example of such a case (Price et al. 1989; Schejter and Shilo, 1989).

The lines A5 2nd 10 and A2 2nd 27 (hereafter referred to as gc 1 for convenience, based on the complementation data suggesting the mutations affect the same locus) are particularly interesting because they are essentially 100% grandchildless--the progeny of homozygous females are always sterile. However, in looking at the embryos from homozygous females, some embryos are seen to form pole cells which migrate to the embryonic gonad late during embryogenesis. Since essentially all fertilized embryos hatch as sterile larvae the pole cells that are formed must at some point die or become incorporated into other tissues. We do not know which of these occurs. The abdominal segment deletions characteristic of members of the maternal-effect-gap mutant class are not present in embryos which do not hatch from these lines. However, because we do not have deficiencies which uncover the locus we cannot rule out the possibility that these lines are hypomorphic mutations which would display the abdominal segment deletion phenotype in the null condition.

The twograndchildless mutants E3 2nd 24 and gc 1 are distinct from members of the maternal-effect-gap class in that pole cells do sometimes form in the grandchildless mutants, whereas they do not form in embryos from strong alleles of the maternal-efect gap class of mutations. The observation that these pole cells do not contribute to the adult gonad suggests that the gene functions identified by these mutants are required following pole cell formation for some

aspects of maintenance of the pole cell phenotype. There is no paternal rescue of these mutants, however, indicating that whatever maintenance function is provided must have a maternal component.

Could theses grandchildless mutations, particularly gc 1, be identifying loci which are directly involved in pole cell determination? The evidence currently available is consistent with this possibility, the primary observation being that both lines that have the gc 1 grandchildless phenotype appear to only affect pole cell formation, or in cases where pole cells do form, their subsequent fate. Other embryonic structures are normal and the hatch rate from homozygous mutant females essentially wildtype. As noted above, however, we have no idea what the null phenotype of the locus identified by these mutants is. From the fact that many embryos do not form pole cells, we suspect, but have not proven, that a strong mutant would be completely unable to form pole cells. What other defects such a null mutant might have is unknown. One simple possibility is that maternally synthesized gene products from the affected locus, which is only required maternally for pole cell determination, is localized to the posterior pole during oogenesis, becomes active following fertilization, and in some unknown way initiate a program of pole cell development. Mutations in such locus would only affect pole cell formation. At this point, however, we have no reason to rule out the possibility that elements required for pole cell determination are also required zygotically and that mutations in such loci would thus be zygotic lethals. Defining the role of the loci identified by the grandchildless mutations described will clearly require more genetic and molecular analysis.

Table 1

Line	Site of P-element Insert
E3 2nd 24	26A
A5 2nd 10	26C (allelic to A2 2nd 27?)
A2 2nd 27	59A
A5 3rd 9	35BC (vasa)
fs 1	47B
fs 4	47B

Figure 4.1 Morphology of E3 2nd 24 ovaries, embryos, and lacZ staining pattern. (A) Wildtype ovaries (wt) are compared to comperably aged homozygous E3 2nd 24 ovaries. (B) Morphology of wildtype compared to E3 2nd 24 eggs. Note the small size and droopy chorionic appendages of the E3 2nd 24 eggs. (C) lacZ expression pattern of E3 2nd 24 heterozygotes. Note expression in the border cells at the anterior oocyte pole (arrow) and the posterior pole follicle cells (arrowhead) at around stage 10 of oogenesis. (D) lacZ expression pattern of homozygous E3 2nd 24 ovaries. Much more expression is seen than would be expected simply from doubling the dose. Also note that there are very few egg chambers that have advanced to stage 10 of oogenesis. Many egg chambers look abnormal.

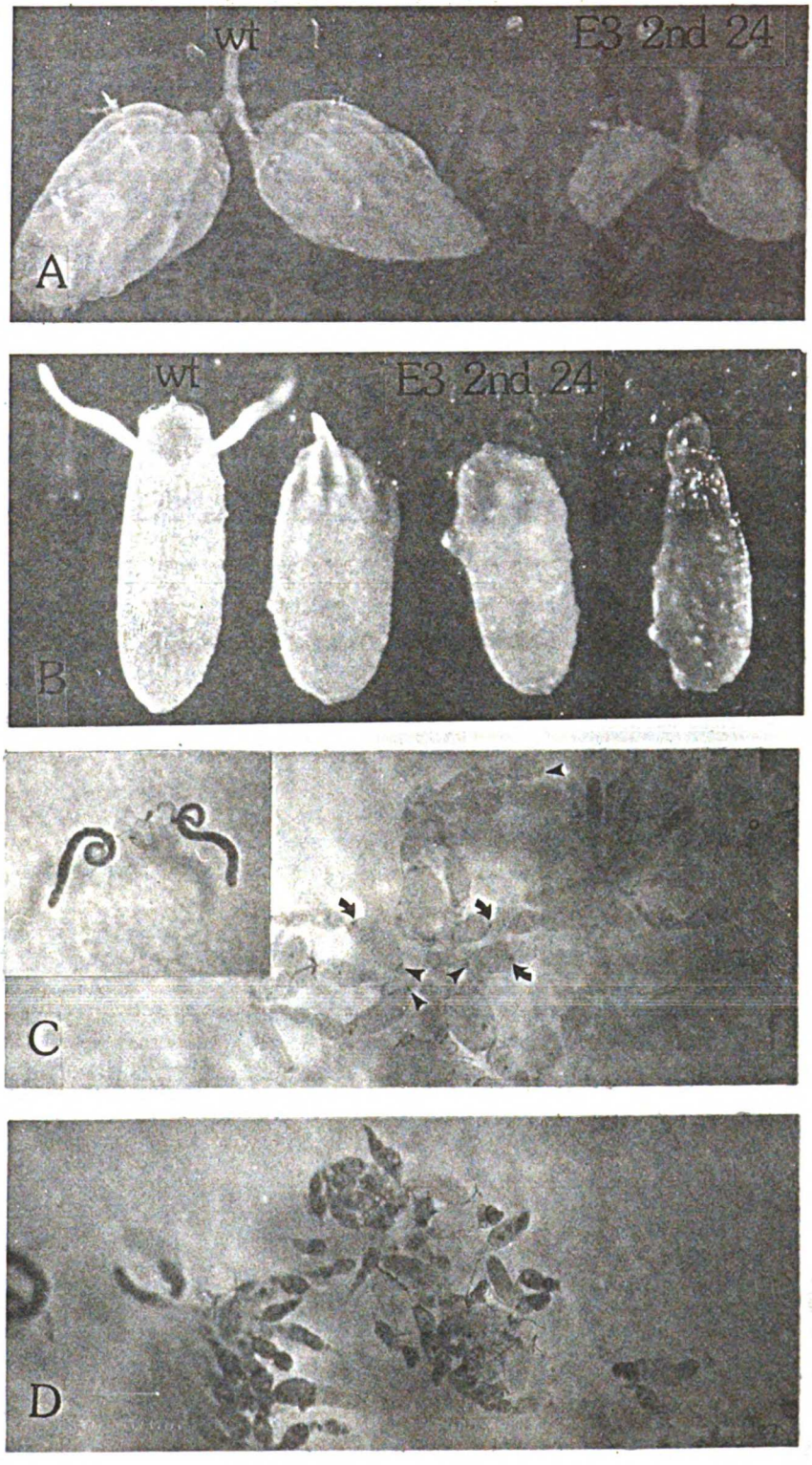
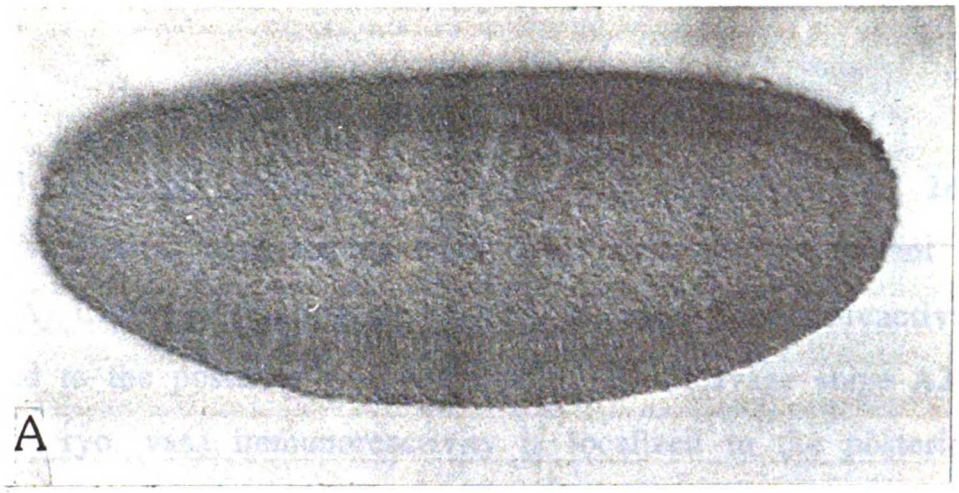
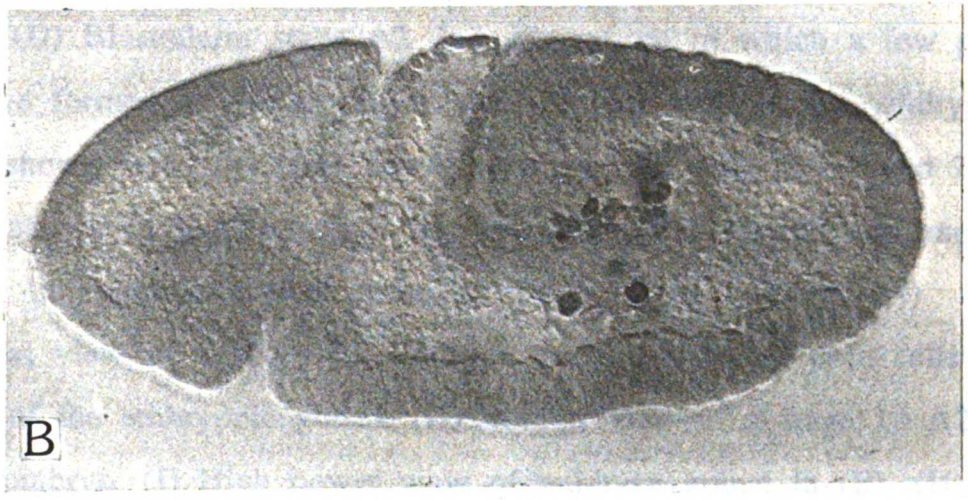

wt
E3 2nd 24
A
wt
E3 2nd 24
B
C
D

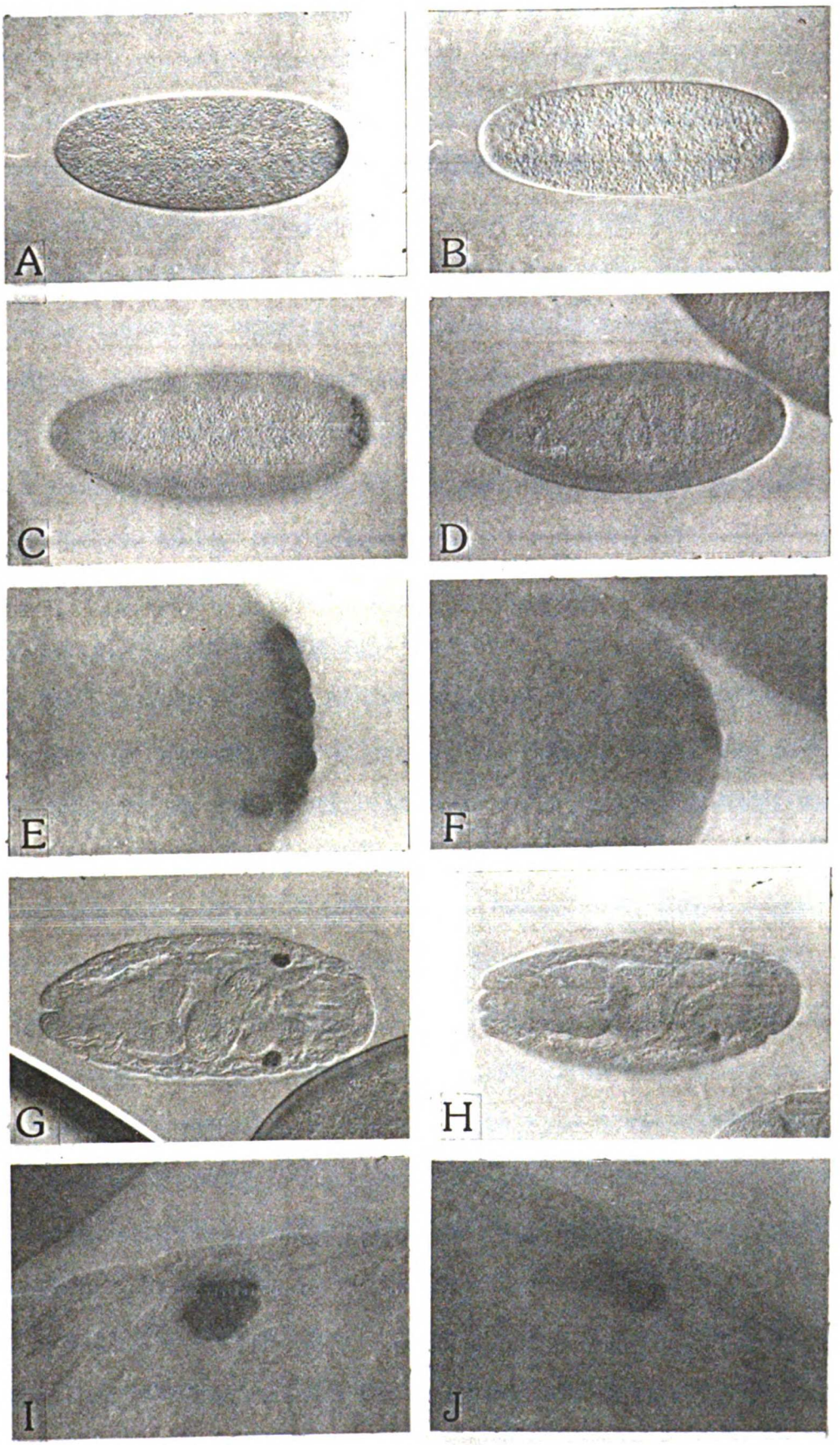
Figure 4.2. Anti-vasa staining of eggs from homozygous E3 2nd 24 embryos. (A) Blastoderm stage embryo which has formed a few pole cells. (B) Embryo during germ band extension. Pole cells are in the midgut and beginning to migrate through it. A few pole cells are present. There are also a number of immunoreactive anucleate blebs in and around the midgut. (C) Stage 13 embryo in which some pole cells have migrated to the site of the embryonic gonad. Lost cells are also apparent. These cells look nore or less normal.


A


B


C

Figure 4.3. Anti-vasa staining of embryos from homozygous A2 2nd 27 females. Similar observations have been made for the mutant A5 2nd 10. (A) Cleavage stage wildtype embryo. vasa immunoreactivity is localized to the posterior embryonic pole. (B) Cleavage stage A2 2nd 27 embryo. vasa immunoreactivity is localized to the posterior pole. (C) Blastoderm stage wildtype embryo in which pole cells have formed. (D) Blastoderm stage A2 2nd 27 embryo in which a few pole cells have formed. (E) High power view of pole cells in the wildtype embryo shown in C. (F) High power view of pole cells in A2 2nd 27 embryo shown in D. Note that they are somewhat smaller and more irregular in appearance than those of the wildtype embryo. (G) Horizontal view of a stage 14 wildtype embryo showing pole cells at the site of the embryonic gonad. (H) Similar view of a stage 14 A2 2nd 27 embryo. (I) High power view of wildtype gonad in (E). (H) High power view of A2 2nd 27 gonad in (J). Note the decreased staining intensity relative to wildtype and the small, irregular size of the labeled cells.


A
B
C
D
E
F
G
H
I
J

FUTURE DIRECTIONS

A number of genetic loci (tudor, oskar, staufen, vasa, valois, cappuccino, spire, fs1-4, E3 2nd 24, nanos and pumilio) have been identified that have a consistent phenotype that results from an effect on the localization and/or function of the posterior pole (abdomen and germline inducing) activity. A number of other loci have been identified which have a less well characterized, or weakly penetrant effect on the formation of these embryonic structures. What are these genes doing, and what is the basic job that the posterior pole cytoplasm is trying to perform?

Functions of the vasa gene product.

The primary finding of the present work is that the product of the vasa locus is a component of Drosophila polar granules and other germline-specific structures. Coupled with the observation that embryos from homozygous vasa females fail to form germ cells or abdominal segments (Schupbach and Wieschaus, 1986), this demonstrates that polar granules, or at least the vasa product, as a component of polar granules, is required for some aspect of the determinative functions of posterior pole cytoplasm. Construction of a female that contains a deletion for the vasa locus reveals that vasa also play a role early in oogenesis. Ovaries from such transheterozygous females rarely develop mature oocytes. Most egg chambers remain stuck in early stages of oogenesis.

The predicted product of the vasa locus suggests the protein is likely to function as a DNA or RNA helicase. Based on the sequence

homology vasa shows with eIF4A and p68, known RNA helicases, as well as several other proteins required for splicing (John Abelson, unpublished), it seems likely that it functions as an RNA helicase.

What is the function of the vasa product in the posterior determinant system? As discussed in Chapter 2, a role in RNA localization and/or translational regulation seem the most likely processes in which vasa may function. These points will be considered briefly in turn.

We know that localization of both the nanos and cyclin B transcripts to the posterior pole occur in a vasa-dependent manner. There is no evidence at present, however, that these mRNAs interact directly with the vasa product, or polar granules at all. In general, helicases bind and function in a sequence-nonspecific manner. An exception to this rule, however, is provided by the SV40 large T antigen, which binds specifically to double stranded DNA containing the SV40 origin of replication sequence. This binding is followed by activation of an asociated DNA helicase activity (Goetz et al. 1988). Thus there is no reason to rule out the possibility that vasa is functioning to directly bind and localize specific transcripts.

A second possibility is that vasa binding to specific RNA sequences is mediated through its first binding some other component, which has a high affinity for specific RNA sequences or structures. This would be comparable to the situation with translation initiation factor eIF4A. On its own, eIF4A will bind sequence-nonspecifically to single-stranded RNA and unwind double-stranded regions. However, as a part of the eIF4A initiation complex, which contains the cap binding protein eIF4E, eIF4A becomes specifically targeted to the 5'

end of mRNA, the site where it is thought to play a physiologically significant role. In this model, a second protein would function to bind and localize specific transcripts to the posterior pole, and vasa, as an associated part of this binding complex, might then be expected to function to regulate some aspects of the function of the bound transcripts.

How might vasa be acting to regulate the function of localized transcripts? Several obvious possibilities are 1) that vasa functions to release transcripts from the localization machinery, to allow their translation following fertilization, and 2) that vasa functions primarily to regulate the translatability of the localized transcripts. These two functions may be intertwined. It seems likely that it is important to prevent the translation of localized transcripts, such as those for bicoid and nanos, prior to fertilization. This is because mature oocytes may be held inside the female up to a week, prior to being fertilized, depending on the environmental conditions. The bicoid product (and probably also that of the nanos locus) functions to specify positional value along the body length through the production of a gradient of protein that regulates the level of expression of zygotic genes that determine the identity of particular body regions. Translation of these localized developmental regulators during oogenesis might result in inappropriate diffusion of the protein products, resulting in abberent development. In the case of bicoid such translational regulation occurs. Bicoid RNA becomes localized during the last half of oogenesis, but translation is not seen until following fertilization (Driever and Nusslein-Volhard, 1988a). A similar translational repression mechanism may exist in the case of nanos as well as

potential germ cell determinants, since both these activities become localized late during oogenesis. Thus, one might imagine that localization of transcripts as well as translational repression is achieved by interaction of a binding protein with a specific RNA secondary structure. Following fertilization some unknown signal might allow vasa to unwind this structure, releasing the transcript from its binding protein and making it available for translation. Alternatively, vasa might be functioning following fertilization primarily as a protein translation initiation factor, to increase the rate of translation of specific transcripts, or translation in general.

Testing the hypothesis that vasa functions through RNA binding

Given the association of RNA with polar granules it will be important to try and demonstrate RNA binding activity using purified vasa protein. RNA helicases described to date are sequence-nonspecific in their affinity for RNA. The vasa product may show a similar lack of specificity. Alternatively, it is possible that the vasa protein may only function to bind and unwind specific message sequences. Thus if helicase activity or RNA binding cannot be demonstrated using randomly chosen substrates, it will be important to try and devise a scheme whereby one might be able to identify such unique RNA species. One approach to this search would be to use vasa protein as an affinity matrix for isolating species of RNA that bind vasa from large RNA populations, such as ovarian or early embryo RNA. Obvious candidates for such unique RNA species are the transcripts of the nanos and cyclin B genes, which become localized to the posterior embryonic pole in a vasa-dependent

manner. If an RNA substrate for vasa binding and helicase action can be identified it will also be important to devise some tests of what vasa is doing with this RNA in-vivo. Given the observations that 1) polysomes associate with polar granules following fertilization, 2) that relatively large amounts of protein translation occur in polar plasm during the process of pole cell formation, and 3) that vasa has extensive homology with eIF4A, which plays an essential role in the initiation of protein translation, a good first hypothesis is that the vasa protein plays a role in the regulation of translation of specific maternal transcripts required for pole cell formation, and perhaps germ cell determination. Cell free protein translation systems have been developed from Drosophila embryos (Morato and Sierra, 1988). If mRNA does bind vasa, it will be interesting to see whether the presence of vasa protein has an effect on the translatability of the RNA. Alternative possible roles for vasa function as a component of polar granules are discussed below.

Localization of a ligand to the posterior pole during oogenesis is likely to require a number of different components.

As discussed in the Introduction and Chapter 3, localization of any macromolecular complex to a particular region of the egg will involve a number of components. In a simple system, a ligand, moving randomly, might bump into localized receptors often enough, and stick with a high enough affinity to account for localization to a particular region of the cell. This system requires, in addition to the ligand and the receptor, a mechanism for localizing the receptor to the posterior pole. Interestingly for this model, during the second

half of oogenesis, as the nurse cells are dumping their contents into the oocyte, cytoplasmic mixing occurs that might be expected to move large amounts of the oocyte contents past a particular point (Gutzeit and Koppa, 1982). In a more complex system, in which directed organelle transport occurs, a mechanism for coupling the ligand to the transport machinery, the transport machinery itself, as well as mechanisms for stabalizing the transport machinery so that it goes in the right direction, must exist. Which of these methods of determinant localization occurs at the posterior pole is unknown.

Functions of the grandchildless-knirps class of genes and Bicaudal-D in the determinative activity of posterior pole cytoplasm.

A clue as to the function of the genes that show a grandchildless-knirps type defect (failure to form abdomen and pole cells) is provided by study of double mutants with Bicaudal-D (Bic-D). Bic-D embryos contain abdominal determinants (the nanos transcript) localized inappropriately to the anterior embryo pole. In combination with Bic-D, vasa, valois, oskar, and tudor result in a supression of the bicaudal phenotype. In contrast, double mutants of Bic-D with staufen, cappuccino, and spire result in an increase in the bicaudal phenotype. One possibility consistent with these observations is that the products of loci in the first group (vasa, valois, oskar, and tudor) are required for providing an environment permissive for mislocalization of abdominal determinants, or an activity necessary for the function of these determinants in their anterior localization. These might be polar granule components or cytoskeletal elements that are required for function of a Bic-D-dependent localization mechanism, as well as

polar granule localization or stabilization at the posterior pole in normal embryogenesis. Staufen, cappuccino and spire, on the other hand, may define loci whose products are normally required for transport of these determinants to the posterior pole. In the absence of these gene functions abdominal determinants are still able to function (because vasa, valois, oskar and tudor activities are still present), but are mislocalized by a Bic-D-dependent mechanism.

With regard to this classification scheme, it would be very useful to know if the nanos transcript is localized (but not functional) in double mutants of Bic-D with oskar, tudor, vasa and valois, or if it is simply not localized. If it is localized but nonfunctional in a particular double mutant, this would suggest that the products of these genes are required for the function of the nanos transcript. A failure to see localization in double mutant combinations, on the other hand, suggests that these gene products are required for some aspect of the localization process.

What role does the Bic-D gene product play in normal oogenesis. The only clue available is that revertants of the Bic-D locus are recessive female steriles, in which 16 nurse cells and no oocyte are formed Mohler and Wieschaus, 1986). This suggests that one of Bic-D's functions during oogenesis is to provide a signal that distinguishes the oocyte from its sibling nurse cells. Since in the germarium the oocyte ends up on one side of the egg chamber and the nurse cells on the other, this signal may also be involved in establishing oocyte polarity.

A role for E3 2nd 24 in the function of the posterior activity.

All of the mutants which have been demonstrated to result in a loss of polar granules at the posterior pole are germline-dependent, which is to say that normal gene function is only required in cells of the germ cell lineage and not the somatic follicle cell lineage, for a normal egg to be produced. It is possible that information required for setting up anteroposterior polarity is all germline derived, set up perhaps when the oocyte is still in the germarium. It also may be, however, that follicle cells play some role in determination of anteroposterior polarity. That this might be the case seems particularly likely in light of experiments on dorsal-ventral embryo patterning which indicate that the oocyte and nurse cells communicate positional information to each other (see Introduction and Chapter 3).

Why have genes that effect anteroposterior polarity, which act in the somatic follicle cells, not been isolated. One possibility is that such genes do not exist. Another possibility is that, while such genes do exist, the phenotypes of mutations at these loci result in more general defects in oogenesis or viability (lethality), such that their effects on anteroposterior axis formation during oogenesis are not apparent. As discussed in Chapter 3, the P-element containing line E3 2nd 24 may identify such a locus, since as a homozygote, females show a female sterile/grandchildless phenotype, whereas females transheterozygous for the P-element and a deficiency for the P-element containing region are completely female sterile. The lacZ staining pattern of this line includes a concentration of staining in the most posterior follicle cells, consistent with a requirement for relatively high levels of gene expression in these cells. A gene such

as that identified by E3 2nd 24 might be postulated to act at the posterior pole, perhaps by sending a signal to the oocyte that effects the localization of the underlying receptors, or, if the receptors are generally distributed, the affinity of those receptors at the posterior pole, such that they are able to bind posterior determinant-associated molecules.

What are the germ cell determinants?

This discussion has focused primarily on the localization and function of abdominal determinants since, as opposed to germ cell determinants, much more is known about them. The question of what are germ cell determinants is no less interesting, however. As discussed throughout this work, a sort of guiding hypothesis in the field has been that polar granules are germ cell determinants, or are asociated with them. The discovery that the product of the vasa locus codes for a component of polar granules, and that mutants at this locus fail to form germ cells, provides strong support for this idea. A very simple hypothesis as to how germ cell determinants work is that these molecules function only maternally, to control germ cell determination early in embryogenesis. Mutants at such loci should be isolated as grandchildless: the progeny of homozygous females should be normal, except that they lack functional germ cells. The P-element line gc-1 displays such a phenotype. Thus it is possible that this line identifies the site of a gene directly involved in germline determination. If this model is correct one might imagine that the product of this locus would become localized to the posterior pole along with the nanos transcript during oogenesis, where it

subsequently becomes activated following fertilization. Alternatively, the determinative transcript might be dispersed throughout the embryo, but only become active at the posterior pole, in association with posterior pole cytoplasm constituents (the polar granules?).

A model in which the functions of the genes discussed is presented in figure 5.1. This model contains a number of steps, which include 1) genes for polar granule components (tudor, vasa) which are required for localization and function of the determinants, 2) genes for elements which may be polar granule components or molecules required for their proper localization or stabilization of this localization (valois, oskar), 3) genes for elements required for transport to the posterior pole (staufen, cappuccino, spire), 4) genes for follicle cell proteins that directly or indirectly provide a posteior-specific influence on the underlying oocyte (E3 2nd 24), and 5) the determinants themselves (nanos, gc-1).

Deriving a model for the functions of genes involved in the function of the posterior cytoplasm has been difficult because for most of these, members of the grandchildless-knirps group, it has not been possible to establish epistasis relationships between the genes in the pathway, using cytoplasmic transplantation or genetic means. Thus, in double mutant combinations of grandchildless-knirps mutants with each other the pole cell and abdominal segment deletion phenotype is always that of the strongest mutant (Schupbach and Wieschaus, 1986), and posterior pole cytoplasm from any particular mutant will not rescue any of the other mutants (Ruth Lehmann, unpublished). These observations suggest that all the grandchildless-knirps group genes function together in the

production of a single output. The absence of polar granules in all these mutants is consistent with this idea. Our analysis of vasa localization in different grandchildless-knirps mutants is an attempt to extract more information from these mutants with regard to what steps exist in the pathway to polar granule function, and which mutants are blocked at what steps. As discussed in Chapter 3, our interpretation of this data is quite limited by the facts that we are following the fate of only one product, whose function and physical relationship to the other components is not clear, and that the mutants we are studying are hypomorphic. Thus far, the only feature that clearly distinguishes members of the grandchildless-knirps class from each other is their interaction with Bic-D.

Testing this model will obviously be greatly facilitated by cloning of the genes associated with these mutations, and the production of antibodies to their products. Homologies to RNA binding proteins, cytoskeletal proteins, or membrane proteins will be useful in creating more informed guesses as to where different genes fit in the model. Production of antibodies to these gene products will be particularly usefull, in that it will tell us which products are polar granule components, which are distributed throughout the cell, and which are localized to the posterior pole and thus may be acting as specific receptors. At this point in time it seems likely that this sort of a brute-force molecular analysis of the gene products involved, in which the physical location of these products in wildtype and single and double mutant backgrounds is directly observed, is the most rapid path to understanding the mechanisms by which the posterior activity becomes localized and functional.

What are polar granules, nuage and nuclear bodies, and why might they be conserved in the mature germline over a wide phylogenetic range?

Given the observation that polar granules, nuclear bodies and nuage are recognized by anti-vasa antibodies, and that vasa, probably a structural component of the granules, is likely to function in some context as an RNA helicase, it seems likely that these structures have something to do with RNA metabolism. In thinking about these structures do it is important, though perhaps inhibitory to the imagination, to remember that structures similar to nuage, nuclear bodies, and polar granules have only been seen in the germ cell lineage of organisms in which they have been identified. Thus one wants to ask, what is the germline doing differently that it needs these big blobs floating around in its cytoplasm and nucleus for? Things that germline cells do differently from most cells is to synthesize and store large amounts of RNA for future use and undergo meiosis. A potential role of these structures in meiosis will not be discussed further.

One possibility, already discussed extensively above, is that the granules are involved in RNA storage and translational regulation of associated transcripts. In this context the presence of granular material on the cytoplasmic face of the nuclear envelope of nurse cells and spermatocytes is significant, since this is the location at which one could control access of transcripts to the cytoplasmic translation machinery. What other sorts of proteins might be associated with the granule in a translational regulation scenario? These might include other RNA binding proteins that function to

prevent access to the translation machinery. As discussed in Chapter 2 and Davidson (1986) there is a general inhibition of translation in unfertilized versus fertilized eggs, and translational repression is also likely to be important during spermatogenesis (Iatrou and Dixon, 1977). Unfortunately there are no proteins isolated which are good candidates for being involved in translational repression in these systems, or in Drosophila. Thus, demonstrating that the granules play some role in this process, short of isolating the relevent transcripts and adding them to purified polar granules in an in-vitro translation system, seems unlikely. Alternatively, these structures might function, not primarily as storage sites for mRNA, but rather as storehouses of translation initiation factors which are required for the high levels of translation that occur in germline cells such as nurse cells, pole cells early in embryogenesis, and spermatids. If this hypothesis is correct, one might hope to use antibodies directed against Drosophila translation initiation factors or conserved epitopes of mammalian initiation factors, and see if these antibodies recognize these structures. If the granules contain eIF4E, the cap binding protein, one should also be able to enrich for them (and thus the vasa protein, for which antibodies exist) by affinity chromatography on 7-methyl-G agarose.

A second hypothesis is that the granules, in particular the nuage and the nuclear bodies, might play some role in RNA processing, perhaps premRNA splicing or assembly of snRNPs in the nucleus. Recently Gall and Gant (1989) have described structures they call spheres in the nuclei of oocytes from a number of different animals, including newts, frogs, spiders and crickets. These spheres are large

nuclear organelles, distinct from nucleoli. Interestingly, they stain with antibodies specific for the Sm antigen of snRNPs, and a monoclonal antibody (mAB K121) specific for the trimethylguanosine cap found on the major snRNAs, suggesting that these organelles contain snRNPs. They propose that the sphere organelle might be the site of assembly of spliceosomes, comparable to the way in which the nucleoli assembles ribosomes for the cytoplasm. The Drosophila oocyte nucleus does not show any anti-vasa staining or polar granule like structures, but this is perhaps not suprising since the oocyte nucleus is relatively inactive (Mahowald, 1968). The nurse cells show lots of punctate anti-vasa staining on the cytoplasmic face of the nuclear envelope. We have not seen any nuclear immunoreactivity associated with these cells. Large nuclear bodies are present in pole cells during the first half of embryogenesis and in spermatocytes, however. Why there should be such large amounts of spliceosome assembly in pole cells that macroscopic granules are seen in these cells, but not in the surrounding blastoderm cells, is unclear. Perhaps polar granules function to store transcripts which are unspliced. Following fertilization, after the cleavage nuclei have moved into the posterior pole cytoplasm, these premRNA-RNP particles move to the nucleus where splicing and subsequent export for translation occurs. This does seem like sort of a cumbersome strategy for keeping specific transcripts out of the translation pool when simply postulating a translational repressor protein would accomplish the same purpose.

During early spermatocyte stages increased synthesis of splicing components makes some sense, since these cells are involved in rapidly growing and storing mRNA for use during the spermatid

stage. Perhaps assembly of splicing machinery or loading of these complexes on pre-mRNA is rate-limiting in these cells, and thus large structures, not normally seen in less active cells, are now visible. In any case, because both the Sm antigen and trimethylguanosine cap are evolutionarily conserved epitopes it should be straightforward to look for the presence of snRNP components in the germline granules using these antibodies.

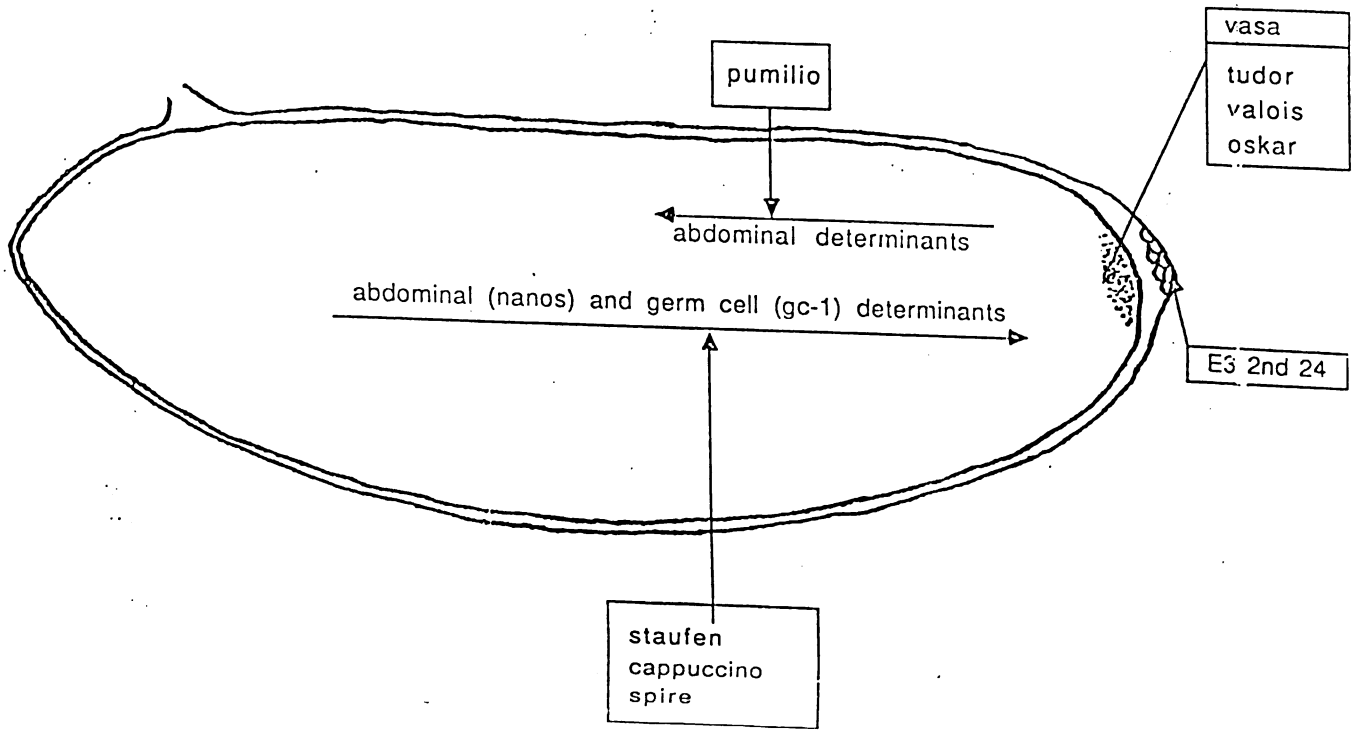
From the above discussion it is clear that the extent of our knowledge of about polar granules, nuage or nuclear bodies is quite small. The total of this knowledge can be summed up by saying that they appear to be germline-specific structures, the Drosophila melanogaster structures all contain the vasa product, and similar structures can be found in the germline of phylogenetically diverse groups. Which cellular processes these structures are important for is unknown, except that it probably has something to do with RNA processing, mRNA localization or translation.

Figure 5.1. Model for the function of genes involved in abdomen specification and germ cell determination. This model schematically illustrates steps at which genes that are important for the determinative functions of the posterior pole cytoplasm may be acting. Shown is a stage 14 oocyte, with anterior located to the left, and dorsal up. Based on observations described in the text the product of the nanos locus is thought to be responsible for abdomen determination; the product of the gc-1 locus is important for germ cell determination. Pumilio function is thought to be required for the transport of the nanos product from the posterior pole to the more anterior site of the prospective abdomen. Note that while the figure is meant to represent processes occuring during oogenesis, pumilio function does not become apparent until following fertilization.

Members of the grandchildless-knirps group of mutations have been divided into two classes, based on their interactions with the dominant mutation Bic-D, which causes mislocalization of the nanos transcript to the anterior pole, and anti-vasa stainings of wild-type embryos, and embryos from mothers homozygous mutant at these loci (see text for details). Based on this information, staufen, cappuccino, and spire are proposed to play a role in the transport of determinants to the posterior pole. Vasa, tudor, valois, and oskar, are proposed to be polar granule components, or molecules in some other way required for the localization or stabilization of localization of polar granule components. The vasa protein is known to be a polar granule component.

The function of the locus identified by the P-element containing line E3 2nd 24 may be required in follicle cells. In particular, high levels of lacZ staining in follicle cells at the posterior oocyte pole in this line suggest that the locus identified might be important for communicating positional information to the underlying oocyte posterior pole.

Model for the function of genes involved in abdomen specification and germ cell determination



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