Distribution of tudor protein in the Drosophila embryo suggests separation of functions based on site of localization

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SUMMARY

Mutations in the tudor locus of Drosophila affect two distinct determinative processes in embryogenesis; segmentation of the abdomen and determination of the primordial germ cells. The distribution of tudor protein during embryogenesis, and the effect of various mutations on its distribution, suggest that tudor protein may carry out these functions separately, based on its location in the embryo. The protein is concentrated in the posterior pole cytoplasm (germ plasm), where it is found in polar granules and mitochondria. Throughout the rest of the embryo, tudor protein is associated with the cleavage nuclei. Mutations in all maternal genes known to be required for the normal functioning of the germ plasm eliminate the posterior localization of tudor protein, whereas mutations in genes required for the functioning of the abdominal determinant disrupt the localization around nuclei. Analysis of embryos of different maternal genotypes indicates that the average number of pole cells formed is correlated with the amount of tudor protein that accumulates in the germ plasm. Our results suggest that tudor protein localized in the germ plasm is instrumental in germ cell determination, whereas nuclear-associated tudor protein is involved in determination of segmental pattern in the abdomen.

Key words: germ plasm, abdominal segmentation, embryogenesis

INTRODUCTION

The early development of multicellular organisms involves a programmed sequence of events by which cells become restricted to specific fates. In Drosophila and other organisms, potential cell fate differences in the egg, where maternally encoded gene products are localized to specific cytoplasmic regions during development of the oocyte (Frohnhofer et al., 1986; St. Johnston and Nüsslein-Volhard, 1992). These molecules provide localized information for zygotic gene expression, and thus the initiation of pattern development in the embryo.

Instruction of zygotic gene expression along the antero-posterior axis in the Drosophila embryo requires maternally derived factors that are localized at the egg poles. Information provided by the anteriorly localized bicoid RNA allows for development of anterior structures (head and thorax; Frohnhofer and Nüsslein-Volhard, 1986; Berleth et al., 1988), whereas posteriorly localized molecules direct formation of abdominal segmentation and germ-line development (Fröhnhofer et al., 1986; Lehmann and Nüsslein-Volhard, 1986, 1991; Wang and Lehmann, 1991). Several maternally encoded gene products have been identified that contribute to the proper functioning of the posterior pole plasm, both for its germ cell determination function and for its role in establishing pattern in the embryonic abdomen (tudor, staufen, valois, vasa, oskar, cappuccino, spire, and mago nashi; Boswell and Mahowald, 1985; Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986; Manseau and Schüpbach, 1989; Boswell et al., 1991). Two additional loci, pumilio and nanos, are required for the formation of the embryonic abdomen but not for germ cell formation (Lehmann and Nüsslein-Volhard, 1987; Lehmann and Nüsslein-Volhard, 1991). These ten loci form a class of maternal-effect genes termed the posterior group, and are said to affect the functioning of the ‘posterior organizing center.’

It has been postulated that all posterior group genes affect the same signal for abdominal segmentation, encoded by the nanos locus, whose transcript becomes localized to the posterior pole cytoplasm by the time of egg deposition (Wang and Lehmann, 1991). Transplantation experiments suggest that nanos activity spreads anteriorly from this localized source, and is capable of inducing abdominal structures (Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991). The wild-type function of pumilio was initially reported to be required for the transport of nanos protein to the prospective abdominal region (Lehmann and Nüsslein-Volhard, 1987), but more recently has been shown to be essential for nanos function outside of the posterior pole plasm (Barker et al., 1992). In contrast, all other...
posterior group genes have been implicated in posterior nanos localization and/or function. The posterior organizing center, presumably through the action of nanos, allows for abdominal development through a negative posttranscriptional interaction with maternally encoded hunchback product (Hülskamp et al., 1989; Irish et al., 1989; Struhl, 1989; Wang and Lehmann, 1991; Wharton and Struhl, 1991). Nanos protein spreads in a graded fashion from the posterior pole (Gavis and Lehmann, 1992; Smith et al., 1992), repressing translation of hunchback mRNA, and thereby preventing the accumulation of hunchback protein in nuclei in the posterior of the embryo. Hunchback protein therefore forms a reciprocal gradient, decreasing in concentration in nuclei from the anterior of the embryo to the posterior. The change from a uniform distribution of mRNA to a nuclear gradient of protein is the result of the activity of the posterior group genes (Tautz, 1988), through their effect on the functioning of the posterior organizing center. The hunchback protein gradient serves to organize the transcription domains of other gap genes in the embryo, specifying abdominal development (Hülskamp et al., 1990; Struhl et al., 1992).

This paper describes the distribution of tudor protein in the developing oocyte and embryo, and discusses the implications of this distribution for the possible role of the tudor locus in the functioning of the posterior organizing center. Mutations in tudor are associated with an allele-specific reduction in the amount of assembled polar granule material present in the posterior pole cytoplasm, and result in the loss of the ability of this cytoplasm to induce the formation of the primordial germ cells. The severity of the defect in the germ plasm (the amount of polar granule material), can be correlated with the severity of abdominal patterning defects caused by the same allele (Boswell and Mahowald, 1985). These observations implicate tudor, along with most genes of the posterior group, in a process common to both germ cell determination and specification of the embryonic abdomen. Here we describe the localization of tudor protein in the egg and developing embryo, and determine the effect of mutations in posterior group genes on this localization. We show that the accumulation of tudor protein at the posterior pole requires the activity of all posterior group genes affecting germ cell determination. The presence of tudor protein in the posterior pole plasm (the germ plasm) is correlated with the ability of this cytoplasm to induce germ cell formation. Tudor protein is also found around nuclei in wild-type embryos, but is not associated with nuclei in embryos that fail to form abdominal segments due to mutations in posterior group genes. The association of tudor protein with nuclei is apparently related to determination of segmental pattern in the abdomen. Our results suggest that the tudor protein carries out distinct developmental functions that allow these two processes to diverge.

MATERIALS AND METHODS

Fly strains and egg collections

The wild-type stock for all experiments was Oregon R. Mutant stocks are listed in Table 1. For collections of embryos from mutant mothers, homozygous mutant females were collected and crossed with wild-type males for 4 days while feeding on wet yeast and molasses. Eggs were collected on molasses-agar plates for the designated time periods.

Production and purification of fusion protein and antiserum

A 947 bp PvuII-EcoRI fragment of tudor (tud) cDNA clone 9A1 was subcloned into the expression vector pGEX2T (AMRAD Corp). The resulting plasmid encodes a hybrid protein consisting of the C-terminal portion of glutathione S-transferase (GST; Smith and Johnson, 1988) fused to a internal fragment of the tud protein (amino acids 886-1199; 35 × 10^3 M_r). This protein was expressed in E. coli following induction with IPTG, and was isolated from other bacterial proteins by affinity adsorption to glutathione-agarose beads (Sigma). The fusion protein was further purified by SDS-PAGE, followed by excision of the protein band and electroelution in an ELUTRAP device (Schleicher & Schuell). The eluted protein was dialyzed against phosphate-buffered saline (PBS) and stored at −20°C.

Rabbits were bled repeatedly for pre-immune sera, and injected twice at two week intervals with 0.5-1.0 mg purified fusion protein emulsified with incomplete Freund’s adjuvant. Rabbits were bled after 8 weeks, and bled weekly beginning at week 10. Rabbits were housed, immunized, and bled at Pocono Rabbit Farm and Lab, Canadensis, PA. Serum was stored at −20°C until use, at which point any unpurified serum was stored at 4°C with 0.02% sodium azide.

Immunoblotting

Protein extracts were prepared from ovaries and staged embryos by homogenization in extraction buffer (2.5% SDS, 0.1 M Tris-HCl, pH 6.8, 10 mM EDTA) with the addition of protease inhibitors (2 mM-phenylmethylsulphonyl fluoride (PMSF), 10 μM leupeptin, 10 μM pepstatin, and 10 mM sodium metabisulfite). An equal volume of 2× gel loading buffer (120 mM Tris, pH 6.8, 2% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.01 mg/ml bromophenol blue) was added, the samples were boiled for 5 minutes and spun at full speed for 2 minutes in an Eppendorf microfuge. 7.5% SDS-PAGE gels were run according to Laemmli (1970), using a modified transfer buffer for high molecular mass proteins (50 mM Tris, 380 mM glycine, 0.1% SDS (w/vol.), 20% methanol). Blots were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl, pH 7.6) with 0.1% Tween 20, and all subsequent incubations and washes were carried out in this solution.

Antibody staining of tissues

Embryos were collected in microcentrifuge tubes, washed with distilled water, dechorionated for 2 minutes in 50% bleach, and rinsed three times with distilled water. They were then fixed in 1:1 heptane: formaldehyde fix (100 mM Hepes, 2 mM MgSO4, 1 mM EGTA, 4% formaldehyde) for 20 minutes at room temperature on a rocking platform. The embryos were devitellinized by vigorous...
shaking in 1:1 heptane:methanol, followed by several rinses in absolute methanol. Embryos were used immediately for antibody staining, or stored in methanol at −70°C.

Embryos were taken from methanol to 1:1 methanol:PBT (PBT = PBS+0.1% Tween 20), then into PBT. Following several rinses in PBT, embryos were blocked for 3 hours at room temperature in PBT. Affinity purified α-9A1 antibody preparation was diluted 1:20 in PBT, and embryos were incubated in this solution overnight at 4°C on a rocking platform. Washes in PBT were performed over a 3-5 hour period, involving at least five changes. The secondary antibody was used was Texas Red-conjugated donkey anti-rabbit IgG (Amersham). Incubations were for a period of 3-5 hours at room temperature, or overnight at 4°C. Final washes were in PBT (at least 5 changes over 3-5 hours at room temperature). Embryos that were double-labeled with α-9A1 and a monoclonal antibody to histones (Chemicon International, Inc.) were treated simultaneously with both the Texas Red-conjugated anti-rabbit IgG, and a fluorescein-conjugated goat anti-mouse IgG (Sigma). For DAPI staining, embryos were incubated in 0.1 µg/ml solution of DAPI in PBT for 5 minutes at room temperature in the middle of the washing procedure. For mounting, samples were taken through a glycerol series (30%, 60% and 90% glycerol) and then were mounted in 90% glycerol with 2.5% n-propyl gallate as an anti-fading agent. Slides were viewed on a Zeiss Axioskop photomicroscope.

Ovaries were fixed according to Xue and Cooley (1993), stained with affinity-purified α-9A1 antibody diluted 1:100 in PBSTB (PBSTB = PBS+0.1% Triton X-100+0.8% bovine serum albumin), and processed with secondary antibody as above.

Determination of staining intensity

Embryos from wild-type and nanos\(^{l7}/Df(3R)DfFX^3\) females (the latter referred to as nanos\(^{DF}\)embryos) were stained with α-9A1 and Texas Red-conjugated secondary antibody as described above. Twelve embryos from each sample were selected at random and photographed under identical conditions on TMAX p3200 film (Eastman Kodak Co.). Negatives were digitized with a Photometrics STAR I, cooled-CCD camera as described by Devaud et al. (1992). Transmitted light intensity was converted to optical density. Areas for measurement were selected by tracing the stained posterior pole region and subtracting the background intensity of an area within the embryo, just outside the stained crescent. Tracings were done by an independent investigator with no knowledge of the genotypes of the embryos being scanned. Data were analyzed with the IMGINTENS program (Paul Furcinitti, unpublished), which calculates the average intensity per pixel within the selected area, the overall intensity of the area, and the size of the selected area (in number of pixels). The mean intensity per area (which takes into account both intensity per pixel and the number of pixels in the area) was calculated for the 12 wild-type embryos and compared with the 12 nanos\(^{DF}\) embryos. Standard errors were calculated using Statview 512+ (Abacus Concepts, Inc.).

Pole cell counts

Wild-type (Ore R) and nanos\(^{DF}/Df(3R)DfFX^3\) females were mated with Ore R males, and allowed to lay eggs onto collection plates for 30 minutes at 25°C. Plates were set aside to allow embryos to age for 90 minutes, in order to obtain collections of embryos between 90 and 120 minutes old. These embryos were fixed for antibody staining as described above, then treated with an antibody recognizing vasa protein (courtesy of Paul Lasko) followed by a horseradish peroxidase-coupled goat-anti-rabbit secondary antibody (Pierce). During this period of development of the embryo, the anti-vasa antibody detects vasa antigen in pole cells exclusively, facilitating the counting of pole cells using Nomarski optics.

Electron microscopy and immunolabeling

Oregon R embryos were collected at timed intervals and the chorion removed by washing for 2 minutes in 50% bleach solution. After rinsing thoroughly with tap water, the embryos were transferred to the specimen holder of a Balzer HPM 010 High Pressure Freezer, frozen and transferred to liquid nitrogen for storage.

Embryos were freeze-substituted in 0.1% glutaraldehyde in acetone or methanol for 3 days at −90°C. They were then warmed to −35°C over a period of 6 hours and rinsed with 100% acetone or methanol (3 times for 20 minutes each). All subsequent steps were performed at −35°C. The samples were infiltrated with Lowicryl K4M over a period of 6 hours and allowed to remain overnight in 100% Lowicryl K4M. The resin was then polymerized by exposure to 360 nm UV light for 24 hours, after which time the samples were allowed to warm to room temperature. Sections (80 nm) were cut on a Reichert Ultratrac E microtome and transferred to nickel grids.

Grids were incubated in blocking buffer (BB = 0.1% cold water fish gelatin, 0.8% bovine serum albumin and 0.08% Tween 80) for 30 minutes, incubated in primary antibody for 1.5 hours in a moist chamber, and rinsed once with BB and twice with PBS over a total of 5 minutes. Rinses were followed by incubation for 1.5 hours in secondary antibody (goat anti-rabbit IgG conjugated to 10 nm gold particles) diluted in BB in a moist chamber, rinsing as above, and fixation in 0.5% glutaraldehyde in PBS for 5 minutes. Grids were then rinsed in PBS followed by distilled water for 2 minutes, and air dried. Grids were post-stained with uranyl acetate (5 minutes) and lead citrate (2 minutes) and observed in a Philips CM10 electron microscope operating at 80 kV. Most micrographs were taken at a magnification of 15,500× on the microscope. The primary antibodies used were α-9A1 anti-tudor, and a rabbit polyclonal antibody against the amino-terminal two-thirds of the vasa protein (kindly provided by Dr Paul Lasko).

RESULTS

Expression of tudor protein in wild-type ovaries and embryos

In order to study tudor protein in ovaries and embryos, we raised antibodies to a bacterially expressed fragment of tudor. Previous molecular analysis indicated that the tudor locus is capable of producing a protein of approximately 285×10\(^3\) M\(_r\) (Golumbeski et al., 1991). Affinity-purified antiserum prepared against a 35×10\(^3\) M\(_r\) internal portion of the tudor protein (referred to as α-9A1) recognizes a protein of the predicted size in extracts of wild-type ovaries (285×10\(^3\) M\(_r\), see Fig. 1). In extracts of early embryos (0-2 hours of development), proteins of 205×10\(^3\) M\(_r\) and 135×10\(^3\) M\(_r\) are observed, and the level of the 285×10\(^3\) M\(_r\) form declines. The 205×10\(^3\) M\(_r\) polypeptide is the predominant form in later embryogenesis. Although the ovaries and embryos were homogenized in buffer containing standard protease inhibitors, it is possible that some of the proteins detected result from proteolytic degradation. Several lines of evidence support the presumption that at least some of these polypeptides represent processed forms of the tudor protein: (1) the developmental profile seen on immunoblots is highly reproducible with regard to sizes of the major bands; (2) these samples do not exhibit general proteolysis, as indicated by probing the same samples on immunoblots using an anti-lamin or anti-vasa antiserum (data not shown); and (3) a second antiserum directed against the carboxy-
terminal region of the protein recognizes the same bands on immunoblots (not shown). The fact that two separate antisera recognizing distinct domains of the protein detect the same size bands indicates that these polypeptides are related to the tudor protein. Molecular analysis has revealed no alternative splicing of the tudor transcript (Golumbeski et al., 1991), however, alternate translation initiation remains a possibility.

The distribution of tudor protein in developing oocytes and embryos was examined using affinity-purified α-9A1 antiserum. This antiserum detects tudor protein in the germarium of wild-type ovarioles, and in perinuclear rings in the germ-line derived cells of early egg chambers (stages 1-3; see King, 1970; Fig. 2A). In stage 4-6 egg chambers, tudor protein is clearly visible at the anterior margin of the oocyte, where the oocyte is connected to the nurse cells. By stage 6, the protein can be seen on the posterior side of the oocyte nucleus as well as the anterior. In stage 7 and 8 egg chambers, the perinuclear rings of tudor protein in the nurse cells remain, and the protein appears to fill the oocyte cytoplasm. By early stage 9, a small but distinct posterior crescent of tudor protein is visible in the oocyte (Fig. 2B). The posteriorly localized protein is visible in the oocyte at least through late stage 10 (Fig. 2C), after which time the egg chambers become difficult to analyze due to the presence of the vitelline membrane. In the unfertilized egg, tudor protein is detected in a thin crescent at the extreme posterior pole.

After fertilization, the somatic nuclei of the Drosophila embryo undergo 13 nuclear divisions without cytokinesis. After the ninth intravitelline nuclear division, the nuclei reach the cortex and those entering the posterior pole cellularize, forming the pole cells (the primordial germ cells). The remaining nuclei undergo four more nuclear divisions and are cellularized after the 13th nuclear division cycle. In embryos undergoing the first nuclear division (0-0.25 hours after egg deposition at 25°C), the α-9A1 antiserum detects a thin crescent of tudor protein localized in the germ plasm at the extreme posterior pole, as well as lower level staining throughout the embryo (Fig. 3A). As illustrated in Fig. 3B-D, tudor protein steadily accumulates in the posterior pole plasm (the germ plasm) throughout the nuclear cleavage stages, and is incorporated into pole cells when they form (Fig. 3D). From the first nuclear division, tudor protein can also be detected around the embryonic nuclei. During the next eight intravitelline nuclear divisions (approx. 0.25-1.5 hours of development), tudor protein continues to be associated with nuclei, while the low level diffuse staining dis-

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**Fig. 1.** Immunoblot detection of tudor protein. Protein extracts from whole ovaries and early embryos were electrophoresed through 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose and probed with α-9A1 (anti-tudor). The apparent relative molecular masses of the major bands are indicated at the left.

**Fig. 2.** Distribution of tudor protein in wild-type ovaries. Whole ovaries were incubated with α-9A1 followed by secondary antibody conjugated to Texas Red and observed by indirect immunofluorescence. (A) Early stages. The germarium is to the left (g); progressively older egg chambers proceed to the right in an anterior to posterior direction. Egg chambers are numbered according to their stage (see King, 1970). The oocyte is at the right (posterior) of each egg chamber. The long arrows denote anterior staining in the oocyte, and the short arrow indicates the first place in which tudor protein is visible on the posterior side of the oocyte nucleus. In stage 7 the protein fills the oocyte cytoplasm. (B) Stage 9. Tudor protein is distributed throughout the oocyte cytoplasm and is most concentrated at the posterior pole (indicated by arrow). (C) Stage 10. Tudor protein is localized to the posterior pole; general cytoplasmic staining is reduced.
appearance. When embryos are stained with α-9A1 and counterstained with DAPI or an anti-histone monoclonal antibody, two areas of tudor protein localization are apparent on either side of the chromosomes during metaphase (Fig. 4A,B). This indicates that the protein is concentrated around the nucleus, and that it is not directly associated with the DNA, since the detectable α-9A1 staining is clearly at a distance from the chromosomes. At interphase, tudor protein surrounds the nucleus, appearing as a ring around the decondensed chromatin (Fig. 4C,D). Immunogold labeling indicates that tudor protein is excluded from the nucleus during interphase (not shown). The antibody labels mitochondria in the vicinity of the nucleus, and immunogold particles appear in small clumps in the cytoplasm, not associated with any visible organelles. By light microscopy, distinctly punctate staining can be seen throughout the embryo during the late cleavage stages, and may represent mitochondrial labeling. At the pole cell stage (approx. 1.5-1.75 hours) tudor protein is associated with all nuclei, and is most concentrated in the pole cells (Fig. 3D). The concentration of tudor protein in pole cells appears to diminish shortly after cellularization of the somatic nuclei, ultimately declining to a level similar to the nuclear staining of the somatic cells.

Distribution of tudor protein in mutant backgrounds

In embryos laid by females homozygous for any of the tudor mutant alleles (hereafter referred to as tudor embryos), the size of the wild-type tudor protein is unchanged, although it is slightly less abundant in the more severe tudor alleles (tud1 and tud3; not shown). Although the protein is present at approximately normal levels in these embryos, its distribution is clearly altered (Table 1 and Fig. 5A). Whole-mount staining of tudor embryos with α-9A1 indicates that tudor protein does not accumulate at any of the wild-type localization sites. Instead, only a general low level staining throughout the embryo is evident (compare Fig. 5A with Fig. 3). This low level staining never takes on the punctate appearance seen in wild-type embryos.

Genetic analysis of the tudor locus and the distribution of tudor protein suggest that tudor+ function is necessary for establishing the germ plasm of the Drosophila embryo. If the posterior localization of tudor protein plays a role in germ cell determination, mutations in some posterior group genes essential for germ cell determination (cappuccino, mago nashi, oskar, staufen, spire, tudor, vasa and valois) should disrupt this localization. Although tudor protein is present in embryo extracts from these mutants, its localization in the embryo is disrupted. Tudor protein localization in the germ plasm and around cleavage nuclei is eliminated by mutations in any of the posterior group genes required both for germ cell formation and segmentation of the embryonic abdomen (see Table 1 and Fig. 5B). In contrast, mutations in nanos and pumilio (both of which are required for abdominal segmentation, but not germ cell formation), do not alter the posterior localization of tudor protein (Table 1 and Fig. 5C). We assessed tudor protein distribution in embryos from nanosL7 mutant females, as well as embryos from females trans-heterozygous for the nanosL7 allele and a deletion of the nanos locus (Df(3R)DlF81). The nanosL7 allele is clearly not a null mutation of the nanos gene, because nanos protein is produced in embryos laid by nanosL7 mutant females (Smith et al., 1992). Embryos from

Fig. 3. Distribution of tudor protein in early wild-type embryos. Whole embryos were stained with α-9A1 as described in Materials and methods. In all cases anterior is to the left and dorsal is at the top. Nuclear cleavage stages were determined by counterstaining with the fluorescent DNA-specific dye 4,6-diamino-2-phenylindole (DAPI; not shown). (A) 0-0.25 hour embryo, undergoing the first nuclear division; (B) 0.25-0.5 hour embryo, at second nuclear division; (C) mid-cleavage stage embryo; (D) higher magnification of a pole cell stage embryo. The arrow indicates the position of the pole cells and the arrowhead indicates the position of a yolk nucleus.
homozygous nanos\textsuperscript{L7} mothers have approximately normal levels of tudor protein at the posterior pole (compare Fig. 5C with Fig. 3B). However, if half of the mutant nanos\textsuperscript{L7} product is eliminated by a deletion of the locus, tudor protein levels increase at the pole relative to wild type (Fig. 6A,B). Image digitization and densitometry data indicate that there is a significant increase in the intensity of tudor staining in the posterior of nanos/\textit{Df} embryos as compared to wild type, and that the overall area of staining is greater in the mutant (Fig. 6C, see also Materials and methods). By observation of wild-type and nanos/\textit{Df} embryos stained with anti-vasa antibody, it is evident that embryos lacking normal nanos function actually produce extra pole cells at the posterior pole (an average of 17.7 pole cells in wild-type and 26.2

Table 1. Distribution of tudor protein in mutant embryos*

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*Embryos were derived from females homozygous for mutations in the indicated maternal effect genes (with the exception of nanos\textsuperscript{L7}/\textit{Df}, which were from females transheterozygous for nanos\textsuperscript{L7} and \textit{Df(3R)DlFX3}, a deletion of the \textit{nanos} locus). Whole-mount preparations were immunostained as described in Materials and methods.

†mago nashi mutant females produce some embryos with double abdomens (DA) when grown at 25°C. At 17°C, these females produce embryos displaying abdominal defects typical of the posterior group, with no incidence of head defects or double abdomens (Boswell et al., 1991). Anti-tudor staining is the same at both temperatures.

pole cells in mutant embryos; see Table 2). These data indicate that the number of pole cells produced in an embryo is correlated with the amount of tudor protein present at the posterior pole, and is influenced by the activity of nanos.

We also analyzed the effect of the mis-localization of oskar message on the distribution of tudor protein. Ephrussi and Lehmann (1992) demonstrated that functional germ cells could be induced at the anterior pole by mislocalizing oskar RNA to the anterior. The 3′ untranslated region (3′UTR) of the oskar mRNA, which directs the normal posterior localization of the message, was replaced with the 3′UTR of the bicoid transcript, which contains the bicoid anterior localization signal. This construct (osk-bcd3′UTR) was used to transform flies, creating females whose eggs have oskar message localized at both poles. Embryos developing from these eggs lack head and thoracic structures, and instead have abdominal structures duplicated in mirror-image symmetry in the anterior (Ephrussi and Lehmann, 1992). Pole cells, capable of functioning as germ cells, develop ectopically at the anterior pole. We examined these embryos at the EM level, and found polar granules clearly visible in the anterior pole cytoplasm (Fig. 7A). When such embryos are stained with α-9A1, both anterior and posterior poles exhibit an accumulation of tudor protein (Fig. 7B). It is evident that concentration of tudor protein in the pole plasm is associated with the ability of this specialized cytoplasm to direct the formation of primordial germ cells.

The pool of tudor protein in the pole plasm is likely to be directly involved in the determination of germ cells. Since nanos and pumilio are not required for germ cell determination, it is not surprising that mutations in these genes do not negatively affect this pool of tudor protein. However, in embryos collected from homozygous mutant nanos and pumilio mothers, the association of tudor protein with nuclei is disrupted (Fig. 5C). Other members of the posterior group, all of which are involved in abdominal segmentation as well as germ cell determination, are also required for tudor nuclear localization (Fig. 5B and Table 1). Maternal genes, such as torso, trunk, and bicoid (Frohnhöfer and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986; Klingler et al., 1988), necessary for establishment of the anteroposterior axis, but not required for germ cell formation or abdominal segmentation, have no effect on the distribution of tudor protein (Table 1). Thus, only genes known to be required for germ cell formation disrupt the posterior localization of tudor protein, whereas genes required for abdominal segmentation disrupt the association of tudor protein with nuclei.

**EM localization of tudor protein in the posterior pole**

Since mutations in tudor affect the amount of assembled polar granule material in the germ plasm, it was of interest to examine whether anti-tudor antibodies would detect a component of polar granules in thin sections of embryos examined by electron microscopy. The protein product of the posterior group gene vasa has been identified as a component of polar granules, and has been shown, using anti-vasa antibodies, to be localized to the posterior pole cytoplasm around stage 10 of oogenesis (Hay et al., 1988a). Polar granules are first visible in the developing oocyte at stage 10 (Counce, 1963; Mahowald, 1968), and shortly thereafter, the posterior pole plasm becomes capable of inducing germ cell formation if injected into cleavage stage embryos (Illmensee et al., 1976). The presence or absence of polar granules thus correlates with the functioning of the pole plasm in the specification of the germ line. EM immunolocalization of the posterior pole region of early embryos using the α-9A1 antiserum shows that some, but not all, tudor protein is associated with polar granules. Inter-

![Fig. 5. Tudor protein localization in embryos from females homozygous for posterior group mutations. Whole embryos were stained with the α-9A1 tudor antibody as described in Materials and methods. In all cases anterior is to the left and dorsal is at the top. All embryos are mid-cleavage stage. (A) tudor, a severe allele of tudor (although not a protein null). (B) oskar, a null allele of oskar. This is representative of most members of the posterior group (see Table 1). (C) nanos. Note the posterior pole staining. This staining pattern is also typical of tudor distribution in embryos from pumilio females (see Table 1).](image)

### Table 2. Numbers of pole cells in wild-type and nanos/Df embryos

<table>
<thead>
<tr>
<th>Maternal genotype</th>
<th>Number of embryos</th>
<th>Number of pole cells (average)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oregon R</td>
<td>32</td>
<td>17.7</td>
<td>8-29</td>
</tr>
<tr>
<td>nanos/Df(3R)Df</td>
<td>35</td>
<td>26.2</td>
<td>16-46</td>
</tr>
</tbody>
</table>

Interestingly, a significant amount of tudor protein is also found within the mitochondria (Fig. 8A). This is in contrast to the distribution of the vasa protein, which is localized exclusively in polar granules at the posterior pole (Fig. 8B). Both the mitochondrial and polar granule staining by the anti-tudor antiserum persists after formation of the pole cells. In embryos from osk-bcd3′UTR females, which have a functional germ plasm, including polar granules, at the anterior (Fig. 7), tudor protein is found in polar granules and mitochondria at both poles (Fig. 9A,B). Vasa protein is present in polar granules in the anterior germ plasm as well (Fig. 9C).

The function of tudor protein in mitochondria in the germ plasm may be specific to this region and may be related to the presence of polar granules. Nonetheless, the mitochondrial staining is not restricted to the posterior pole; rather, mitochondria throughout the embryo contain tudor protein. Control experiments indicate that the results depicted in Fig. 8 are not due to non-specific staining, but reflect the distribution of the tudor epitope recognized by α-9A1. Neither pre-immune serum nor antibody recognizing the vector-derived portion of the fusion protein detected any material by these procedures. The sections shown in Figs 8 and 9 are adjacent sections of the same embryo stained separately with α-9A1 and anti-vasa antiserum.

**DISCUSSION**

In *Drosophila*, genetic data indicate that development of segmental pattern in the embryo, and the establishment of the germ line, are initially programmed by molecules sequestered in the egg during oogenesis. Genes involved in localization of these factors are said to have a ‘maternal effect,’ since the contribution of their products to the zygote is required solely from the mother. Eight maternal-effect genes have been identified in *Drosophila* that affect the localization or function of the germ cell determinant(s) (Boswell and Mahowald, 1985; Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986; Manseau and Schüpbach, 1989; Boswell et al., 1991), factors that become localized to the posterior pole cytoplasm, or germ plasm, during the late stages of oogenesis (Illmensee et al., and Schüpbach, 1989).
Fig. 8. EM immunolocalization of antigens in the posterior pole plasm. (A) Tudor protein detected with \( \alpha \)-9A1. Mitochondria (m) and polar granules (p) are approximately equally labeled. Polar granules are also indicated with arrowheads. (B) Vasa protein, detected with anti-vasa antibodies, accumulates only in polar granules; no labeling of mitochondria is seen. In both cases the secondary antibody is goat anti-rabbit IgG coupled to 10 nm gold beads.

Fig. 9. Tudor and vasa proteins are present in the anterior germ plasm of osk-bcd3'UTR embryos. A and B are one section and C and D are an adjacent section of a single embryo from an osk-bcd3'UTR female. Polar granules (p) and mitochondria (m) are present in both anterior and posterior germ plasms. Polar granules are also indicated with arrowheads. (A,B) Detection of tudor protein with \( \alpha \)-9A1. (A) Anterior pole. (B) Posterior pole. (C,D) Detection of vasa protein. (C) Anterior pole. (D) Posterior pole.
1976). We have studied the involvement of the tudor locus in germ cell determination, as well as its role in establishing segmental pattern in the embryonic abdomen.

Assembly of the germ plasm

Mutations in tudor disrupt the ultrastructure of the germ plasm. This disruption is manifested as a visible decrease in assembled polar granule material (Boswell and Mahowald, 1985). Polar granules are RNA/protein organelles found only in the germ plasm (Mahowald, 1962; Counce, 1963), and have been correlated with the presence or functioning of the germ cell determinant(s) (Hathaway and Selman, 1961; Jazdowska-Zagrodzinska, 1966; Bownes and Kalthoff, 1974; Brown and Kalthoff, 1983). Consistent with this correlation, the polar granule defect caused by tudor mutations is associated with the complete loss of pole cells (primordial germ cells) in embryos derived from homozygous mutant tudor mothers. Approximately 40% of the progeny of such females die during embryogenesis, exhibiting defects in the formation of abdominal segments. The severity of defects in abdominal segmentation varies with the mutant allele, and can be correlated with the degree of disruption of the polar granules. Although other posterior group genes do not exhibit this allele-specific effect on polar granule assembly, all of the mutations affecting germ cell formation (staufen, valois, vasa, oskar, cappuccino, spire, and mago nashi) affect the size, number, or morphology of polar granules (Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986; Manseau and Schüpbach, 1989; Boswell et al., 1991), and all are associated with disruption of abdominal segmentation. The presence of an intact germ plasm in the egg thus affects determinants for both germ line differentiation and abdominal development. These determinants are likely to be transported to, and sequestered in, the posterior pole cytoplasm by a common process involving the products of the posterior group genes.

The developing oocyte of Drosophila is situated at the posterior tip of the egg chamber, connected by cytoplasmic bridges to its 15 sister germ-line cells. These ‘nurse cells’ serve as the source of metabolites, RNA, and protein for the growing egg cell, that are sent into the egg through connections situated at its anterior pole (King, 1970). In comparison to mechanisms anchoring cytoplasmic molecules, such as bicoid RNA, at the anterior egg pole, the arrangement of the egg chamber necessitates more complex localizing mechanisms for cytoplasmic components that must traverse the oocyte to become fixed in the posterior pole plasm. Nonetheless, it is clear that many of the posterior group maternal gene products are transported to, and sequestered in the posterior pole plasm of the egg. Of those genes for which probes are available, each has some product, either mRNA or protein or both, that is concentrated in the posterior pole cytoplasm at some point in development (Hay et al., 1988b; Lasko and Ashburner, 1990; Ephrussi et al., 1991; Golumbeski et al., 1991; Kim-Ha et al., 1991; St. Johnston et al., 1991; Wang and Lehmann, 1991; Macdonald, 1992). The posterior group genes have been ordered in a pathway based on the effect of posterior group mutations on the localization of each characterized gene product to the germ plasm (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992). From this type of analysis, it is clear that at least some of the posterior group genes are involved in the germ plasm localization process. However, localization data do not necessarily imply that germ cell determination takes place via the temporal functioning of gene products in a linear pathway. An alternative possibility is that the posterior group gene products are assembled into a functioning unit in a cooperative manner, and that the absence of one component renders the assembly non-functional.

Localized tudor protein is required for germ plasm function

In this study, we analyzed the distribution of tudor protein in oocytes and embryos. Similar to other posterior group gene products, tudor protein becomes concentrated at the posterior pole of the developing oocyte, and accumulates in the germ plasm of early embryos until it is incorporated into pole cells (Fig. 3). Localization of tudor protein is dependent on the activity of all posterior group genes whose function is required for germ cell formation, since tudor protein is absent from the germ plasm of all posterior group mutants in which germ cell determination is disrupted (see Table 1 and Fig. 5). The presence of tudor protein in the germ plasm is associated with the ability of this cytoplasm to induce germ cell formation, whether at the posterior pole or at an ectopic site.

We assessed tudor protein localization in embryos derived from females carrying a hybrid transgene that mis-directs the oskar message to the anterior pole of the egg (the construct is referred to as osk-bcd3′UTR; see Ephrussi and Lehmann, 1992). These females also carry the wild-type oskar gene, which normally directs localization of oskar mRNA to the posterior pole (Ephrussi et al., 1991; Kim-Ha et al., 1991). Thus, embryos laid by osk-bcd3′UTR females have oskar mRNA at both poles, and develop two abdomens in mirror image symmetry, with pole cells forming at the anterior as well as the posterior pole. Anteriorly localized oskar protein is evidently capable of recruiting the germ plasm components necessary for the formation of primordial germ cells and abdominal segments. Both vasa and tudor are required for the functioning of the anterior germ plasm, whereas other posterior group genes that affect germ cell determination are not (Ephrussi and Lehmann, 1992). We have shown that the anterior pole of osk-bcd3′UTR embryos contains polar granules (Fig. 7A) and that this ectopic germ plasm contains tudor protein (Figs 7B, 9A). Normal tudor function is also required for the development of ectopic pole cells that have been observed in embryos from females carrying six copies of the wild-type oskar gene (Smith et al., 1992). These results are consistent with the requirement for localized tudor protein for the formation of germ cells, and suggest that tudor is directly involved with the functioning of the germ cell determinant(s).

Nanos activity affects tudor protein levels and pole cell number

In addition to tudor’s role in germ cell determination, mutations in tudor also affect the functioning of the determinant for abdominal segmentation. This determinant has been identified as the nanos gene product (Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991), which is crucial for the production of abdominal structures, but is
apparently dispensable for formation of the primordial germ cells. Localization of tudor protein to the germ plasm does not require nanos* function; in fact, reduction of normal nanos function results in an increase in the amount of tudor protein at the posterior pole (Fig. 6). An apparent consequence of this increase in localized tudor protein is that a greater than normal number of pole cells are formed in the embryo (see Table 2). This indicates a potential inhibitory effect of wild-type nanos product on the formation of pole cells, a possibility suggested previously by studies on the effect of increasing oskar gene dosage (Smith et al., 1992), which increases nanos protein levels in the posterior of the embryo. An increase in oskar gene dosage alone serves to increase pole cell number (Ephrussi and Lehmann, 1992; Smith et al., 1992). Embryos with extra doses of oskar also appear to have an increased amount of tudor protein at the posterior pole (A.B., unpublished observations). However, if nanos function is disrupted in embryos with extra doses of wild-type oskar, pole cell number is increased even further (Smith et al., 1992). In early embryos, and through the cleavage stages, nanos mRNA is localized in the germ plasm (Wang and Lehmann, 1991). Nanos protein levels are highest at the posterior pole, and diminish in a graded manner towards the anterior (Gavis and Lehmann, 1992; Smith et al., 1992). Later, nanos protein becomes highly concentrated in the pole cells, and is not detectable in the rest of the embryo. The protein remains in pole cells during their migration into the posterior midgut at gastrulation. In fact, anti-nanos antibody has been used as a pole cell marker (Ephrussi and Lehmann, 1992; Smith et al., 1992). It is intriguing that nanos protein, which is not involved in pole cell determination or formation, is concentrated and retained in pole cells, while disappearing from regions of the embryo in which it originally functioned.

The role of tudor protein in abdominal segmentation

Whereas nanos* function is clearly not required for tudor germ plasm localization, it does appear to be necessary for the association of tudor protein with nuclei. In embryos derived from nanos mutant mothers, tudor protein does not accumulate around embryonic nuclei, although localization of tudor protein in the germ plasm is unaffected (Fig. 5C). These results suggest that the two processes known to require tudor+ function (germ cell determination and abdominal segmentation) may involve differentially localized tudor protein and/or distinct forms of the protein. The fact that nanos+ function is required for nuclear localization of tudor protein implies that the two gene products may function interdependently to instruct abdominal segmentation. Recent evidence indicates that pumilio and nanos probably act cooperatively in determining hunchback expression in the embryo, thereby directing the spatial distribution of the zygotic gap gene products (Barker et al., 1992). tudor may be involved in this process as well. The fact that tudor protein is associated with all nuclei, and not just those in the prospective abdominal region, suggests that it is not nanos alone that regulates the nuclear accumulation of tudor. The pumilio protein is evenly distributed throughout the embryo (Macdonald, 1992), and therefore may be a candidate for such a function.

It has been reported that all of the posterior group genes except pumilio function to localize (or maintain the localization of) nanos activity in the posterior pole (Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991). Mutations in some posterior group genes directly affect the localization of nanos mRNA to the posterior pole (Ephrussi et al., 1991), however, evidence of the requirement for tudor* function results in nanos mRNA localization is inconclusive. In our hands, in situ hybridization of nanos cDNA in tudor mutant embryos resulted in posterior localization of the probe in 24-28% of the population, as compared to 55-57% in wild-type embryos (A.B., unpublished results). This apparent decrease in the frequency of embryos with localized nanos message may reflect the variability of the abdominal defects observed in tudor embryos. The observation that early tudor embryos (up to syncytial blastoderm stage) often exhibit localized nanos message, whereas cellular blastoderm embryos generally do not (A.B., unpublished data), suggests that tudor plays a role in maintaining the posterior localization of nanos mRNA. However, we do not consider this to be the primary function of tudor. If homozygous mutant tudor females also carry the oskbcd3’UTR transgene (which normally allows for abdomen and pole cell development at both poles), no pole cells form, but some nanos activity remains at both poles of the embryos (Ephrussi and Lehmann, 1992). The localization of tudor protein in nanos embryos, described above, suggest that the role of tudor in abdominal segmentation differs from the roles of other posterior group genes in that it is not involved solely in the localization of nanos to the posterior pole, but rather in a process taking place outside the germ plasm, in cooperation with the nanos product.

Subcellular localization of tudor protein

By EM immunolocalization, we have determined that at least a portion of the tudor protein in early embryos is located in mitochondria (Fig. 8). The mitochondrial-localized protein may represent a proteolytically processed form, as proteins destined to enter mitochondria are often proteolytically modified upon entry into the mitochondrial matrix (Glick and Schatz, 1991). Immunoblot analysis has suggested that tudor protein may be processed during development (Fig. 1), yielding distinct smaller forms of the protein which may serve to carry out separate functions, or allow some of the protein to be transported to or sequestered in different egg regions. The multiple sites of tudor protein localization and dynamic nature of its distribution, as well as the very large size of the protein (285×10^3 M_r), are all consistent with the possibility of tudor acting as a multifunctional protein in the developing embryo.

At this point the role of tudor protein in mitochondria is unclear. The existence of tissue-specific nuclear-encoded proteins within mitochondria is well established (Attardi and Schatz, 1988). These tissue-specific proteins within mitochondria can be involved in electron transport, regulation of mitochondrial protein synthesis, and many other mitochondrial related functions. The tudor protein present in germ plasm mitochondria may be responsible for altering the local pH of the posterior pole plasm necessary for proper maintenance of the polar granules. Mitochondrial tudor protein may also be involved in the regulation of local Ca^{2+} levels,
thus mediating the functioning of a second messenger pathway (Augustine, 1987; Bansal and Majerus, 1990). Mitochondrial activity is high at the posterior pole and in pole cells (Akiyama and Okada, 1992). While RNA synthesis is low in pole cells, protein synthesis is high relative to the rest of the embryo (Zalokar, 1976). Increased protein synthesis could reflect the translation of stored maternal RNAs, and may require locally enhanced mitochondrial activity. Mitochondria are found associated with polar granules in the germ plasm during oogenesis (Mahowald, 1968), and have been shown to accumulate in the germ plasm of amphibians (Mahowald and Hennen, 1971). The significance of the association between mitochondria and polar granules is thus far unknown, but is likely to involve tudor function. Tudor protein is present in both organelles, and is required for assembly or maintenance of polar granules. One component of polar granules, the vasa protein, shares sequence similarity with eukaryotic initiation factor eIF4A (Lasko and Ashburner, 1988; Hay et al., 1988b), and thus may be responsible for translating specific maternal mRNAs, including tudor. Wild-type vasa function is required for accumulation of tudor protein in the germ plasm. It is possible that some tudor mRNA is translated by vasa protein in polar granules, where it remains, and some of the protein is transported into mitochondria during the association of the organelles during oogenesis. We do not yet know if tudor is present first in mitochondria or polar granules. This will require careful dissection of oogenesis at the EM level. Unfortunately, preservation of morphology in egg chambers and late stage oocytes sufficient for immuno-EM has not yet been achieved. Nevertheless, the staining of early embryos at both the light and EM levels suggests that region-specific translation of the uniformly distributed tudor message could account for the apparent accumulation of tudor protein in the germ plasm relative to the rest of the embryo. There are approximately twice as many mitochondria at the poles (K.M., unpublished results), however, tudor staining is approximately equal in mitochondria throughout the embryo. This suggests that the high level of tudor protein in the germ plasm is due to its accumulation in polar granules. Results of staining embryos that lack polar granules is consistent with this interpretation (see Fig. 5 and Table 1). There may also be a cytoplasmic component, in neither mitochondria nor polar granules, that is recognized by the α-9A1 antibody at the light microscope level but not at the EM level. Staining at the EM level, while providing higher resolution, is less sensitive than immunofluorescent staining because only those epitopes that are at the top surface of the section can be labeled (Kellenberger and Hayat, 1991). By comparison, immunofluorescent probes in whole mount preparations have access to many more antigenic sites, and this may account for the conspicuous accumulation of protein observed at the light microscope level.

Conclusions

We have shown that the tudor protein has at least two distinct functions in the developing embryo, and that these functions are separable based on the interactions of tudor with genes required for germ cell formation, and those required solely for abdominal segmentation. The different sites of tudor localization may represent compartmentalization of specific functions of the tudor protein. This separation of functions may involve different forms of the protein, containing distinct functional domains. Alternatively, a single polypeptide may exist which is capable of performing distinct functions based on interactions with local factors at different sites within the embryo. The fact that a developmentally relevant gene product is found within mitochondria is also intriguing. Other studies have suggested a role for mitochondria in germ cell formation (Kobayashi and Okada, 1989; Akiyama and Okada, 1992), although currently little is known about the nature of mitochondrial function in any determinative process. Continued biochemical analyses of tudor function in mitochondria and nuclei, and use of antibodies recognizing distinct protein regions, may identify specific forms and/or functional domains of the tudor protein. The accumulation of tudor protein in the posterior pole cytoplasm is critical for germ plasm assembly, and thus for the determination of the germ line. All known genes involved in germ cell determination are required for localizing tudor protein in the germ plasm, suggesting that tudor plays a pivotal role in the functioning of the germ cell determinant. In addition, the requirement for nanos+ and pumilio+ function in the nuclear localization of tudor protein suggests a cooperativity between these gene products for the determination of abdominal segmentation. Further study of the nature of the interaction between tudor, nanos, and pumilio will be required to define their relative contributions to this process.

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