

# The *shut-down* Gene of *Drosophila melanogaster* Encodes a Novel FK506-Binding Protein Essential for the Formation of Germline Cysts During Oogenesis

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## ABSTRACT

In *Drosophila melanogaster*, the process of oogenesis is initiated with the asymmetric division of a germline stem cell. This division results in the self-renewal of the stem cell and the generation of a daughter cell that undergoes four successive mitotic divisions to produce a germline cyst of 16 cells. Here, we show that *shut-down* is essential for the normal function of the germline stem cells. Analysis of weak loss-of-function alleles confirms that *shut-down* is also required at later stages of oogenesis. Clonal analysis indicates that *shut-down* functions autonomously in the germline. Using a positional cloning approach, we have isolated the *shut-down* gene. Consistent with its function, the RNA and protein are strongly expressed in the germline stem cells and in 16-cell cysts. The RNA is also present in the germ cells throughout embryogenesis. *shut-down* encodes a novel *Drosophila* protein similar to the heat-shock protein-binding immunophilins. Like immunophilins, *shut-down* contains an FK506-binding protein domain and a tetratricopeptide repeat. In plants, high-molecular-weight immunophilins have been shown to regulate cell divisions in the root meristem in response to extracellular signals. Our results suggest that *shut-down* may regulate germ cell divisions in the germarium.

THE production of gametes in multicellular organisms is initiated with the establishment and division of germline stem cells. These cells are characterized by their unusual property—the ability to self-renew and to differentiate into a mature gamete. *Drosophila* oogenesis is a well-established system in which to study the processes of stem cell division and differentiation; the lineage, from stem cell to gamete, can be easily followed using a number of available markers. In addition, many female-sterile mutations affecting the differentiation of germ cells have been identified.

Each *Drosophila* ovary is divided into ~15 ovarioles, each of which contains two to three germline stem cells located at the apical region of the germarium in contact with the terminal filament. The formation of the egg chamber involves a highly ordered series of events that begins with the asymmetric division of a germline stem cell (see Figure 1A). This results in both self-renewal of the stem cell and the production of a daughter cell, which will become committed to a pathway of differentiation. The daughter cells of the germline stem cells become cystoblasts that undergo four mitotic divisions, each with incomplete cytokinesis, to produce a cyst of 16 germline cells interconnected by actin-rich ring canals (reviewed by DE CUEVAS *et al.* 1997). One of the 16 cells

will become an oocyte, while the other 15 will develop as accessory nurse cells.

Little is known about the molecular mechanisms that regulate these events. It has been proposed that signals originating from the somatically derived terminal filament regulate the stem cell divisions (LIN and SPRADLING 1997; KING and LIN 1999). The *Drosophila* gene *decapentaplegic* (*dpp*), a member of the TGF- $\beta$  family, has also been shown to regulate the division of germline stem cells in the ovary (XIE and SPRADLING 1998); however, the source of this signal remains unclear. Other growth factors and hormones are also likely to be required, but as yet their function in germline development has been more extensively studied in mammals (reviewed by LIN 1997). The role of cell cycle regulatory proteins in germline cyst formation is also largely unexplored, due to their essential requirements in other tissues. Currently, two genes, *bag of marbles* (*bam*) and *benign gonial cell neoplasm* (*bgn*), are known to be required for the differentiation of the cystoblast from the stem cell daughter (MCKEARIN and OHLSTEIN 1995; LAVOIE *et al.* 1999). The Bam protein is an essential component of the fusome, a germline-specific organelle rich in membranous vesicles and skeletal proteins. The fusome develops from an analogous organelle in the stem cell, the spectroosome (LIN *et al.* 1994), which determines the orientation of the stem cell divisions by anchoring one pole of the mitotic spindle. The fusome regulates the formation of the 16-cell cyst from the cystoblast and controls the number of cystocyte divisions. Mutants in *hu-li-tai-shao* (*hts*) and *lis1*, which both fail

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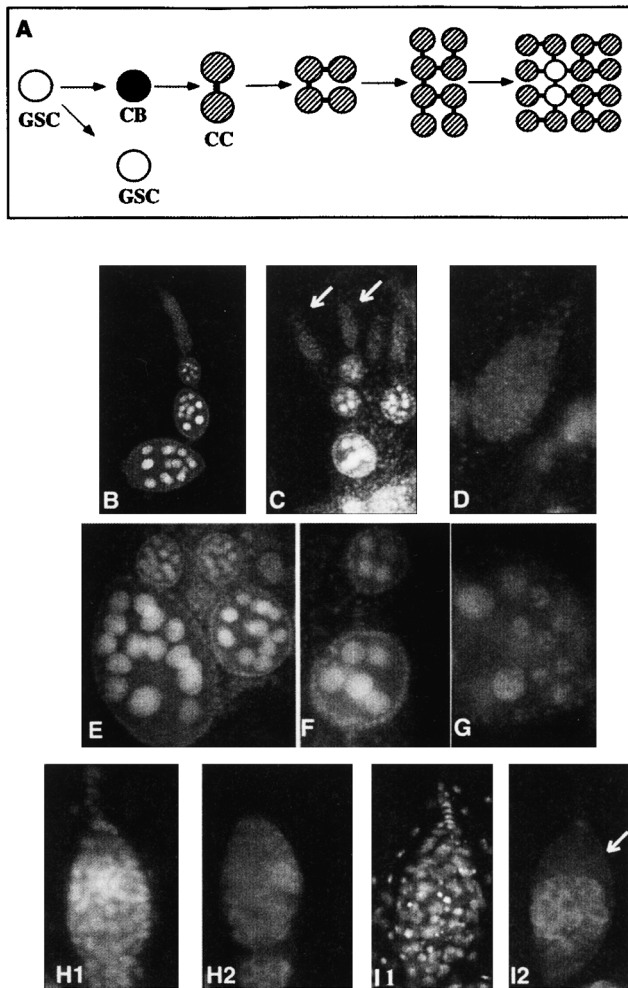


FIGURE 1.—(A) Schematic representation of the germline stem cell lineage. In region 1 of the germarium the stem cell (GSC) divides asymmetrically to renew itself and to produce a cystoblast (CB) that undergoes four mitotic divisions, each with incomplete cytokinesis, to produce a germline cyst of 16 cystocytes (CC) interconnected by ring canals. One of the two oldest cells with four ring canals will develop as the oocyte. (B–G) Hoechst-stained images of wild-type (B) and *shu*<sup>WQ41</sup> ovaries. The wild-type ovariole shown in B consists of several egg chambers at successive stages of development. In contrast, the *shu*<sup>WQ41</sup> ovarioles are associated with only 1–3 egg chambers (C). About one-third of the germaria have no associated egg chambers (arrows in C). (E and F) Mutant egg chambers with the normal number, or less than the normal number, of nurse cells. (G) A mutant egg chamber in which germline cysts of different developmental stages have been mispackaged. Hoechst (H1 and I1) and anti-Vasa (H2 and I2) stains of wild-type (H) and mutant (I) ovaries demonstrating a loss of germ cells from the apical region of the mutant germarium (arrow). This is consistent with a failure to maintain stem cells.

to make a normal fusome, produce cysts with variable numbers of germline cells (YUE and SPRADLING 1992; LIU *et al.* 1999). In both *hts* and *lis1* mutants, cysts with the correct number of germline cells fail to develop an oocyte, indicating an additional requirement for a functional fusome in oocyte determination. Two additional genes, *Bicaudal-D* (*Bic-D*) and *egalitarian* (*egl*), are

essential for the determination of the oocyte (SCHÜPBACH and WIESCHAUS 1991). The localization of *Bic-D* and *Egl* proteins to the oocyte is one of the earliest markers of oocyte specification (SUTER and STEWARD 1991; MACH and LEHMANN 1997). Analysis of mutants in both genes has revealed that they fail to localize determinant mRNAs to the presumptive oocyte, but it remains unclear whether their role in RNA localization is direct because the establishment of the microtubule organizing center is affected by mutations in both genes (THEURKAUF *et al.* 1993). Further, the homology of the *Bic-D* protein to myosin (SUTER *et al.* 1989) suggests that its role in RNA localization may be mediated indirectly through the cytoskeleton. The differentiation of one of the 16 cells of the cyst into an oocyte appears, therefore, to depend on the establishment of a correctly organized cytoskeleton during cyst formation.

We were interested in identifying genes that function upstream of *Bic-D* in the determination of the oocyte and found that in the *shut-down* (*shu*) mutant *Bic-D* protein was made, but mislocalized. Mutants in *shu* were originally isolated in a screen for genes on the second chromosome affecting oogenesis (SCHÜPBACH and WIESCHAUS 1991). Previously, TIRRONEN *et al.* (1993) proposed that mutations in *shu* affect the cystocyte integrity. We characterized the phenotype produced by a complete loss-of-function allele of *shu*. Our results demonstrate that the primary requirement for *shu* is for the normal development and maintenance of the germline stem cells. We have cloned the *shu* gene. The expression pattern of both the RNA and protein is consistent with a function in the germline stem cells. *shu* RNA also accumulates in the germplasm of blastoderm-stage embryos in pole cells and the embryonic gonad. *shu* encodes a novel protein that shows homology to the high-molecular-weight immunophilins. The immunophilins are a family of proteins characterized by the presence of an FK506-binding domain, which has been shown to exhibit peptidyl-prolyl isomerase activity. In *Arabidopsis thaliana*, immunophilins regulate cell divisions in the meristem in response to plant hormones (VITTORIOSO *et al.* 1998). Germline stem cell divisions in the *Drosophila* ovary are thought to be regulated by extracellular signals, raising the intriguing possibility that the *Shu* protein may function in the control of the stem cell divisions in response to growth-promoting signals.

## MATERIALS AND METHODS

**Fly stocks:** All fly stocks were maintained under standard culture conditions. *shu*<sup>WQ41</sup>, *shu*<sup>WM40</sup>, *shu*<sup>PB70</sup>, *bgcn*<sup>2W34</sup> (also known as *pep*<sup>2W34</sup>), and *tud*<sup>WCS</sup> flies were provided by Trudi Schüpbach and are described by SCHÜPBACH and WIESCHAUS (1989, 1991). The deficiency lines *Df(2R)bwS46*, *Df(2R)tid*, *Df(2R)b23*, and *Df(2R)OVI*, and the FRT stocks *y w P[ry<sup>+</sup>; FLP]<sup>12</sup>*; *CyO/ Sco, w; P[mini w<sup>+</sup>; FRT]<sup>2R-G13</sup>/CyO, w; P[mini w<sup>+</sup>; FRT]<sup>2R-G13</sup> L/CyO* and *w; P[mini w<sup>+</sup>; FRT]<sup>2R-G13</sup> P[mini w<sup>+</sup>; ovoD1]<sup>2R-32X9</sup>/S Sp Ms(2) M bw<sup>D</sup>/CyO* (CHOU and PERRIMON 1996) were obtained from the Bloomington and Umea stock centers and are de-

scribed at <http://flybase.bio.indiana.edu>. In all experiments *Oregon-R* (*Ore-R*) and *w<sup>118</sup>* flies were used as wild-type controls.

**Germline transformation:** *P*-element-mediated transformation was performed as described by SPRADLING and RUBIN (1982). Plasmids for injection were constructed as follows. The 2.6-kb *Hind*III fragment isolated from a genomic clone in the *shu* region was inserted in the *Hind*III site of pBlueScript KS<sup>+</sup>. This was then isolated as a *Kpn*I-*Not*I fragment and ligated to the corresponding sites of the transformation vector pCaSpeR2 (PIROTTA 1988). To express *shu* under the control of the *ovarian tumor* (*otu*) promoter, an *Eco*RI-*Not*I fragment representing the full-length *shu* cDNA was inserted into the corresponding sites of the germline transformation vector, pCOG (ROBINSON and COOLEY 1997). For all experiments, at least three independent lines were used.

**Germline clonal analysis:** For the generation of germline clones, each *shu* allele was crossed into the *w<sup>118</sup>* background and then recombined onto the P[*mini w<sup>+</sup>*; FRT]<sup>2R-G13</sup> chromosome. *w*; P[*mini w<sup>+</sup>*; FRT]<sup>2R-G13</sup> *shu*/Cyo females were mated with males of the genotype *y w* P[*ry<sup>+</sup>*; FLP]<sup>12</sup>/Y; P[*mini w<sup>+</sup>*; FRT]<sup>2R-G13</sup> P[*mini w<sup>+</sup>*; *ovo<sup>D1</sup>*]<sup>2R-32X9</sup>/Cyo and eggs were collected for 24 hr. Heat shock was administered for 2 hr in a 37° water bath for 2 consecutive days during larval or pupal stages. Female progeny of the genotype *y w* P[*ry<sup>+</sup>*; FLP]<sup>12</sup>/*w*; P[*mini w<sup>+</sup>*; FRT]<sup>2R-G13</sup> P[*mini w<sup>+</sup>*; *ovo<sup>D1</sup>*]<sup>2R-32X9</sup>/P[*mini w<sup>+</sup>*; FRT]<sup>2R-G13</sup> *shu* were mated with wild-type males and observed for the production of eggs. Eggshells were prepared as described by WIESCHAUS and NUSSLEIN-VOLHARD (1986) and examined by dark-field microscopy. After 2–4 days, the ovaries were dissected, stained with Hoechst 33258 (Molecular Probes, Eugene, OR), and examined by fluorescence microscopy. Control females of the same genotype, which had not been subjected to heat shock, and female progeny from mothers of the genotype *y w* P[*ry<sup>+</sup>*; FLP]<sup>12</sup>/*w*; P[*mini w<sup>+</sup>*; FRT]<sup>2R-G13</sup>/Cyo were analyzed in parallel.

**Cloning and sequence analysis:** DNA isolation, Southern blotting, and library screenings were performed using standard techniques, as described by SAMBROOK *et al.* (1989). *In situ* hybridization to squashes of salivary gland chromosomes from larvae of the genotypes +/+ and *Df*/+ was performed using digoxigenin-labeled probes. Cosmid clones were obtained from the European *Drosophila* Genome Project. Genomic phage clones were isolated from a λFIXII library made from *cn bw* genomic DNA (courtesy of Beat Suter). cDNA clones were isolated from an *Ore-R* ovarian library (gift of Beat Suter) made in λZAPII. Sequencing of cDNA clones was performed using an automated ABI (Columbia, MD) dye termination sequencer by the University of Medicine and Dentistry of New Jersey—Robert Wood Johnson Medical School DNA Synthesis and Sequencing Laboratory. Sequencing of genomic DNA was done by cycle sequencing of overlapping PCR fragments. Genomic DNA from *Bic-D<sup>P106</sup>* flies was used as a control for the sequencing of *shu<sup>PB70</sup>*, as both mutations were generated on the same parental chromosome (*cn bw*). Sequence comparisons were made using gapped blasts with the NCBI BLAST program at <http://www.ncbi.nlm.nih.gov>. Protein motifs were identified using the PROSITE database (HOFMANN *et al.* 1999) at <http://www.expasy.ch/sprot/prosite/>.

**Antibody production and immunoblotting:** For the production of antisera against the Shu protein, the *SalI-XhoI* fragment, representing amino acids 31–392, was subcloned into the corresponding sites of the vector pET30b (Novagen). The 6XHis-tagged Shu protein was prepared by SDS polyacrylamide gel purification of induced cultures of *Escherichia coli* strain BL21 (DE3) carrying the pET30b/*shu* plasmid. Polyclonal rat antisera were generated by Pocono Rabbit Farms and Laboratories (Canadensis, PA). For Western blotting, protein extracts were electrophoresed through 10% SDS-polyacrylamide gels

and transferred to nitrocellulose membranes. Blocking was carried out overnight at 4° in 5% nonfat dried milk in phosphate buffered saline (PBS)/0.4% Tween-20. Anti-Shu serum and the anti- $\alpha$ -tubulin monoclonal antibody (clone no. DM1A; Sigma, St. Louis), were used at dilutions of 1:500 and 1:2000, respectively, in blocking solution. Anti-rat and anti-mouse secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at a dilution of 1:2000 and 1:3000, respectively, and were detected using chemiluminescence (Pierce Chemicals, Rockford, IL).

***In situ* hybridization and immunostaining:** *In situ* hybridization to ovaries, testes, and embryos was performed as described by TAUTZ and PFEIFLE (1989) using riboprobes synthesized using the Genius 4 RNA labeling kit (Boehringer Mannheim, Indianapolis). For antibody staining, ovaries were treated as described by LANTZ *et al.* (1994). Affinity-purified anti-Shu antiserum (preabsorbed overnight at 4° with *shu<sup>WQ41</sup>* ovaries) was used at a dilution of 1 in 500, anti-Bic-D monoclonals 1B11 and 4C2 (SUTER and STEWARD 1991) were used at 1 in 40 each, anti-Orb monoclonals 4H8 and 6H4 (LANTZ *et al.* 1994) were used at 1 in 30 each, anti-Sxl monoclonal 18 (BOPP *et al.* 1993) was used at 1 in 10, anti-Bam C monoclonal c2 (MCKEARIN and OHLSTEIN 1995) was used at 1 in 10, anti-Vasa (LASKO and ASHBURNER 1990) was used at 1 in 500, and anti-adducin 87 monoclonal 1B1 (ZACCAI and LIPSHITZ 1996) was used at 1 in 2. Cy3-conjugated donkey anti-rat or donkey anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories) were used at 1 in 1000. FITC-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) was used at 1 in 150. For nuclear staining, ovaries were incubated in Hoechst 33258 (Molecular Probes) at a concentration of 1  $\mu$ g/ml in PBS for 5 min. For labeling of actin, FITC-conjugated phalloidin (Molecular Probes) was used at a concentration of 40 units/ml in PBST for 20 min. Ovaries were mounted in 70% glycerol/PBST or Vectashield (Vector Laboratories, Burlingame, CA) mounting media.

## RESULTS

All three *shu* alleles cause recessive female sterility with no effects on zygotic viability (SCHÜPBACH and WIESCHAUS 1991). The two strong loss-of-function alleles, *shu<sup>WQ41</sup>* and *shu<sup>WM40</sup>*, also result in male sterility while the weaker allele, *PB70*, does not affect male fertility. Comparison of the phenotypes of *shu<sup>WQ41</sup>* and *shu<sup>WM40</sup>* as homozygotes and hemizygotes (over *Df(2R)b23*) reveals that *shu<sup>WQ41</sup>* is likely to be a null allele while *shu<sup>WM40</sup>* is a very strong loss-of-function allele. Due to the presence of a closely linked recessive lethal mutation on the *shu<sup>WQ41</sup>* and *shu<sup>PB70</sup>* chromosomes, few homozygous adults could be isolated. The phenotype of *shu<sup>WQ41</sup>* was therefore studied in hemizygous flies [over *Df(2R)b23*], but will be referred to in the text as *shu<sup>WQ41</sup>*, while that of *shu<sup>PB70</sup>* was analyzed in hemizygotes or in the heteroallelic combination with *shu<sup>WM40</sup>*.

***shu* function is required for the normal function of the germline stem cells:** To analyze the *shu<sup>WQ41</sup>* ovarian phenotype, ovaries were dissected from 0- to 1- and 2- to 4-day-old females and stained with the nuclear stain Hoechst and with antibodies recognizing several different markers of germ cell differentiation. The severity of the phenotypes observed in *shu<sup>WQ41</sup>* can vary between females, and even between ovarioles, suggesting that

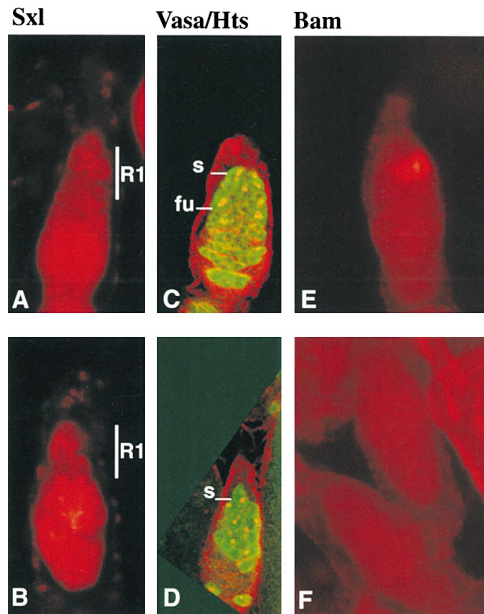


FIGURE 2.—*shu* function is required for the normal differentiation of the stem cell progeny. Staining of wild-type and *shu*<sup>WQ41</sup> germaria, respectively, with anti-Sxl (A and B), anti-Hts/anti-Vasa (C and D), and anti-Bam (E and F). R1, region 1; s, spectrosomes; fu, fusomes.

there may be some functional redundancy for *shu*. In contrast to wild-type ovaries (Figure 1B), 40–60% of the ovarioles from newly eclosed *shu*<sup>WQ41</sup> females do not contain any developing egg chambers (Figure 1, C and D). Usually, the other ovarioles contain only one to three egg chambers and their numbers fail to increase as the females age. Although some egg chambers with 15 nurse cells and an apparently normal oocyte nucleus are observed, subsequent egg chambers have fewer than 16 germ cells, *e.g.*, 2, 4, 8, 10, or 12 (Figure 1, E and F). None of the egg chambers develop into normal eggs but instead degenerate by mid- to late oogenesis. Those egg chambers with the correct number of germ cells may have arisen through the differentiation of prestem cells, *i.e.*, germ cells that developed as cystoblasts without first being established as stem cells (KING 1970). The remainder of the *shu*<sup>WQ41</sup> ovarioles contain no developing egg chambers (Figure 1D), only germaria-containing clusters of germline cells that can sometimes appear pycnotic, indicating that they may be dying.

The strong cytoplasmic staining of these germ cells with antibodies recognizing the Sex Lethal protein (Figure 2, A and B) indicates that *shu* function is dispensable for the establishment of the female mode of sexual differentiation of the germ cells. Staining of these germ cell clusters with antibodies that recognize spectrosomes and fusomes (ZACCAI and LIPSHITZ 1996) reveals that they do not develop branched fusomes characteristic of older cysts (Figure 2, C and D). To determine the developmental stage of these *shu* germ cells, they were further analyzed using an antibody specific for the cytoplasmic form of the Bam protein (MCKEARIN and

OHLSTEIN 1995). In wild-type germaria, the cytoplasmic form of Bam accumulates in mitotic cystoblasts and cystocytes (Figure 2E). However, in newly eclosed *shu*<sup>WQ41</sup> females no staining was observed in any of the germaria analyzed (>50; Figure 2F). This suggests that, after the first few egg chambers are formed, no further mitotic cystoblasts develop. Instead, these germ cells appear to redivide to form clusters of abnormal germ cells that degenerate or occasionally produce tumorous cysts. In older females (>4 days), ovarioles in which the cells at the tip do not express Vasa, apparently failing to maintain germline cells at the tip (Figure 1I), are frequently observed consistent with defects in stem cell renewal or maintenance. In addition, we have also observed some mispackaged egg chambers (Figure 1G). Collectively, these phenotypes suggest that *shu*'s function is essential for the normal activity of the stem cell. The absence of wild-type function could affect the first asymmetric division of the stem cell, resulting in an abnormal cystoblast that is compromised in its ability to undergo the normal four rounds of mitotic division. In addition, this abnormal asymmetric division would affect stem cell renewal, resulting in an abnormal stem cell that divides several times to produce ill-fated germ cell clusters.

**Weak loss-of-function alleles of *shu* reveal later requirements in oogenesis:** Females heteroallelic for the weak loss-of-function allele PB70 and the strong loss-of-function allele WM40 lay only ~30% as many eggs as wild-type females, none of which developed. Typically, these eggs are abnormally shaped and dorsal appendages, if present, are reduced or fused (see Figure 4B). Inspection of the ovaries of these females revealed at least five developing egg chambers in the majority of ovarioles. About 80% of these egg chambers contain the correct 15:1 ratio of nurse cells to oocyte (Figure 3B) but never develop into wild-type eggs. Although an oocyte is established, as seen by the localization of Bic-D and Orb proteins to the posterior-most cell in the cyst (Figure 3F), its identity is not maintained and the oocyte appears to fail in its further development. Subsequent to stage 2 or 3, the distribution of the two proteins becomes diffuse and accumulation of the proteins is observed in nurse cells (Figure 3F). This pattern is particularly striking for the Bic-D protein (Figure 3G) and suggests that the transport system from the nurse cells to the oocyte has collapsed in these egg chambers. Subsequently, oocyte development fails and the egg chambers degenerate. These observations corroborate the results of TIRRONEN *et al.* (1993). We have also observed at low frequency (5–10%) egg chambers in which the oocyte was not correctly positioned (Figure 3D) and egg chambers in which the oocyte nucleus appears polyploid rather than diploid, although not to the same extent as the nurse cells. This is similar to phenotypes observed in *Bic-D* partial loss-of-function mutations (J. Y. OH and R. STEWARD, unpublished results). These observations suggest that there may be a later requirement for *shu* function during egg chamber development.

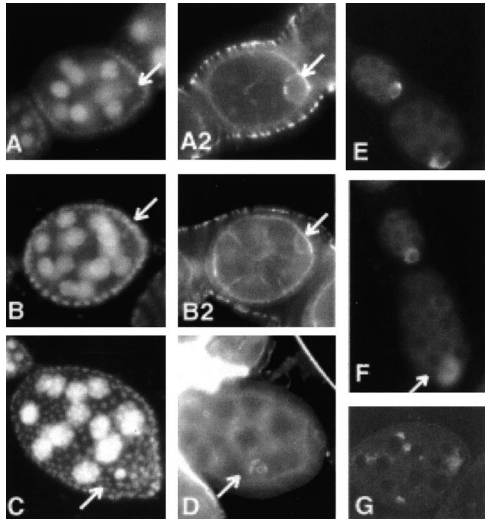


FIGURE 3.—*shu* function may be required at later stages of egg chamber development. In ovaries from 0- to 2-day-old *shu<sup>PB70</sup>/shu<sup>WM40</sup>* females, oocyte identity is established. A and B show Hoechst and phalloidin-stained egg chambers from wild-type and *shu<sup>PB70</sup>/shu<sup>WM40</sup>* females, respectively. The oocyte is the cell with the diploid oocyte nucleus and four ring canals positioned at the posterior of the egg chambers (arrows). In C, the oocyte appears to have undergone endoreduplication and no longer looks diploid. The egg chamber shown in D has a mispositioned oocyte (arrow). In wild-type egg chambers the Bic-D and Orb proteins both localize to the presumptive oocyte and show overlapping patterns of localization. (E) Wild-type egg chambers stained with anti-Orb. (F) Corresponding stages of *shu<sup>PB70</sup>/shu<sup>WM40</sup>* ovaries stained with anti-Orb. Both Bic-D and Orb proteins localize to the oocyte but their localization is not maintained and the focus of staining within the oocyte becomes diffuse (arrow). (G) *shu<sup>PB70</sup>/shu<sup>WM40</sup>* egg chamber stained with anti-Bic-D showing diffuse staining in the oocyte and patches of accumulation among the nurse cells.

***shu* functions in the germline:** The phenotype of *shu* indicates that its function is required for the normal activity of the germline stem cell. To address whether *shu* functions autonomously in the germline, we made use of the FRT-mediated, dominant female sterile (DFS) technique and generated mutant clones in the germline by mitotic recombination (CHOU and PERRIMON 1992). Females heterozygous for autosomal insertions of the DFS mutation *ovo<sup>D1</sup>* fail to lay eggs and their ovaries contain no egg chambers that have developed beyond stage 4–5 of oogenesis (Figure 4C). In contrast, females of the genotype *y w P[FLP]/w; FRT ovo<sup>D1</sup>/FRT shu<sup>PB70</sup>*, which were subjected to heat shock, lay abnormal eggs (Figure 4B). These eggs are similar to those laid by females hemizygous or heteroallelic for the PB70 allele. Inspection of ovaries from females in which germline clones of the strong loss-of-function alleles WQ41 and WM40 were induced revealed the presence of abnormal egg chambers that appear, by Hoechst staining, to be degenerating by mid- to late oogenesis (Figure 4D). These results indicate that *shu* functions in the germline. They do not, however, exclude the possibility of an additional somatic function.

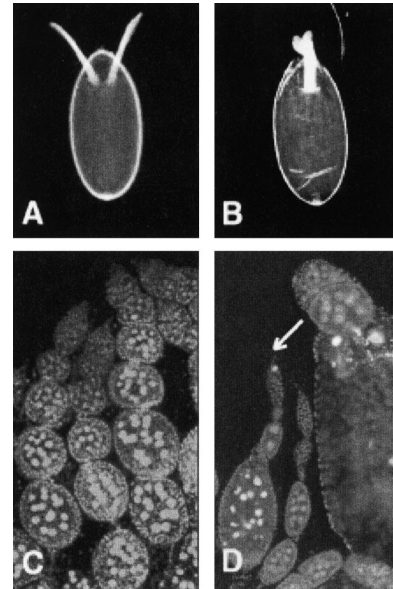


FIGURE 4.—*shu* function is required in the germline. Germ-line clones were generated by mitotic recombination in the *ovo<sup>D1</sup>* background. (A and B) Dorsal views of eggshells produced by germline clones induced in mothers of the genotype *y w P[FLP]/w; FRT ovo<sup>D1</sup>/FRT* (A) and *y w P[FLP]/w; FRT ovo<sup>D1</sup>/FRT shu<sup>PB70</sup>* (B). (C and D) Hoechst-stained ovarioles from mothers of the genotype *y w P[FLP]/w; FRT ovo<sup>D1</sup>/FRT shu<sup>WM40</sup>* without (C) and with (D) heat-shock treatment. The arrow in D indicates an ovariole in which a clone has produced an egg chamber containing only two polyploid cells. In the germarium an individual germ cell that has undergone premature endoreduplication to produce a polyploid cell is present. Also shown to the right of this is an older degenerating egg fused to two younger egg chambers.

**Cloning of the *shu* gene:** Previous studies have localized *shu* to genetic map position 2-105 in the cytological interval 59D8–60A2 (SCHÜPBACH and WIESCHAUS 1991). To refine its position, we performed complementation tests between the *shu* alleles and deficiencies in the region. Our results placed *shu* in the interval defined by the proximal breakpoint of *Df(2R)b23* and the distal breakpoint of *Df(2R)OVI* (Figure 5A). Simultaneously, we mapped *shu* genetically relative to the female-sterile mutant *bgn* that is also uncovered by *Df(2R)b23*. We screened females for recombination events between the *cn bw shu<sup>WQ41</sup> sp* and *cn bw bgn<sup>QW34</sup>* chromosomes that would result in females that were fertile *in trans* to *Df(2R)b23*. Of 6500 females screened, 2 fertile females were obtained. Both of these females were mutant for *sp*, placing *shu* 0.063 cM proximal to *bgn*, *i.e.*, within ~30 kb. We mapped the position of *bgn* (probe provided by D. McKearin) and the distal breakpoint of *Df(2R)OVI* (Figure 5B), which lies between *shu* and *bgn*, using a cosmid contig obtained from the European *Drosophila* Genome Project (data not shown).

On the basis of the *shu* phenotype we would expect the gene to be expressed in early stages of oogenesis and during spermatogenesis. We therefore looked at

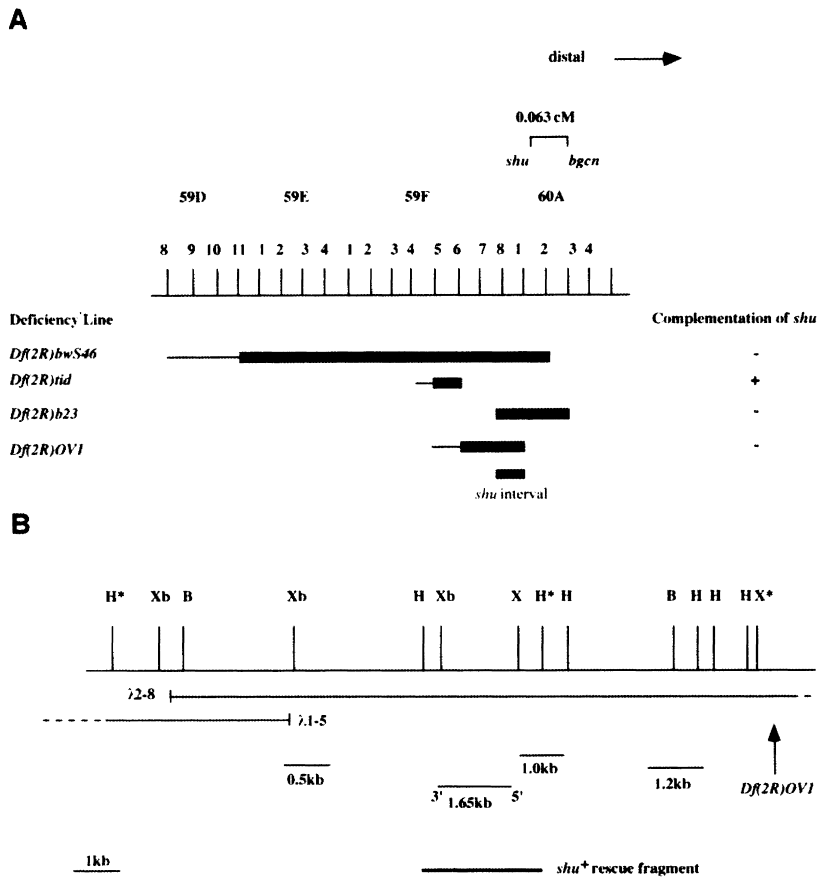


FIGURE 5.—Genetic and molecular map of the region containing the *shu* locus. (A) Genetic mapping of *shu*. DNA deleted by the deficiency chromosomes is represented by black boxes, with the uncertainties in the breakpoints indicated. The region defining the *shu* interval is shown by a stippled box. (B) Restriction map of an ~16-kb region around the *shu* locus showing the position of the distal breakpoint of *Df(2R)OVI*. Overlapping bacteriophage  $\lambda$  clones covering the region are shown underneath. Ovarian transcripts identified by Northern analysis are indicated. The 2.6-kb *HindIII* fragment, which encodes *shu* but no other transcripts, was used for transformation rescue of the *shu* phenotype. Both the 1.65-kb transcript and the adjacent 1-kb transcript are transcribed in the distal to proximal direction. B, H, X, and Xb represent *Bam*HI, *Hind*III, *Xho*I, and *Xba*I sites, respectively. Polymorphisms identified in genomic DNA from *Ore-R*, and the *cn bw*, *cn bw sp*, and *Df(2R)tid* chromosomes are indicated (\*).

the expression pattern of candidate ovarian cDNAs that were isolated using genomic fragments in the region proximal to the *Df(2R)OVI* breakpoint. A 1.65-kb transcript in this region (Figure 5B) is expressed in the germarium of the ovary and in the apical tip of the testes (see below and Figure 6). This transcript is of identical size on Northern blots of ovary and testes mRNA. The 2.6-kb *HindIII* fragment of genomic DNA containing this transcript, plus 700 bp of upstream sequence, can rescue the sterility of *shu* males and females (see MATERIALS AND METHODS). This fragment encodes no other complete transcript. In addition, we have identified mutations in the open reading frame of this transcript in genomic DNA from all three *shu* alleles (see below and Figure 7). These data confirm that this transcript encodes *shu*.

#### *shu* is expressed in a dynamic pattern in the germline:

We examined the expression pattern of *shu* during oogenesis, spermatogenesis, and embryogenesis using whole mount *in situ* hybridization with RNA probes made from the cDNA clone corresponding to its transcript. As shown in Figure 6, A and C, the mRNA can first be detected at the apical tip of the germarium in the germline stem cells and cystoblasts. The level of expression decreases in the remainder of region 1, where the cystocytes are dividing to produce 2-, 4-, 8-, and 16-cell cysts. However, in region 2b, where 16-cell

cysts are present, strong levels of expression are again seen. In region 3, equivalent levels of expression can be seen in all 16 germ cells of the stage 1 egg chamber. This pattern of expression corresponds well with the earliest phenotypes of *shu* and confirms the results of our clonal analysis that indicated that *shu* function is required in the germline. Subsequent to stage 4 of oogenesis, there appears to be an abrupt downregulation of *shu* expression until stage 10, where the transcript can be detected at high levels in the nurse cells (Figure 6B). In early cleavage-stage embryos uniform levels of *shu* mRNA are detected (Figure 6E), suggesting a possible maternal function for *shu*. Interestingly, by the cellular blastoderm-stage *shu* mRNA is exclusively found in the pole cells (Figure 6F). The mRNA can be detected in the germ cells throughout their migration through the midgut and into the gonadal mesoderm (data not shown) and is present in the embryonic gonad of stage 15 embryos (Figure 6G). No staining above background level can be detected in any tissues other than the gonads. The *shu* mRNA can also be detected at the apical tip of the testes where the stem cells are dividing to produce cysts of primary spermatocytes (Figure 6D).

#### *shu* encodes a FK506BP-like protein with a TPR motif:

Sequence analysis of the longest ovarian cDNA clones corresponding to the *shu* transcript revealed an open reading frame of 1236 bp. This is predicted to encode

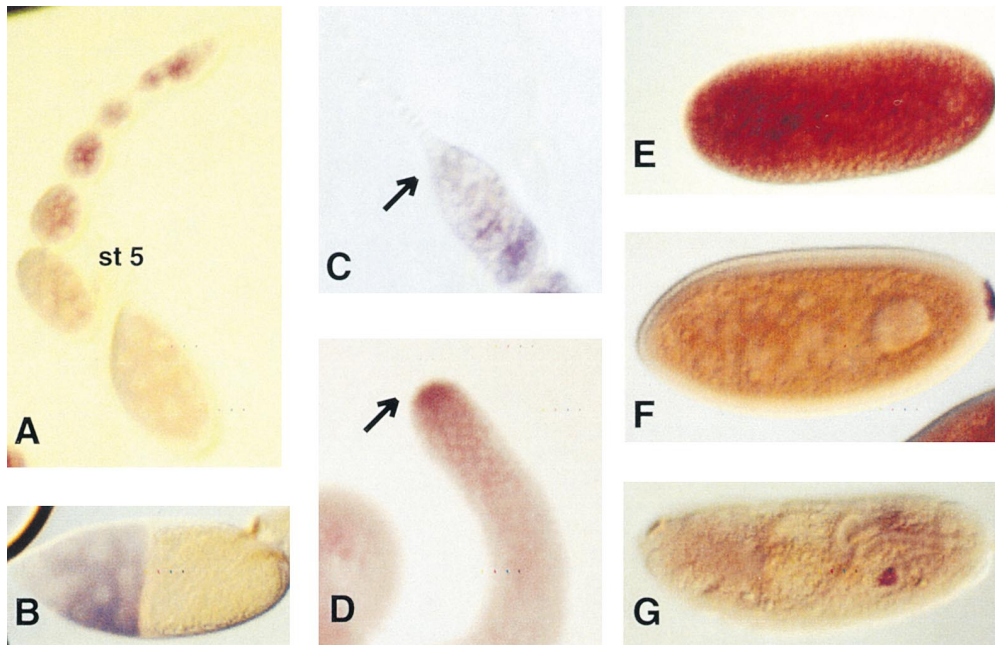


FIGURE 6.—*In situ* hybridization to wild-type tissue using *shu* antisense riboprobes. (A) Wild-type ovary showing strong germline expression of *shu* from germarial stages through stage 4. The level of expression is reduced subsequent to this and increases again in the nurse cells of the stage 10 egg chamber (B). In the germarium (C), low levels of *shu* expression can be detected in the germline stem cells and cystoblasts. The level of expression appears to be reduced in dividing cystocytes in the posterior of region 1. Similarly, in the testes (D), *shu* mRNA can be detected in the dividing stem cells at the tip of the testes. Uniform levels of the RNA are present in cleavage-stage embryos (E). During the cellular blastoderm stage, the RNA is incorporated into the pole cells and can be detected in the embryonic gonad at stage 15 (G).

a 392-amino-acid protein (Figure 7A) with an expected molecular weight of 45 kD. The first conceptual translation initiation site, at position 58, has 2/4 matches (GATA) to the *Drosophila* consensus site (CAVENER 1987). The 57 bp upstream of this ATG does not contain an in-frame stop codon. The longest cDNA is apparently full length because its size, 1682 bp, corresponds well with the size predicted by Northern analysis (data not shown), and an expressed sequence tag available from the Berkeley *Drosophila* genome project, which was isolated from a different cDNA library (0- to 3-hr embryo), has an identical 5' end. Also, this cDNA can rescue the sterility of *shu* females and males when expressed under the control of the *ovarian tumor (otu)* promoter (data not shown; ROBINSON and COOLEY 1997).

A search for recognized protein motifs, using the Prosite database, revealed a peptidyl-prolyl *cis-trans* isomerase (PPIASE) domain at amino acids 103–193 and one tetratricopeptide repeat (TPR) at amino acids 303–334. PPIASE domains are protein-protein interaction motifs that have been shown, *in vitro*, to catalyze the *cis-trans* isomerization of the peptide bond of proline residues, resulting in changes in protein folding (FISCHER and SCHMID 1990). Their activity is inhibited by binding of the drugs FK506 and rapamycin. Hence, proteins containing these domains are commonly referred to as FK506-binding proteins (FK506BP). The TPR motif is a degenerate 34-amino-acid sequence, which is also thought to mediate protein-protein interac-

tions (LAMB *et al.* 1995). In addition, the Shu sequence contains a putative nuclear localization signal (NLS), and 18 predicted phosphorylation sites, although the functional significance of these, if any, remains unknown. A schematic representation of the protein is shown in Figure 7B.

Comparison of the predicted amino acid sequence of Shu with other proteins in the database, using the Gapped BLAST search program (ALTSCHUL *et al.* 1997), revealed that it is a novel *Drosophila* protein that shows similarity to proteins of the high-molecular-weight immunophilin family. This class of protein typically contains both PPIASE and TPR motifs. They are evolutionarily conserved, multifunctional proteins, examples of which have been identified in mammals and plants. Human FKBP6 (p36) (MENG *et al.* 1998) shows 28% identity and 46% similarity (probability  $10^{-24}$  with 3% gaps) to Shu along the length of the entire protein sequence (Figure 7C). A similar level of homology is also observed with human FKBP4 (SANCHEZ *et al.* 1990), FKBP52 (the mammalian p59 heat-shock protein-binding immunophilin; PEATTIE *et al.* 1992), and several plant immunophilins. Functional studies of human FKBP12, the best-characterized FKBP, have identified 14 residues important for enzymatic activity and drug binding (KAY 1996). As shown in Figure 7C, Shu retains 8 of these residues and a further amino acid is also conserved, suggesting that this domain is functionally important. Similarly, comparison of the Shu TPR motif

## A

CGCCAACTCAGCGCATTCCGGAATTCGTTTCGTAATAATTTCTGTAATTTTGCATA **ATG**  
M

61/2 ∇  
GAA GAA AAC TTC GAA CCG TAC ACC CCA **CAG** TTG CTC AAG AAT CCC TTG TCC TAT TCG GAT  
E E N F E P Y T P Q L L K N P L S Y S D

121/22  
CTC GTG AAA AAG GGC GTT GAG TTC **CAG** GTC GAC AAT TCG CAG CAG AAC CAT GCC AGG GAT  
L V K K K G V E F E V D N S Q Q N H A R D

181/42  
CTG GGT CTC GAC AGC GAC AGC GAC **AGC** GAC TAC GAG GAT GCG CTG GAC GTG GAC GGC GAG  
L G L D S D S D S D Y E D A L D V D G E

241/62  
GAG CTA CGT TCA CCG TGG ACA TAT TCA TTC GAC GAA CTG CGT GCG CTG ATG AGC GAG ATC  
E L R S P W T Y S F D E L R A L M S E I

301/82  
GAC GAA AAC ATT TAC AAG CCG ATC ACT CGC ACC GGT CAC GTG GAT CGG GAG GCG GTG CCG  
D E N I Y K R I T R T G H V D R E A V P

361/102  
AAC AAG GCT AGG GTC TCG GTT CGG TAC AGT GGC TAC TGG GAG GGC GAG ACC GCT CCC TTC  
N K A R V S V R Y S G Y W E G E T A P F

421/122  
GAC TCC TCC CTG CTG CGG GGC AGC AAA TTC GTG TTT **GAA** ACT GGC CAG GGG GAA CGG TGG  
D S S L L R G S K F V F E T G Q G E R W

481/142  
TTG AAG GCC TGG AGG TCG CCG TCC GCA AGC ATG CGT CCT TAC GAG CAG GCC GAG TTT ATA  
L K A W R S P S A S M R P Y E Q A E F I

541/162  
ATC TCC TAC AAG CTG CTT TTC GGA GAA CTC GGT TGC CCT CCG CGG ATC AAG CCC AAG GCG  
I S Y K L L F G E L G C P P R I K P K A

601/182  
GAT GCG CTC TTT AAA GTG GAG GTA ATT GAC TAC TCG CTC ATA GGA GAT GCG AAG GGC ATC  
**D A L F K V E V I D Y G** L I G D A K G I

661/202  
GAT GCT ATC CCC CAA GAA GAC CGC GAC AAG TTT TGT GTG GTC TAT CCG AAG GCT GTC GAC  
D A I P Q E D R D K F C V V Y P K A V D

721/222  
TTG CAC CTA CAT GGC AAG GAC TCG GTG AAG CTC GGA CGC TAT CAG AGT GCA GCA ACT GCC  
L H L H G K D S V K L G R Y Q S A A T A

781/242  
TTC GAA CCG GCA GTG AGC TCA CTG AAC TAC TGC CGA ATG GCC AAT GAC GAG GAG GAG CGC  
F E R A V S S L N Y C R M A N D E E E R

841/262 ∇  
AAG CAA ACT GAA CTG CTT ATT ACC CTG AAC CAA AAC CTG ATG ATC GTT TAC AAC AAG ATG  
K Q T E L L I T L N Q N L M I V Y N K M

901/282  
AAC **AAG CCG AAG CCG** GCC TGC ATC ATG ATG AAG GCC TTA CGC CAC CTG ACC ATG GGC AAT  
N **K P K R** A C I M M K A L R H L T M G N

961/302  
CCG TCT TGC AAG GCG CTC TTC CAG GAG GGA CGC GCC CTG GCC GCT CTG GGC GAG TAC AAC  
P S C K A L F Q E G R A L A A L G E Y N

1021/322  
TTG GCC CGC AAC GCT TAC CTG CAG CCG CAG **GCC** AAG CAG CCG GCG AAC AAG GAG ATC AGC  
L A R N A Y L Q A Q A K Q P A N K E I S

1081/342  
GAC CAG ATT ATC AGC ATG AAC AAG AGA ATC AGC AAG TAT GAG GAG GCC AGT CGC GAT ATA  
D Q I I S M N K R I S K Y E E A S R D I

1141/362  
**TGG** GCA CGT GCC TTC TCG TTA AAG AAC TCA AAG TCA GAC GTC CGC AAA ACG CCT GCT CAG  
W A R A F S L K N S K S D V R K T P A Q

1201/382  
CTG GAG AAG GAG GCA AAA GGA ACA GGA TTT CAA **TGA** CAAAATGGAAGATTTGATTAGCGGTTTAAG  
L E K E A K G T G F Q \*

1261  
AACACTTCAGATCAACAAGTGAGCTTTTCACGTAAATCTTATTCAAACGCTCAGTTCGACGCGACTTGCAAAATGGCCA  
AGGAACACAACCTGAAGCTAACTTTGTCTCCATCCAAGAGGATGTTCTGACATGTGCTAAGCCGATGTTAAGTTCCG  
TTGAGCTTACTAAGAACCCCAAGTATTTTCATATTTTCATATCTTACTCAAAAAAAAAAATACCAACATGAAGGTAA  
TTTAGTTCCAAGTTCTAGAAGAGCAGTATCATTAGTTATTTTCATATAAGCAACATGAATATCGTAAGCCAGACGAAT  
GTTAACGTTTTTTGTTATTTAGAGCAACGTAGACCTTAAGTTGTTAAAAACCACAATAAAGTAATGCACGGCAGCTAC  
ATAAAAAAAAAAAAAAAAAA 1682

FIGURE 7.—(A) Sequence of *shu* cDNA and predicted protein. The DNA sequence of the longest cDNA clone is shown with the amino acid sequence indicated below the open reading frame. The ATG start codon and the TGA stop codon are shown in bold, and the polyadenylation signal is underlined. Introns were identified by comparison of the cDNA and genomic sequences and are indicated above the sequence (∇). The introns at positions 116 and 870 are 59 and 68 bp in size, respectively, and both have the eukaryotic consensus splice sites. Underlined amino acid sequences represent the PPIASE domain (103–193) and TPR motif (304–337). The putative NLS at amino acid 283 is boxed. The codons mutated in each of the three *shu* alleles are underlined and the altered nucleotide is shown in bold. (B) Schematic representation of the structure of the protein with the two domains and positions of the mutations indicated. The PPIASE and TPR domains are indicated by black and hatched boxes, respectively. (C) Alignment of the Shu sequence with that of hFKBP6 (accession no. AF038847). Identical residues are indicated as white on black, and conserved residues are shown as black on gray. Dashes represent gaps introduced to maximize the alignment. The positions of the PPIASE domains and TPR motifs in each protein are indicated by lines above (Shu) and below (FKBP6) the sequence. Functionally important residues of hFKBP12 and the consensus TPR residues are indicated above the sequence (the consensus TPR residues of hFKBP6 are indicated below its additional two repeats), with residues conserved in Shu shown in boldface type.

with the consensus TPR sequence reveals that 5 of 6 consensus residues are conserved (Figure 7C).

To investigate the nature of the *shu* mutations, we sequenced the genomic DNA from flies hemizygous for each allele (shown in Figure 7, A and B). The *WQ41* allele has a C to T transversion at position 88 that creates a premature stop codon (Q11 to STOP). This confirms our prediction, based on genetic analysis, that *WQ41* is a complete loss-of-function allele. The *WM40* allele has

a G to A transversion at position 1142 that creates a premature termination of translation 31 amino acids before the end of the protein (W342 to STOP). The weakest loss-of-function allele of *shu*, *PB70*, is due to a G to A transversion at position 1051 that results in an alanine (332) to threonine substitution within the TPR motif. This result suggests that the TPR motif is likely to be important for *shu* function. Interestingly, this mutation does not affect male fertility.





sumably arisen through the differentiation of prestem cells, *i.e.*, germ cells that developed as cystoblasts without first being established as stem cells (KING 1970). These early egg chambers develop until midoogenesis and then degenerate. Germline stem cells are clearly established in the absence of *shu* function, because they express Sex Lethal protein and have spectrosomes. Although the stem cells appear to have undergone several divisions, their progeny do not become committed to the normal pathway of germline differentiation. Instead, they presumably redivide to produce clusters of ill-fated germ cells. This phenotype contrasts with that of mutants such as *bam* and *bgen* (GATEFF 1982; MCKEARIN and SPRADLING 1990), which specifically block cystoblast differentiation and result in tumors of mitotically active cells that continue to behave like stem cells. As *shu* females age, a loss of germ cells is observed, resulting in agametic ovarioles. This indicates that stem cell renewal is also affected by the loss of *shu* function. One way in which these phenotypes could have arisen is through an aberrant asymmetric division of the stem cell.

A similar asymmetric division controls the production of primary spermatocytes from male germline stem cells during spermatogenesis. Strong loss-of-function alleles of *shu* also result in male sterility. The mutant testes contain fewer than normal elongating sperm bundles and the apical tip of the testes, where the germline stem cells divide, appears reduced compared to wild type, and many of the cells are degenerating (TIRRONEN *et al.* 1993; K. MUNN and R. STEWARD, unpublished data). These observations indicate that *shu* also has an essential function in germline stem cell regulation in males.

Recently, mutations in *decapentaplegic* (*dpp*), *pumilio* (*pum*), *nanos* (*nos*), *piwi*, and *fs(1)Yb* that affect stem cell maintenance and asymmetric division have been described (LIN and SPRADLING 1997; COX *et al.* 1998; FORBES and LEHMANN 1998; XIE and SPRADLING 1998; BHAT 1999; KING and LIN 1999). While the source of the *dpp* signal remains unknown, the function of both *piwi* and *fs(1)Yb* is thought to be required in the somatic cells of the terminal filament. Both *piwi* and *fs(1)Yb* have been proposed to be required for the production of a somatic signal that regulates the germline stem cells. In contrast, *pum* and *nos* have been reported to function in the germline. Our analysis of ovarian germline clones indicates that *shu* also functions in the germline autonomously. This is supported by the observation that we can rescue both male and female sterility using a transgene whose expression is controlled by the germline-specific *otu* promoter. As some of the phenotypes observed in *shu* are similar to those described for *piwi* and *fs(1)Yb*, it is possible that *shu* is required for the response of the germline cells to these somatic signals. We have observed no effects on fertility in transheterozygous combinations of *shu* with *piwi*, *pum*, or *nos*. In contrast to *shu*, mutations in *dpp*, *piwi*, *pum*, and *nos* allow the

production of mature eggs. The other phenotypes observed in *shu* egg chambers, including a loss of oocyte identity and occasionally mispositioned oocytes, are therefore likely due to later requirements for *shu* in egg chamber development. Consistent with this possibility, *shu* expression increases in newly formed 16-cell cysts.

The expression pattern of the *shu* mRNA suggests that *shu* may function in germline development during embryogenesis. The mRNA is incorporated into the pole cells and can be detected in the germ cells throughout their migration into the embryonic gonad. A number of RNAs have been identified that are incorporated into the pole cells including *cyclin B*, *germ cell less*, *hsp83*, *nos*, *orb*, *oskar*, *pum*, *tudor*, and *vasa*. As discussed above, mutations in some of these genes also affect early germline development (for review see RONGO and LEHMANN 1996). As the existing alleles of *shu* fail to produce normal eggs that are fertilized, we were unable to study the possible effects of zygotic *shu* function on germ cell migration or proliferation.

The Shu protein shows significant homology to an evolutionarily conserved class of proteins, the immunophilins. Although these proteins have been shown, *in vitro*, to catalyze changes in protein folding (FISCHER and SCHMID 1990), their function *in vivo* remains unclear. The best characterized of the FK506BPs is the low-molecular-weight immunophilin human FKBP12. It is a cytosolic protein that has been implicated in signal transduction (reviewed by MARKS 1996). In vertebrates, FKBP12 has also been proposed to regulate translation through its association with the FKBP12-associated rapamycin-binding protein (reviewed by BROWN and SCHREIBER 1996). This complex regulates the binding of translation initiation factors to the 5' end of the mRNA. The *Drosophila* gene *vasa* encodes a germline-specific homologue of the translation initiation factor eIF4A (LASKO and ASHBURNER 1988). Null alleles of *vasa* produce a variety of phenotypes that include atrophied germaria containing reduced numbers of developing germline cysts (STYHLER *et al.* 1998; TOMANCAK *et al.* 1998). We have found no genetic evidence that *shu* functions in this particular pathway, as females transheterozygous for a null allele of *shu* and a deficiency that removes *vasa* show no defects in oogenesis.

In addition to its FK506-binding domain, Shu contains a predicted TPR motif. This is a protein-protein interaction motif that was originally identified in cell cycle regulatory proteins but has since been found in a number of different proteins with no common biochemical function (LAMB *et al.* 1995). We have identified a mutation in an allele of *shu* within the TPR, indicating the importance of this motif for *shu* function. This allele does not cause male sterility, suggesting that the protein might function differently in spermatogenesis, perhaps through interacting with different partners. Alternatively, it is also possible that spermatogenesis is less sensitive to reductions in levels of wild-type function.

The presence of a TPR motif, in addition to the FK506-binding domain, is characteristic of the high-molecular-weight immunophilins, an evolutionarily conserved family of proteins whose function remains uncharacterized. Interestingly, there are no examples of this type of immunophilin in yeast or *Caenorhabditis elegans*. Mammalian immunophilins were originally identified in complexes of HSP90 with unliganded steroid hormone receptors (OWENS-GRILLO *et al.* 1996). It has been proposed that immunophilins function in the cytoplasmic to nuclear targeting of these complexes (PRATT *et al.* 1993). Interestingly, the germline expression pattern of the *Drosophila* homologue of HSP90, *Hsp83*, is strikingly similar to that of *shu* (DING *et al.* 1993). Biochemical experiments will confirm whether Shu interacts with HSP83 in the germline or with alternative partners.

Unfortunately, the lack of mutants in the mammalian immunophilins has prevented the identification of their *in vivo* functions. Human FKBP6 maps to a common 1-Mb deletion in patients with William's syndrome (MENG *et al.* 1998), a developmental disorder associated with a haploinsufficiency at chromosome 7q11.23 (EWART *et al.* 1993). William's syndrome has multiple associated phenotypes. Together with the large size of the deletions, this has made it difficult to correlate specific gene functions with a particular aspect of the disease. Human FKBP6 and rodent FKBP52 are both expressed at particularly high levels in testes (NAIR *et al.* 1997; MENG *et al.* 1998), suggesting that immunophilins may have a conserved function in germline development. Interestingly, germline stem cells of the mammalian testes, like *Drosophila* germline stem cells, undergo asymmetric, self-renewing divisions.

Some insight into the function of immunophilins was recently obtained through the analysis of the *pasticcino-1* (*pas-1*) mutant in *A. thaliana*. The *pas-1* mutant was isolated in a screen for mutants that showed an abnormal response to the cell division-promoting plant hormones, cytokinins. The mutants have defects in cell division and elongation in the cotyledons and the apical root meristem (FAURE *et al.* 1998). Cloning of *pas-1* revealed that it is a homologue of mammalian FKBP52 (VITTORIOSO *et al.* 1998). FKBP52 has been shown to co-localize with the mitotic apparatus and to copurify with cytoplasmic dynein, suggesting that it too may be required for cell divisions (PERROT-APPLANAT *et al.* 1995; SILVERSTEIN *et al.* 1999).

The *shu* phenotypes support the possibility that *shu* may also function during germline cell divisions. Specifically, its function seems to be important for the divisions of the germline stem cells. In support of this, we can detect expression of the protein in the stem cells while the levels are greatly reduced in the dividing cystocytes. The future identification of Shu-interacting proteins, coupled with the potential of genetic analysis in *Drosophila*, should greatly increase our understanding

of how germline stem cells are regulated and provide valuable information about the function of the immunophilins.

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