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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Manuscript received January 15, 2000
Accepted for publication May 23, 2000

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 alleles 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 alleles that

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Genetics 156: 245–256 (September 2000)
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 (egf), 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. shuWQ41, shuG41, shuWQ41, bgcnW47 (also known as peyG47), and tubG16 flies were provided by Trudi Schüpbach and are described by Schüpbach and Wieschaus (1989, 1991). The deficiency lines Df(2R)b23, Df(2R)b23, and Df(2R)OV1, and the FRT stocks y w P(2)y; FLP]12, CyO/Sco, w; P(mini w); FRT]12; CyO/Sco, w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; P(mini w); FRT]12; CyO and w; and SSp Ms(2) M bx+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;h flies were used as wild-type controls.

**Germline transformation:** Pelement-mediated transformation was performed as described by Spradling and Rubin (1982). Plasmids for injection were constructed as follows. The 2.6-kb HindIII fragment isolated from a genomic clone in the shu region was inserted in the HindIII site of pBlueScript KS'. This was then isolated as a KpnI-NcoI fragment and ligated to the corresponding sites of the germline transformation vector pCaSpeR2 (Pirrotta 1988). To express shu under the control of the ovarian tumor (tum) promoter, an EcoRI-NcoI fragment representing the full-length shu DNA 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^{1118} background and then recombined onto the P[w^+]; FRT^{2R4G} chromosome. w^+/w^+; FRT^{2R4G} shu/CyO females were mated with males of the genotype y^{+}/y'; FLP^{12}/Y; P[w^+]; FRT^{2R4G} P[w^+/w^+]; FRT^{2R4G} P[w^+]; FRT^{2R4G} multiple of the germline transformation vector, and eggs were collected for 24 hr. Heat shock was administered for 2 hr in a 37°C water bath for 2 consecutive days during larval or pupal stages. Female progeny of the genotype y{+}/y'; FLP^{12}/Y; P[w^+]; FRT^{2R4G} P[w^+/w^+]; FRT^{2R4G} 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 genotype y{+}/y'; FLP^{12}/Y; P[w^+]; FRT^{2R4G} P[w^+/w^+]; FRT^{2R4G} shu were analyzed in parallel.

**Cloning and sequence analysis:** DNA isolation, Southern blotting, and library screens 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 clones were isolated from an AFIXII library made from 11 bw genomic DNA (courtesy of Beat Suter). cDNA clones were isolated from an Ore-R ovarian library (gift of Beat Suter) made in λ ZapII. Sequencing of cDNA clones was performed using an automated ABI (Columbia, MD) dye terminator sequencing machine. 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 PGR fragments. Genomic DNA from BicD^D606 flies was used as a control for the sequencing of shu^{D60}, as both mutations were generated on the same parental chromosome (cn bw). Sequence comparisons were performed using gapped blasts on the Prosite database (Hoffmann et al. 1999) at http://www.expasy.ch/sprot/prosite/.

**Antibody production and immunoblotting:** For the production of antisera against the Shu protein, the Surf-Xbld 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°C in 5% nonfat dried milk in phosphate buffered saline (PBS)/0.4% Tween-20. Anti-Shu serum and the anti-α-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 Genus 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°C with shu^{Q2} ovaries) was used at a dilution of 1 in 500, anti-Bic-D monoclonals 1B11 and 4C2 (Suter and Stewart 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 (Zaccari 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 μg/ml in PBS for 5 min. For labeling of actin, FITC-conjugated phallolidin (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 (Schröb and Wieschaus 1991). The two strong loss-of-function alleles, shu^{Q2} and shu^{Q3}, also result in male sterility while the weaker allele, PB70, does not affect male fertility. Comparison of the phenotypes of shu^{Q2} and shu^{Q3} as homozygotes and hemizygotes (over Df(2R)b23) reveals that shu^{Q2} is likely to be a null allele while shu^{Q3} is a very strong loss-of-function allele. Due to the presence of a closely linked recessive lethal mutation on the shu^{Q2} and shu^{Q3} chromosomes, few homozygous adults could be isolated. The phenotype of shu^{Q2} was therefore studied in hemizygous flies (over Df(2R)b23), but will be referred to in the text as shu^{Q2}, while that of shu^{Q3} was analyzed in hemizygotes or in the heteroallelic combination with shu^{Q3}.

**shu function is required for the normal function of the germline stem cells:** To analyze the shu^{Q2} 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^{Q2} can vary between females, and even between ovarioles, suggesting that
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* 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 pre-stem cells, i.e., germ cells that developed as cystoblasts without first being established as stem cells (King 1970). The remainder of the *shu* ovarioles contain no developing egg chambers (Figure 1D), only germaria containing clusters of germ line 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 (Zaccaci and Lipshttz 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* 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 II), 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.
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Figure 3.—shu function may be required at later stages of egg chamber development. In ovaries from 0- to 2-day-old shuPB70/shuWM40 females, oocyte identity is established. A and B show Hoechst and phallolidin-stained egg chambers from wild-type and shuPB70/shuWM40 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 shuWM40/shuWM40 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) shuWB1/shuWM40 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 ovdP1 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 ovdP1/FRT shuPB70, 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.

Cloning of the shu gene: Previous studies have localized shu to genetic map position 2-105 in the cytological interval 59D8–60A2 (Schübbach and Wieschaus 1991). To refine its position, we performed complementation tests between the shu alleles and deficiencies in 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 ovdP1 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 ovdP1/FRT shuPB70, 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.

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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
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Figure 6.—In situ hybridization to wild-type tissue using shu antisense riboprobes. (A) Wild-type ovariole 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 germ-line 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 interactions (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 10−22 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...
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 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.

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 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.

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.
Shu protein is expressed in stem cells and 16-cell cysts: Rat antisera were raised against a 6× His-tagged protein containing amino acids 31–392 of Shu. Western analysis revealed that the antisera specifically recognize a 52-kD band in wild-type ovary extracts (Figure 8A, lane 1). This protein is not detected in ovary extracts from hemizygous \textit{shu}\textsuperscript{WQ41} females (Figure 8A, lane 2), evident in all cells of the newly formed 16-cell cysts. Like the \textit{shu} mRNA, this persisted until stage 4–5 egg confirming the specificity of the antisera. To determine the subcellular localization of the Shu protein, affinity-purified antibodies were used for immunofluorescent staining of ovaries. The Shu protein is expressed in the germline stem cells and cystoblasts (Figure 8B). Low levels of staining were observed in dividing cystocytes in the posterior of region 1. In region 2b, staining was evident in all cells of the newly formed 16-cell cysts. Like the \textit{shu} mRNA, this persisted until stage 4–5 egg chambers. The staining was present in the cytoplasm of the germ cells. Only background levels were detected in the follicle cells. Germline staining was eliminated in \textit{shu}\textsuperscript{WQ41} ovaries. Cytoplasmic staining was also seen later in the nurse cells of stage 10 and older egg chambers (data not shown). However, as the strong loss-of-function alleles of \textit{shu} do not develop normal eggs at this stage it is difficult to ascertain if this later staining is specific. We have been unable to detect staining in the germ cells of embryos with this antiserum. This could be due to technical reasons, such as the antibody sensitivity or specificity, as we have only been able to detect very low levels of the protein on immunoblots of embryonic extracts.

**DISCUSSION**

Our analysis of the \textit{shu} phenotype, using a complete loss-of-function allele, has revealed requirements for \textit{shu} in the normal behavior and differentiation of the germline. The few developing egg chambers with 16 germ cells present in newly eclosed \textit{shu}\textsuperscript{WQ41} females have pre-
sumably arisen through the differentiation of pre-stem cells, i.e., germ cells that developed as cystoblasts without first being established as stem cells (King 1970). These early egg chambers develop until midogenesis 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 agamic 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 germine 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 germ line 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 germ line 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 (Naif et al. 1997; Meng et al. 1998), suggesting that immunophilins may have a conserved function in germ line development. Interestingly, germ line stem cells of the mammalian testes, like Drosophila germ line stem cells, undergo asymmetric, self-renewing divisions.

Some insight into the function of immunophilins was recently obtained through the analysis of the pasticino-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 (Vittorioiso 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-Apllanat et al. 1995; Silverstein et al. 1999).

The shu phenotypes support the possibility that shu may also function during germ line cell divisions. Specifically, its function seems to be important for the divisions of the germ line 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 germ line stem cells are regulated and provide valuable information about the function of the immunophilins.

We are grateful to Trudi Schüpbach for providing us with the shu alleles and to Dennis McKearin for communicating results prior to publication. We also thank the following people who provided reagents: Lynne Cooley, Paul Lasko, Haifan Lin, Howard Lipshitz, Dennis McKearin, Paul Schaedl, and Beat Suter. In addition, we are grateful to Girish Deshpane and Trudi Schüpbach for comments on the manuscript. We also acknowledge Le Nguyen for preparation of fly food and Huey-Seng Huang and Keith Korsun for technical assistance. This work was supported by a grant from the National Science Foundation and by the W. Horace Goldsmith Foundation.

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Communicating editor: T. Schüpbach