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Ectopic expression of the *Drosophila* Bam protein eliminates oogenic germline stem cells

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SUMMARY

The *Drosophila* germ-cell lineage has emerged as a remarkable system for identifying genes required for changes in cell fate from stem cells into more specialized cells. Previous work indicates that *bam* expression is necessary for cystoblast differentiation; *bam* mutant germ cells fail to differentiate, but instead proliferate like stem cells. This paper reports that ectopic expression of *bam* is sufficient to extinguish stem cell divisions. Heat-induced *bam*$^+$ expression specifically eliminated oogenic stem cells while somatic stem cell populations were not affected. Together with previous studies of the timing of *bam* mRNA and protein expression and the state of arrest in *bam* mutant cells, these data implicate Bam as a direct regulator of the switch from stem cell to cystoblast. Surprisingly, ectopic *bam*$^+$ had no deleterious consequences for male germline cells suggesting that Bam may regulate somewhat different steps of germ-cell development in oogenesis and spermatogenesis. We discuss a model for how *bam*$^+$ could direct differentiation based on our data (McKearin and Ohlstein, 1995) that Bam protein is essential to assemble part of the germ-cell-specific organelle, the fusome. We propose that fusome biogenesis is an obligate step for cystoblast cell fate and that Bam is the limiting factor for fusome maturation in female germ cells.

Key words: stem cell, differentiation factor, Bag-of-marbles protein, ectopic expression, *Drosophila*, *bam*, germ cell

INTRODUCTION

Cell differentiation underlies the development of any multicellular organism and factors that regulate a choice between cell fates have been identified in a number of experimental systems. In some cases, the action of these factors is cell autonomous since they are partitioned at cell division (Doe, 1996; Spana and Doe, 1995; Knoblich et al., 1995; Kraut et al., 1996). Cell non-autonomous mechanisms for cell specification have also been described in a variety of systems (Kramer and Cagan, 1994; Zipursky and Rubin, 1994; Henderson et al., 1994; Crittenden et al., 1994; Orkin, 1995). Irrespective of cell autonomy or non-autonomy, asymmetry generally produces a rapid transcriptional response in the targeted daughter cell (Jessell and Melton, 1992; Orkin, 1995; Guo and Kemphues, 1996).

By definition, stem cell divisions are developmentally asymmetric. Stem cells can present a special challenge for differentiation mechanisms since one daughter must remain a pluripotent stem cell while the other commits to specific cell fates (Horvitz and Herskowitz, 1992). The oogenic germ-cell lineage of *Drosophila* is an excellent system for studying stem cell fate and cell differentiation. Analysis of mutations that block early germ-cell differentiation and stem cell survival can identify regulatory molecules with very low abundance or highly restricted temporal expression (Mével-Ninio et al., 1991; Lehmann and Nüsslein-Volhard, 1991; Wang et al., 1994; McKearin and Spradling, 1990). In addition, the effects of mutations that specifically disrupt germ-cell differentiation can be studied directly since gametogenesis is not essential for viability (Spradling, 1993).

In *Drosophila* ovaries, stem cells lie at the anterior tip of the ovariole, a muscular sheath which contains the developing egg chambers (Spradling, 1993). Genetic (Wieschaus and Szabad, 1979; Schüpbach, 1985) and laser ablation studies (Lin and Spradling, 1993) showed that each ovariole contains 2-3 self-renewing germline stem cells. By analogy to the interactions between the distal tip cell and the germline stem cells in *C. elegans* (Ellis and Kimble, 1994), several workers have proposed that somatic cells could influence the activity of germline stem cells during *Drosophila* gametogenesis (Spradling, 1993; Fuller, 1993; Gönczy and DiNardo, 1996). Lin and Spradling (1993) demonstrated that laser ablation of the terminal filament cells altered the rate of germline stem cell divisions. Although this result establishes that somatic terminal filament cells can communicate with germline cells, the basis of this signaling is not known.

Fig. 1A shows that an oogenic stem cell division produces one cystoblast daughter as well as another stem cell. The cystoblast divides precisely four times and with incomplete cytokinesis to produce 16 interconnected cystocytes. The cystocyte cluster, termed the cyst, becomes surrounded by an

epithelial follicle cell layer; the complete unit is referred to as the egg chamber.

Germline stem cells differ from cystoblasts in two principal ways; stem cells separate completely from mitotic daughters and they divide as long as the animal is viable. Morphological studies suggest that the choice between a stem cell and a cystoblast involves coordinated changes of several cell biological features. Contractile ring closure must be modified as a consequence of the commitment to cystoblast fate, since cytokinesis remains incomplete when a cystoblast divides. The cystoblast must also activate a mechanism for counting mitoses since the cells withdraw from the mitotic cycle after four divisions. A third target of cystoblast commitment is the germ-cell-specific organelle called the fusome (Fig. 1B; Storto and King, 1989; Lin et al., 1994). The organelle's precursor, the spectrosome, is found in stem cells and cystoblasts as a large, spherical structure that is rich in membrane skeletal proteins (Lin et al., 1994; de Cuevas et al., 1996). The fusome begins to elongate and branch once cystoblast divisions begin, eventually entering each of the cystocytes through the intercellular bridges. The organelle not only grows during these divisions but also undergoes dramatic increases in the mass of membranous tubules that fill its core (Lin et al., 1994; McKearin and Ohlstein, 1995). Thus it is likely that the production of fusome components, both tubular membranes and membrane skeletal, increases following cystoblast commitment.

To date, the *bag-of-marbles* (*bam*) gene's products are the only molecular markers that distinguish cystoblasts from stem cells. In stem cells, Bam protein localizes to the spectrosome. In cystoblasts, *bam* gene transcription is induced (McKearin and Spradling, 1990) and Bam protein begins to accumulate in the cystoblast's cytoplasm (BamC) as well as remaining enriched in the cystoblast's spectrosome. In mitotically active cystocytes, Bam protein is found in the cytoplasm and the growing fusome (BamF; McKearin and Ohlstein, 1995). Finally, when cystocyte divisions stop, BamC disappears while BamF continues as a fusome component.

Analysis of Bam protein expression in various genetic backgrounds indicates that cytoplasmic Bam can direct cystoblast/cystocyte differentiation (McKearin and Ohlstein, 1995). Inactivating *bam* mutations block germ-cell differentiation, causing the proliferation of thousands of cells with characteristics of stem cells (McKearin and Ohlstein, 1995). Thus, bam^+ is necessary to suppress stem cell fate in stem cell daughters and might directly promote cystoblast differentiation.

This paper reports the results of bam^+ ectopic expression studies that address the role of bam^+ in stem cell versus cystoblast differentiation. Heat-shock-induced bam^+ expression in adult female germline stem cells blocks further self-renewing divisions but differentiated germ cells that were not stem cells at the time of heat shock apparently developed normally. Females that were heat-shocked throughout their life cycle developed normally except that they eclosed as germline-less adults. Together with the results of earlier investigations, these data identify bam^+ as both necessary and sufficient to suppress stem cell behavior and assign to bam^+ the characteristics of a limiting factor for cystoblast differentiation. These conclusions are discussed in the context of a putative biochemical role for Bam protein in regulating fusome biogenesis.

MATERIALS AND METHODS

Fly stocks and genetic crosses

All fly stocks used were maintained under standard culture conditions. w^{1118} served as a wild-type control in all experiments. bam^{86} is a null allele of the *bam* gene (McKearin and Ohlstein, 1995). P[w^+; *lacZ*]BC69 is an enhancer trap insertion near the 5′-end of the *vasa* gene and expresses β-Galactosidase exclusively in germ cells (F. Laski, personal communication). P[w^+; *hsp70-bam*$^+$]^{11d} is a transgene inserted on a wild-type chromosome 3; P[w^+; *hsp70-bam*$^+$]^{18d} is a transgene inserted on an X chromosome marked with w^{1118}. To examine transgenic ovaries that expressed β-Galactosidase in germ cells, progeny were recovered from w^{1118}; P[w^+; *lacZ*]BC69 males mated to w^{1118}; P[w^+; *hsp70-bam*$^+$]^{11d}/ TM3, *Sb ri e* virgin females.

Microscopy and immunohistochemistry

Ovary dissections, fixation and immunohistochemistry were performed as described by McKearin and Ohlstein (1995). X-gal staining was performed as described in Margolis and Spradling (1995) except ovaries were fixed in 0.1% glutaraldehyde. Ovaries were viewed on a Zeiss Axiophot microscope using DIC optics. TdT-mediated dUTP-biotin nick-end labeling (TUNEL) was carried out as described by Gavrieli et al. (1992), with modifications described in Clark and McKearin (1996). Rhodamine-labeled phalloidin was reacted with fixed ovaries as described by the manufacturer (Molecular Probes, Inc.). Confocal microscopy was performed on a BioRad 1024 confocal microscope. Monoclonal supernatant 1B1 (anti-1B1 Ag), which reacts with fusome as well as cytoplasmic antigens, was used to detect the fusome and was the generous gift of H. Lipshitz (Zaccai and Lipshitz, 1996).

Germline transformation

P[*hsp70-bam*$^+$;w^+] was constructed as follows. A fragment that corresponded to most of the structural *bam* gene and 250 bp of genomic DNA downstream of the *bam* gene was recovered from a *bam* genomic clone by PCR. The 3′-end primer was complementary to the T7 RNA polymerase binding site while the 5′-end corresponded to +121 of the *bam* gene (McKearin and Spradling, 1990). Nucleotides for a *Bam*HI site were attached to the 5′-end primer for cloning purposes. The PCR fragment was digested to completion with *Bam*HI, recovered from a gel and cloned into the *Bam*HI site of pCaSpeR-hsp70 (Thummel and Pirotta, 1992).

The final construct, pCaspHSbam, was purified from two cesium chloride gradients and injected into *Drosophila* w^{1118} embryos at a concentration of 1 mg/ml together with pΔ2-3 plasmid DNA (Robertson et al., 1988) at 100 μg/ml as a transposase source (Spradling and Rubin, 1982; Rubin and Spradling, 1982).

Heat-shock protocol

Flies were heat-shocked in plastic vials that had a plug of standard fly culture media in the bottom. The top rayon plug was pushed into the vial to reduce the volume that contained flies. Vials were submerged in a 37ºC water bath to the level of the bottom of the rayon ball for 1 hour followed by transfer to fresh vials containing wetted yeast for recovery. Heat induction regimes of various periodicity, described in the text, were used throughout the course of these experiments.

RESULTS

The *bam* transgene

Previously, we had shown that a 3.0 kb *Sac*I-*Bam*HI fragment of genomic DNA could fully rescue *bam* mutant flies (McKearin and Spradling, 1990). This fragment contains the structural gene, two introns, 250 bp of genomic DNA down-

stream from the 3′-end of the transcript and 900 bp of genomic DNA from the gene's 5′-end. The construct faithfully reproduces the *bam* transcription pattern (B. Ohlstein, unpublished data), such that transgenic *bam* mRNA is detectable in cystoblasts and M2 cystocytes, but not in stem cells (McKearin and Spradling, 1990). To drive ectopic *bam*$^+$ transcription, a fragment that carries most of the *bam* transcription unit, including the full open reading frame, plus approximately 200 bp of genomic DNA that lies 3′ to the gene was inserted into the pCaSPeR transformation vector (Thummel and Pirrotta, 1992) immediately downstream of the *heat shock protein 70* (*hsp70*) promoter.

Three independent transgenic lines were established. The dominant, heat-induced phenotypes described in this paper have been tested in all of these lines and found to be indistinguishable.

Phenotypic rescue with the inducible *bam* transgene

To verify that the transgenes were functional and encoded *bam*$^+$ activity, transgenic chromosomes were crossed into flies that carried chromosomal *bam* null mutations (McKearin and Spradling, 1990). In the absence of heat shock P[*w*$^+$; *hsp70-bam*$^+$]^{18d}/+; *bam* females (Fig. 2A) were sterile and accumulated tumorous egg chambers and proliferating cells typical of *bam* mutant ovaries (McKearin and Spradling, 1990). Females that were heat-shocked daily for 2 days began to lay eggs within 7 days of heat shock. Some of these eggs hatched and produced viable, fertile adults. Although ovaries dissected from heat-shocked females contained a full complement of egg chamber maturation stages, rescued ovaries were smaller than wild type and contained a mixture of normal and aberrant egg chambers.

The time course of rescue was determined by examining ovaries from transgenic females at various times after heat shock. Ovaries were dissected, fixed and stained with DAPI to visualize nuclear DNA and rhodamine-conjugated phalloidin to inspect ring canals (Materials and Methods). When ovaries were dissected 4 days after heat shock, ovarioles contained a broad range of egg chamber types. The most posterior chambers in ovarioles were filled either with *bam*-like tumorous cells or scores of nurse cells. These chambers rarely contained cells that were developing as oocytes. Anterior to the tumorous chambers were more normally formed chambers with between two and fifteen nurse cells and, often, a single oocyte.

Ovaries taken from animals 6-8 days after heat shock contained mostly well-formed egg chambers (Fig. 2B-D); follicle cells formed an epithelial monolayer around a central cluster of polyploid nurse cells and a single oocyte. However, the aggregate number of nurse cells plus oocyte was often less than sixteen. The most common number (~50% of egg chambers) was eight although chambers with between two and fifteen germline cells were found. In all cases, the number of ring canals was one less than the number of nuclei indicating that the germline complement of chambers arose from cystocyte divisions rather than fragmentation of larger clusters. Therefore *bam* cells began dividing as cystocytes once *bam* transcription had been induced by heat shock but cystocyte clusters frequently stopped dividing before completing four mitoses.

Interestingly, oogenic rescue was not reversible; that is,

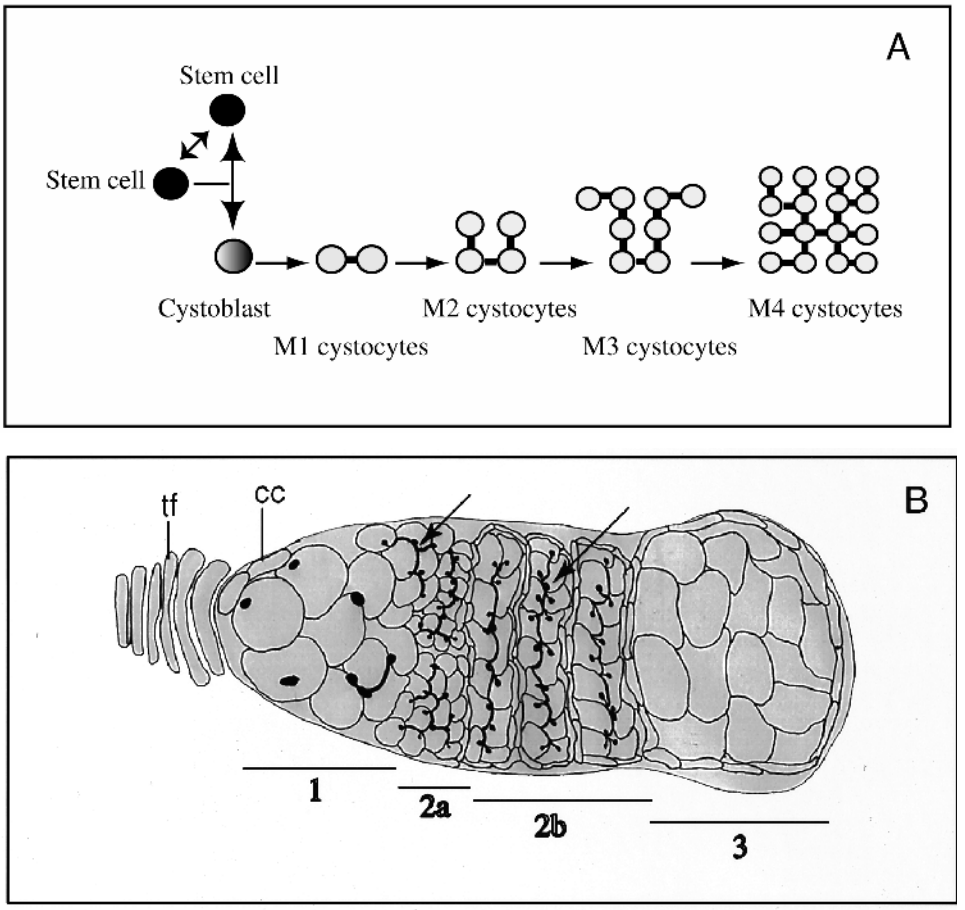

Fig. 1. The *Drosophila* germline lineage and the germarium. (A) A schematic representation of the germ-cell lineage. Stem cell divisions produce stem cell and cystoblast daughters. Cystoblasts divide precisely four times with incomplete cytokinesis, producing clones of two (M1), four (M2), eight (M3) and sixteen (M4) interconnected cells termed 'cysts'. Panel B is an idealized drawing of a germarium. Region 1 contains stem cells, cystoblasts and M1-M3 cysts. M4 cysts, that have completed dividing but are not yet enveloped by a follicular epithelium, are in Region 2a. Region 2b includes the lens-shaped cysts that have been surrounded by follicle cells while Region 3 contains a single spherical egg chamber. Spectrosomes in stem cells and cystoblasts and the elongating and branched fusomes of each germ-cell cyst are drawn in thick, dark lines (see arrows for examples). tf, terminal filament cells; cc, cap cells.

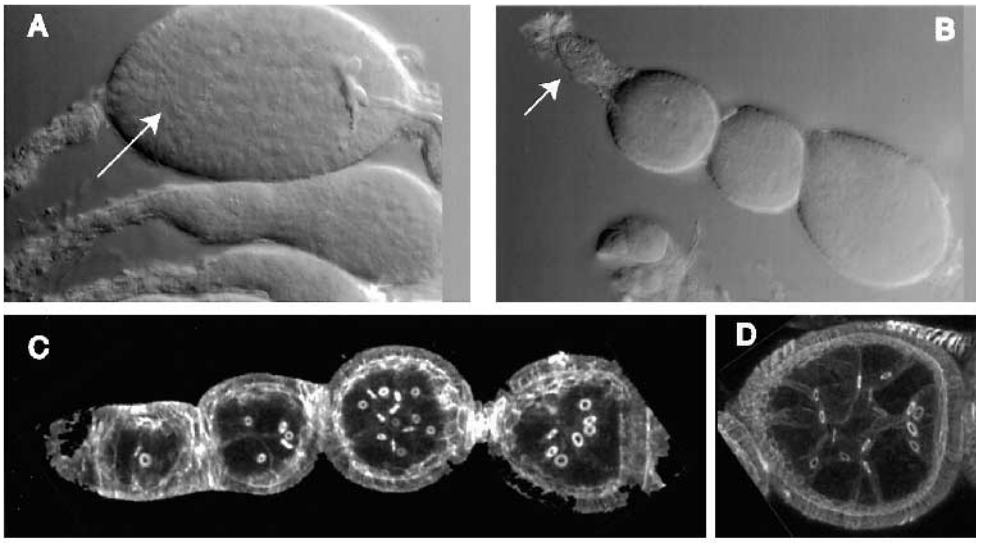

Fig. 2. Transgenic rescue of *bam* phenotype. Anterior is to left in all Panels. (A) An example of ovarioles isolated from P[w^+; *hsp70-bam*$^+$]^{18d}/+; $bam^{\Delta 86}/bam^{\Delta 86}$ females that were not heat-shocked. The ovarioles are bloated by the overproliferation of undifferentiated *bam* mutant germ cells (arrow). (B) An ovariole from a P[w^+; *hsp70-bam*$^+$]^{18d}/+; $bam^{\Delta 86}/bam^{\Delta 86}$ female that was heat-shocked four times in two days. This ovariole contains three morphologically normal egg chambers representing multiple stages of maturation and a shriveled germarium (arrow, see text for explanation). (C) An ovariole (not the same as presented in B) from a P[w^+; *hsp70-bam*$^+$]^{18d}/+; $bam^{\Delta 86}/bam^{\Delta 86}$ female stained with rhodamine-conjugated phalloidin to visualize ring canals connecting germ cells. The ring canals are a measure of the number of times cystocytes divided and whether all germ cells in a cluster are clonally related. Note that, although all the chambers shown contain ring canals, none of the four in this ovariole have a normal number (see text). (D) An example of a fully rescued egg chamber. Note that the chamber contains sixteen germ cells connected by fifteen ring canals. An oocyte lies at the chamber's posterior end with four ring canals displayed on its plasma membrane.

females did not begin to produce tumorous cysts once heat shocks were stopped. In addition, a consistent feature of ovarioles from flies 6-8 days post-heat shock was that germaria were greatly reduced in size (Fig. 2B, arrow). This observation suggested that the production of egg chambers following rescue by *bam* induction was limited. The significance of this observation will be presented in greater detail below.

Taken together, the restoration of germ-cell development and production of egg chambers showed that the P[w^+; *hsp70-bam*$^+$] transgene could supply sufficient wild-type *bam*$^+$ activity for nurse cell and oocyte differentiation and to restore fertility, although rescue was incomplete.

Correlating *bam* mRNA and protein expression patterns

Previous investigations have shown that the cystoblast is the first cell in which *bam* mRNA can be detected (McKearin and Spradling, 1990). Following the appearance of *bam* transcript, Bam protein accumulates in cystocytes' cytoplasm (i.e. BamC; McKearin and Ohlstein, 1995). The number of BamC-positive cells varies depending on the number of incomplete cysts present in a particular germarium (McKearin and Ohlstein, 1995). After inspection of many anti-BamC-stained germaria, we noted some germaria contained only one BamC-positive cell. We scored these single BamC-positive cells (Fig. 3A) as cystoblasts because of their position in the germarium and the placement of the spectrosome within the cell (Lin and Spradling, 1997). By scoring the number of cells near the germarium's tip that had spectrosomes and the cytoplasmic placement of the spectrosomes, it has been possible to determine that not all cystoblasts are BamC-positive.

We considered two possible explanations for finding BamC in only a fraction of cystoblasts; BamC accumulation might be transient, corresponding to a specific part of the cystoblast/cystocyte cell cycle or BamC might accumulate to detectable levels only late in the cystoblast's lifetime, shortly before the cystoblast will divide.

We favor the latter hypothesis because our data do not support cell-cycle-dependent BamC expression. For example, experiments that allowed simultaneous viewing of the fusome and BamC demonstrated that BamC was always present in M1-M3 cysts. If BamC localization was cell-cycle dependent, we would expect some incomplete cysts would have been fixed at a BamC-negative stage. Nevertheless, to test directly for BamC cell-cycle dependence, we stained wild-type cysts with anti-BamC and propidium iodide or DAPI to assay changes in chromosome condensation that accompany the cell cycle. BamC was detected in all M1-M3 cystocytes (Fig. 1A) irrespective of the stage of chromosome condensation (not shown). The BamC staining pattern was altered in mitotic cells in one respect; in cystocytes with condensed chromosomes (in mitosis), BamC was distributed homogeneously throughout the cell whereas BamC was largely excluded from the nucleus in cystocytes with decondensed chromosomes (interphase). We presume that nuclear dissolution during mitosis accounts for the homogeneous distribution.

BamC in stem cells

Germline cells that carry mutations in *α-spectrin* or *hts* genes lack a fusome (DeCuevas et al., 1996; Lin et al., 1994, respectively) but still differentiate into cystocytes and, occasionally, oocytes (Yue et al., 1992). *bam*$^-$ germ cells, however, arrest differentiation well before the cystocyte stage. Thus *bam*$^+$ activity is essential even when formal fusomes cannot form suggesting that cytoplasmic Bam protein is active independent of association with fusomes (McKearin and Ohlstein, 1995). If correct, *bam*$^+$ transcriptional up-regulation and subsequent BamC accumulation in the cystoblast might be pivotal steps in establishing the cystocyte lineage. Since *bam*$^-$ cells arrest near the time of cystoblast differentiation, perhaps *bam*$^+$ expression is necessary and sufficient for cystoblast formation. We set out

to test some predictions of this hypothesis by forcing ectopic and elevated expression of Bam with *bam* transgenes driven by a heat-shock promoter.

The heat-shock regime imposed to induce the transgene had no effect on normal BamC expression; germaria of non-trangenic w^{1118} animals displayed the usual distribution of BamC-positive cysts (Fig. 3B). In order to test for transgene-dependent expression of BamC in germline stem cells, ovaries were dissected from P[w^+; *hsp70-bam*$^+$]^{11d}/+ females and stained with anti-BamC antisera. Fig. 3C shows a typical example of a germarium isolated 3 hours after flies had been heat-shocked. Note that BamC is easily detectable in all cells in the germarium, including the stem cells (compare Fig. 3C to 3B).

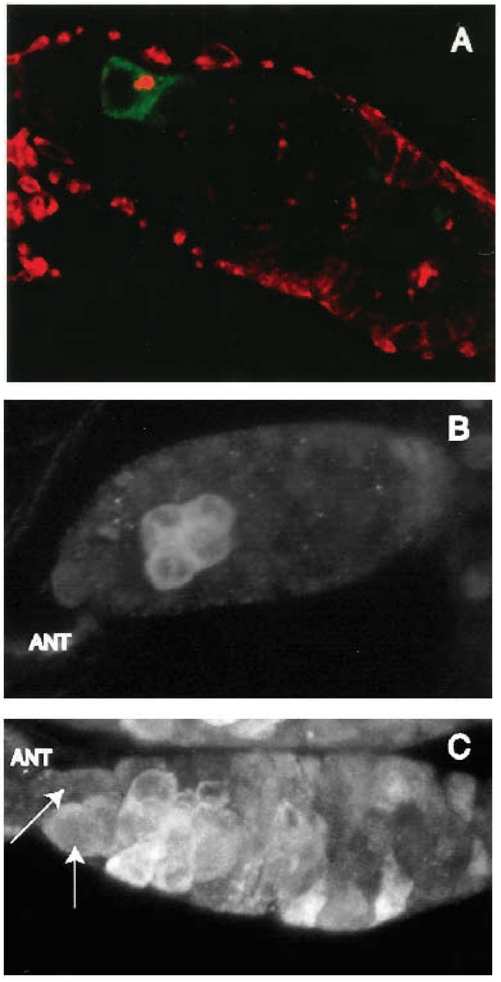

Fig. 3. BamC expression. The germarium in A is an example of BamC expression in a cystoblast. BamC antigen is shown in green while the mAb 1B1 antigen, a fusome component (Zacchai and Lipshitz, 1996), is shown in red. The position of the BamC-positive cell in the germarium and the position of the spectrosome in this cell identify it as a cystoblast. (B) An example of BamC distribution in a wild-type germarium 3 hours after a heat shock. Here a cyst of four cystocytes is accumulating BamC. (C) A germarium from a P[*hsp70-bam*$^+$]^{11d} *bam*$^+$/*bam*$^+$ ovary isolated three hours after heat shock. Note the widespread accumulation of BamC antigen in all germarial cells, including germline stem cells (arrows) at the anterior tip (ANT).

Experiments were conducted to establish the earliest times of BamC production and perdurance of BamC after heat shock. Ovaries dissected 30 minutes after heat shock showed high levels of BamC in somatic and germ cells. Furthermore, Bam remained in the cytoplasm of both somatic and germ cells even after 8 hours, with some exceptions. Occasionally, BamC-negative M4 cysts were found surrounded by BamC-positive cysts (not shown). Prior investigations had indicated that BamC is cleared from wild-type cystocytes shortly after the fourth cell division, suggesting that BamC is actively degraded in M4 cysts (McKearin and Ohlstein, 1995).

Consequences of BamC in stem cells

Previously, we have suggested that *bam* mutant germ cells inappropriately express many characteristics of germline stem cells (McKearin and Spradling, 1990; McKearin and Ohlstein, 1995). Perhaps *bam* gene transcriptional activation in the cystoblast daughter is essential to suppress the expression of stem cell programs. A prediction of this model is that ectopic *bam* transcription, and subsequent BamC accumulation, could block essential stem cell functions and extinguish self-renewing stem cell divisions, causing progressive loss of germ cells. In this context, we had noted that the germaria of heat-shocked P[w^+; *hsp70-bam*$^+$]^{18d} /+ ; *bam*$^{\Delta 86}$/*bam*$^{\Delta 86}$ flies were shriveled (Fig. 2B, arrow).

Wild-type and P[w^+; *hsp70-bam*$^+$]^{11d} *bam*$^+$ /*bam*$^+$ females were heat-shocked daily for 6 days. Ovaries from wild-type females contained a normal assortment of egg chambers and manifested wild-type germarial morphology (Fig. 4A). Staining these ovaries with anti-fusome mAb 1B1 antibody confirmed that wild-type germaria continued to produce all cyst stages (Fig. 4C). On the contrary, we found that heat-induced P[w^+; *hsp70-bam*$^+$]^{11d} *bam*$^+$ /*bam*$^+$ ovaries were dramatically smaller than the heat-shocked wild-type controls or those of non-heat-shocked sibs (not shown). Ovarioles contained maturing egg chambers but none were younger than stage 3 or 4 (Fig.4B). Most significantly, germaria were strikingly shrunken (Fig. 4B).

Germaria of P[w^+; *hsp70-bam*$^+$]^{11d} *bam*$^+$ /*bam*$^+$ animals that were 7 or more days post-heat shock appeared to be completely devoid of germ cells, filled instead with a small number of squamous cells. These cells could be derived from the germarium's somatic cells or could be quiescent germline cells. We used a monoclonal antibody that reacts with fusome proteins (anti-1B1 Ag; Materials and Methods) to distinguish between these possibilities. Ordinarily, all germ cells in the germarium contain a fusome or a related organelle, the spectrosome (Fig. 4C; also, Lin et al., 1994); even *bam* mutant germ cells contain a fusome (McKearin and Ohlstein, 1995). However none of the cells in the shrunken germaria of heat-shocked P[w^+; *hsp70-bam*$^+$]^{11d} *bam*$^+$ /*bam*$^+$ females contained the diagnostic organelle (Fig. 4D).

Progressive depletion of germ cells

Shriveled germaria could have resulted from a mass killing effect caused by *bam*$^+$ induction or from a more targeted effect on developing germ cells. We reasoned that, if ectopic *bam*$^+$ had broad killing effects on oogenic cells, the normally orderly progression of egg chamber stages within germaria would be dramatically disrupted. On the contrary, if ectopic *bam*$^+$ effects were more specific for proliferating germ cells (stem cells, cys-

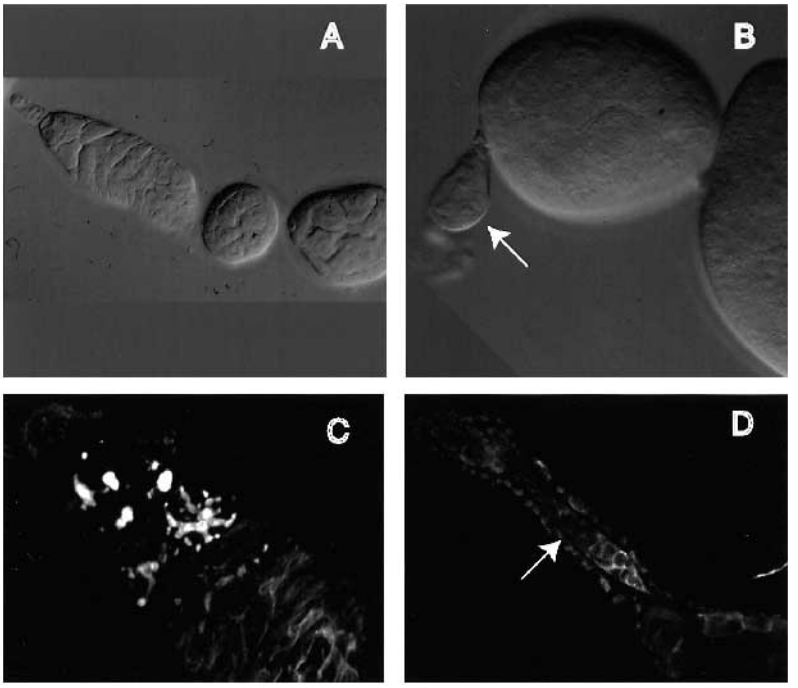

Fig. 4. Ectopic Bam induces stem cell loss. Anterior is to the left in all panels. (A) The anterior end of a wild-type ovariole. The DIC photomicrograph shows a germarium and two egg chambers posterior to the germarium. Note that the germarium is elongated and divided into discrete sections by the developing cysts. (B) An example of the anterior end of an ovariole from a P[w^+; *hsp70-bam*$^+$]^{11d} *bam*$^+$ /*bam*$^+$ female. These animals were heat-shocked four times over a 2 day period and their ovaries were dissected 8 days after the first heat shock. The photomicrograph shows part of a large, more mature chamber and a stage 6 chamber attached to a shriveled germarium. (C) A stack of confocal optical sections taken from a wild-type germarium reacted with mAb 1B1 to detect spectrosomes and fusomes. The projected image shows several fusomes at various stages of elongation. Note also that the mAb 1B1 appears on plasma membranes of somatic cells in the anterior and posterior regions of the germarium. (D) The reaction of mAb 1B1 against a germarium from a female that was heat-shocked as described above. Note that none of the mAb 1B1 antigen appears on a spectrosome or fusome-like structure; all immunoreactive protein is near the plasma membrane of germarial cells.

tocytes), we expected to find progressive loss of egg chamber stages as each extant cluster matured to the next stage and was not replaced by a younger developing cyst.

We tracked the effects of *bam*$^+$ induction on germ cells by dissecting transgenic ovaries at various intervals after heat shock (regime: 1 hour, 37°C/ 2 hours, 25°C/ 1 hour, 37°C). Ovaries were examined at 4, 6, 8, 10, 12, 16, 20 and 24 hours post-heat shock. Antibodies against the germ-cell-specific protein Vasa were used to distinguish germ line from somatic cells in ovaries (Lasko and Ashburner, 1990). The morphological changes of spectrosome/fusome organelle (Lin et al., 1994; Lin and Spradling, 1995) were used to monitor cyst maturation and egg chamber development. Stem cells and cystoblasts both have spherical spectrosomes that are fully contained within single cells. The spectrosome in stem cells, however, occupies a stereotypical position, lying immediately under the stem cell's plasma membrane nearest the cap cells (Lin and Spradling, 1995). Germaria were optically sectioned in a confocal microscope and the state of differentiation of each germ cell was scored according to the criteria outlined above. Examples are shown in Fig. 5; for clarity, each panel is a projection of optical sections taken through 5-7 μm and therefore does not represent a complete projection of the sampled germarium. The projection shown always included the youngest germline cell or cyst found in the sample.

Ovaries isolated from w^{1118} flies 12 hours (Fig. 5A) and 24 hours (Fig. 5B) post-heat shock (PHS) displayed wild-type distribution of stem cells, cystoblasts, developing cysts and maturing egg chambers. Transgenic ovaries isolated at 4, 6 (Fig. 5C), 8 and 10 hours (Fig. 5D) PHS also had normal complements of germ cells, cysts and chambers. We found, however, that germaria from 10 hour PHS ovaries often had only one stem cell (Fig. 5D) instead of the usual two or three. The trend toward stem cell elimination continued in 12 hour PHS ovaries where stem cells were rare. By 16 hours PHS, no stem cells could be found and the most terminal position in the germarium was usually occupied by 2-cell or 4-cell cysts (Fig. 5E).

Even as stem cells disappeared from germaria, the remainder of the germarium maintained normal overall germarial morphology. All the various stages of incomplete cysts characteristic of germarial region 1 were present. Germarial regions 2 and 3 remained filled with maturing cysts and egg chambers. As expected, once stem cells disappeared (between 12 and 16 hours PHS), we observed a processive disappearence of incomplete cysts from youngest to oldest. Thus the youngest cysts in 20 hour PHS germaria were generally 4-celled (Fig. 5F). This trend continued as ovaries from longer intervals after heat shock were examined. We found that ovaries from P[w^+; *hsp70-bam*$^+$]^{11d} *bam*$^+$ /*bam*$^+$ flies 48 hours PHS did not have cystocytes characteristic of germarial region 1 at all but did have cysts typical of regions 2a, 2b and 3. In germaria from 72 hour PHS ovaries, cysts of region 2a were missing but cysts typical of region 2b and 3 were present; 96 hour PHS ovaries were missing region 2b cysts but retained region 3 chambers. Finally, ovaries from 120 hour PHS flies had only shrunken germaria that did not contain any germline cells. Egg chambers representing all post-germarial stages of oogenesis were present in the remainder of the ovariole; in fact, each wizened germarium was connected to a maturing egg chamber (between stages 4 and 6) by a short stack of interfollicular stalk cells.

Is ectopic Bam toxic to stem cells?

Studies of a variety of genes have shown that apoptosis is a common cellular response to inappropriate developmental signals induced by ectopic expression as well as gene inactivation (Abrams, 1996; Asano et al., 1996). Time-course experiments indicated that the majority of stem cell elimination occurred between 6 and 12 hours PHS. In order to test if Bam expression induced apoptosis in germline stem cells, we assayed P[w^+;

hsp70-bam^{+}]^{11d} *bam*$^{+}$ ovaries by TUNEL labeling (Gavrieli, 1992) at 6, 8 and 11 hours after heat shock. Cells in germarial region 1 were not labeled by the TUNEL assay, implying that ectopic *bam*$^{+}$ expression did not induce apoptosis in stem cells, cystoblasts or actively dividing cystocytes (not shown).

Often one group of TUNEL-positive cells was observed in germarial regions 2a or 2b, where postmitotic cystocytes become enveloped by follicle cells. Because of their size, position and proximity to one another, we judged that these TUNEL-positive cells were cystocytes of one cyst undergoing synchronized apoptosis. Ectopic *bam*$^{+}$ expression near the end of cystocyte divisions might disrupt normal cystocyte differentiation because regulated Bam accumulation appears to be important for terminating cystocyte divisions (Hawkins et al., 1996; McKearin, 1997). Irrespective of the reason for occasional post-mitotic cystocyte death, many germarial region 2 cysts were unaffected and appeared to mature normally since egg chamber production continued for 7-10 days after heat shock.

A related explanation for stem cell loss is that *bam*$^{+}$ induction caused these cells to die by means other than apoptosis. Examination of heat-shocked transgenic germaria by visible and confocal microscopy did not reveal any signs of cell necrosis in germarial region 1. Stem cells survived for up to 12 hours PHS; no necrotic cells were observed in these germaria despite examinations at 2 hour intervals. However, necrotic cell death is more variable than apoptosis and detection is less reliable. Visual inspection of the tissue might not reveal necrosis if cell death was limited specifically to stem cells and cellular debris was cleared very rapidly.

General cell function and *bam* expression

The experiments described above showed that adults' germline stem cells had exquisite sensitivity to *bam*$^{+}$ induction. At the same time, the phenotypes of heat-shocked transgenic females suggested that somatic follicle cells were insensitive to Bam expression since cyst formation and egg chamber maturation continued even while stem cells were being eliminated. However, these experiments only tested the consequences of initiating *bam*$^{+}$ induction after adults had eclosed. We therefore tested the effects of *bam*$^{+}$ induction over the entire course of the life cycle by a series of daily heat shocks from embryos through adults (regime: 1 hour, 37°C/ 2 hours, 25°C/ 1 hour, 37°C). Animals that eclosed under these conditions showed somatic development and adult viability that was indistinguishable from wild type.

Although *bam* expression throughout the life cycle did not affect viability or somatic development, heat shocks repeated over the life cycle produced germline-less adult females. The heat-shocked ovaries resembled those produced from a dysgenic cross (Wei et al., 1991) or the progeny of *tudor* or *oskar*^{301} mutant females (Boswell and Mahowald, 1985; Lehmann and Nüsslein-Volhard, 1986).

Since it was necessary to verify that cells within the mutant ovaries were indeed somatic, we repeated the experiment using P[*w*$^{+}$]BC69^{vasa}/+; P[*w*$^{+}$; *hsp70-bam*$^{+}$]^{11d}/+ animals. P[*w*$^{+}$]BC69^{vasa} is an enhancer trap transposon inserted into the *vasa* gene and it expresses β-galactosidase in germline cells but not in somatic cells (F. Laski, personal communication). Daily heat shocks were initiated with 0- to 24-hour-old embryos collected from the appropriate cross (Materials and Methods) and continued until adult flies eclosed. The ovaries from such females were dissected and stained with X-Gal to reveal the pattern of β-galactosidase expression. P[*w*$^{+}$]BC69^{vasa}/+; TM3/+ ovaries were indistinguishable from wild type and all germline cells were X-Gal-positive (Fig. 6A,C). P[*w*$^{+}$]BC69^{vasa}/+; P[*w*$^{+}$; *hsp70-bam*$^{+}$]^{11d}/+ ovaries, in contrast, were developmentally rudimentary and X-Gal-negative (Fig. 6B,D).

When P[*w*$^{+}$]BC69^{vasa}/+; P[*w*$^{+}$; *hsp70-bam*$^{+}$]^{11d}/+ ovaries were dissected from newly eclosed females, we found that every ovary produced a few vitellogenic egg chambers that could complete oogenesis. Very often these chambers contained fewer than 16 germline cells but almost all formed a vitellogenic oocyte and manifested nearly normal nurse cell and follicle cell epithelial morphology. Despite having one or sometimes two egg chambers, the remainder of the ovary contained only ovarioles filled with somatic cells.

Developmental periods sensitive to ectopic Bam

The effects of ectopic *bam*$^{+}$ expression during either pre-adult development or in adults were apparently limited to oogenic germ cells. Yet germ cells go through several stages during pre-adult development. They form first as pole cells and associate with mesodermal cells to form the larval gonad (Williamson and Lehmann, 1996), proliferate by apparently symmetric divisions during larval stages (Spradling, 1993) and begin dividing asymmetrically as stem cells at pupariation (King, 1970; Spradling, 1993). This raises the question of whether sensitivity to ectopic Bam is a feature of germ cells at all stages or do these cells acquire the capacity to respond as they differentiate? To address this issue, we carried out experiments that delivered a heat-induced pulse of *bam*$^{+}$ during specific developmental stages over the fly's life cycle.

P[*w*$^{+}$; *hsp70-bam*$^{+}$]^{11d} transgenic embryos were collected over a 24 hour period and divided into nine groups. Each group received a single heat-shock treatment (Materials and Methods) at one developmental stage. For example, group 1 was heat shocked only as embryos, group 2 only as first instar larvae etc. When the adults that developed from these embryos had eclosed, fertility and ovarian morphological development were examined to determine the effects of the pulse of ectopic *bam*$^{+}$ expression. The results are shown in Table 1.

Table 1. Results of heat-shock treatment

Stage of heat shock	n	Fertile/ovarian morph.
embryo	9	+/WT
1st instar	9	+/WT
2nd instar	9	+/WT
early 3rd instar	9	+/GLL
late 3rd instar	9	+/WIZ
1 day pupae	9	+/WIZ
2 day pupae	9	+/WIZ
3 day pupae	9	+/WIZ
4 day pupae	9	+/WIZ

Animals were heat shocked (regime: 1 hour, 37°C/ 2 hours, 25°C/ 1 hour, 37°C) at the developmental stage indicated in the left column and allowed to develop to eclosion at 25°C. Eclosed females and males were aged for 4-5 days. Fertility was scored on the basis of crawling larvae in the vials that contained the eclosed adults. Ovarian morphology was scored by examination of dissected ovaries by visible light microscopy. WT indicates that ovaries contained all egg chamber stages and displayed normal germarial morphology; GLL indicates that ovaries were rudimentary and contained only a few mature egg chambers attached to shriveled germaria (see Fig. 6B,D); WIZ indicates that ovaries contained variable numbers of mature egg chambers but the germaria were wizened (see Fig. 4B).

All vials of eclosed adults contained viable larvae indicating that each group could produce at least some fertile eggs. Animals that were heat-shocked during the first or second instar stages showed wild-type fecundity and bore ovaries that were morphologically indistinguishable from wild-type ovaries. However adults that developed from larvae that had been heat-shocked during 3rd instar (HS-3I) were only weakly fertile. Ovaries dissected from HS-3I adults were largely germlineless, just as we had observed from females that were heat-shocked throughout their life cycle (Fig. 6). The only reason these females were not completely sterile was that they occasionally contained a few eggs in their otherwise rudimentary ovaries. Bam induction during pupal stages produced adult ovaries with a variable number of egg chambers but without any active germline stem cells as evidenced by shriveled germaria.

Thus, pre-adult germline cells were also sensitive to *hsp70-bam*$^+$ expression. Interestingly, germ cells acquired sensitivity during the 3rd instar, which correlates roughly with the time that germline cells switch from proliferative divisions, producing equal daughters, to germline stem cell divisions, producing unequal daughters (King, 1970; Lin and Spradling, 1997).

Spermatogenesis in P[*w*$^+$; *hsp70-bam*$^+$] *bam*$^+$ /*bam*$^+$ males

bam is unusual among genes of the tumorous egg chamber class since both oogenesis and spermatogenesis are similarly affected. Although RNA in situ hybridization to testes has not revealed the *bam* transcript expression pattern in testes, immunohistochemical experiments have shown that BamC accumulation and BamF fusome association in testes parallels that seen in ovaries (McKearin and Ohlstein, 1995). Thus *bam* is required, probably for closely related processes, in oogenesis and spermatogenesis (McKearin and Ohlstein, 1995; Gönczy et al., 1997).

Suspecting that transgenic *bam* expression in spermatogenic stem cells would have effects similar to those observed for oogenesis, we examined the effects of P[*hsp70-bam*$^+$] expression on male fertility and spermatogenesis. Surprisingly, heat-shocked males were fertile. Furthermore, we determined by morphological examination of testes that ectopic *bam*$^+$ expression had no discernible effect on spermatogenic stem cells or any other spermatogenic cell type. This was true when heat shocks were delivered over the course of the life cycle or when administered daily after eclosion. To exclude the trivial possibility that P[*hsp70-bam*$^+$] was not functional in males, we verified that the transgene could rescue the *bam* spermatogenic

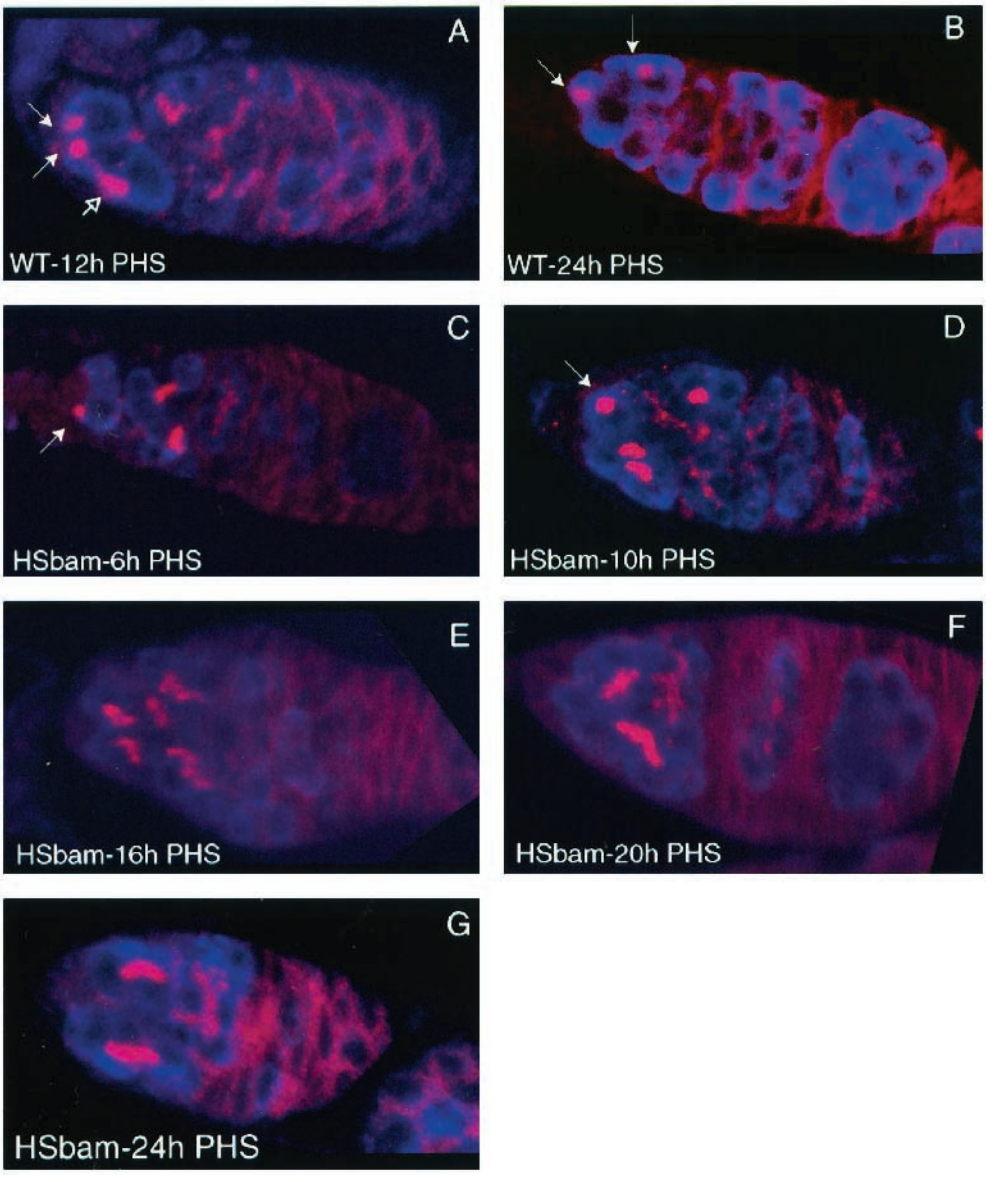

Fig. 5. Time course of stem cell elimination. Anterior is to the left in all panels. Vasa antigen staining is presented in blue, mAb 1B1 antigen staining is in red. (A,B) Wild-type germaria isolated 12 hours and 24 hours after heat shock, respectively. Closed arrows point to likely stem cells while the open arrow in A indicates a probable cystoblast. (C,D) Germline stem cells persist 6 and 10 hours, respectively, after heat shock. In each panel, arrows indicate likely stem cells. [See text for further details.] (E) A germarium isolated 16 hours after heat shock is displayed. At this time, no stem cells can be detected and M2 and M4 cysts occupy the most anterior positions. (F,G) The trend toward increasingly mature cysts at the anteriormost germarial position continues while overall germarial structure remains intact.

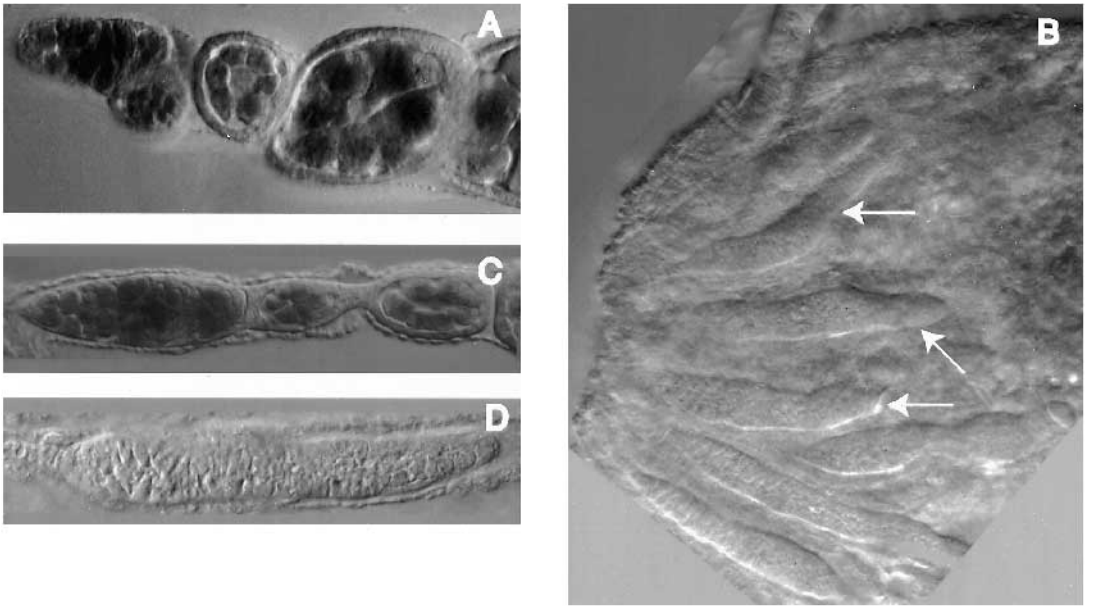

Fig. 6. Ablation of the pre-adult germline. (A) Ovaries from P[w^+; *LacZ*]BC69^{vasa}/+; +/TM3 flies that were heat-shocked daily during development from embryos to adults. These ovaries were fixed and reacted with X-Gal to localize expression of β-Gal. The BC69^{vasa} enhancer trap insertion is an insertion near the 5′-end of the *vasa* gene and expresses β-Gal solely in germ cells (F. Laski, personal communication.). The gonad in B is typical of those isolated from P[w^+; *LacZ*]BC69^{vasa}/+; +/ P[w^+; *hsp70-bam*$^+$] siblings of the flies used for A. It has also been reacted with X-Gal but none of the cells in the germaria or the remainder of the gonad are β-Gal$^+$. Instead the germaria remain undeveloped (arrows). (C) A higher magnification view of a P[w^+; *LacZ*]BC69^{vasa}/+; +/TM3 germarium and a stage two and four egg chamber that are positive for BC69^{vasa} activity. (D) A magnification micrograph of a P[w^+; *LacZ*]BC69^{vasa}/+; +/ P[w^+; *hsp70-bam*$^+$] germarium, tested for β-Gal activity, is shown. As before, these germaria contain only somatic cells.

defect by heat shocking P[*hsp70-bam*$^+$] *bam*$^{\Delta 86}$/*bam*$^{\Delta 86}$ males daily over their life cycles. Males that eclosed from these animals contained normal sperm although sperm production was well below wild-type levels.

DISCUSSION

Comparison of the levels of *bam* mRNA in wild-type germaria revealed asymmetric expression in stem cells and cystoblasts (McKearin and Spradling, 1990). The fact that Bam protein can be detected in stem cell spectrosomes (McKearin and Ohlstein, 1995) suggests that the gene is transcribed, although apparently at a level that remains undetectable by RNA in situ hybridization. In cystoblasts, the transcript becomes readily detectable and Bam protein can be found not only in the cystoblast's spectrosome but also accumulating in the cytoplasm of some cystoblasts (McKearin and Ohlstein, 1995).

Mutational inactivation of *bam* produces germ-cell hyperplasia; *bam* cells behave like ectopic stem cells since they divide completely and remain mitotically active long after they are born (McKearin and Spradling, 1990; McKearin and Ohlstein, 1995). Formally, therefore, *bam*$^+$ acts genetically as a suppressor of stem cell fate and/or a promoter of cystoblast fate. Using inducible transgenes, we found that *bam*$^+$ expression can specifically ablate stem cells, perhaps by converting these cells into cystoblasts.

P[w^+; *hsp70-bam*$^+$] alters *bam* expression

Heat-induced *bam* transcription fulfilled temporal and biochemical requirements for Bam since the transgene rescued otherwise *bam* null flies. However, only a modest fraction of egg chambers were wild type; many were only partially rescued. Most rescued chambers formed nurse cells and a single oocyte but often contained fewer than 16 cells. The simplest explanation for the occurrence of these subnumerary chambers is that rescued cystocytes stopped dividing before completing four mitoses.

We suspect that incomplete rescue by the P[w^+; *hsp70-bam*$^+$] transgene might mean that the timing of *bam*$^+$ expression or the protein's abundance might be critical for its function. For example, full rescue might require *bam* expression within the first few hours after a stem cell daughter is born. In this case, Bam expression might be coordinated temporally or stochiometrically (or both) with that of essential Bam-interacting proteins.

Prior to heat shock, germ cells in newly eclosed P[w^+; *hsp70-bam*$^+$]; *bam* adults are undifferentiated and occur as both free cells within the germarium and cells enclosed within a follicular epithelium. Once *bam*$^+$ was expressed by heat shock, germ cells were released from the differentiation block and progressed to become cystocytes, nurse cells and oocytes. Often the first egg chambers to develop were tumorous nurse cell chambers; these were found when ovaries were dissected within 72 hours post-heat shock. Most striking, however, were the well-formed egg chambers containing a mixture of nurse cells and a single oocyte. Some of these were sufficiently rescued to restore fertility to heat-induced transgenic flies. Many of the rescued chambers contained fewer than 16 cells; we do not know if these could produce viable eggs but the morphological characteristics of the rescued egg chambers would not preclude fertility.

Ectopic *bam* expression blocks extant stem cell function

Experiments using transgenic Bam expression in otherwise wild-type flies convinced us that ectopic *bam*$^+$ could extinguish oogenic stem cell function. Germaria expressing ectopic Bam were similar to germaria from which stem cells had been elim-

inated by laser ablation (Lin and Spradling, 1993). Furthermore, direct examination of germaria expressing ectopic *bam*$^+$ showed progressive loss of all classes of germ cells once stem cells disappeared.

One possible explanation for the germ cell effects of ectopic *bam*$^+$ is that Bam is simply toxic at the levels induced by heat-shock. We consider this unlikely for several reasons; heat-induced *bam*$^+$ expression had no effect on P[*w*$^+$; HS-*bam*$^+$]; *bam*$^+$ males, on larval/pupal somatic development or when animals were heat shocked as embryos, first or second instars. The continued maturation of nurse cells and oocytes within completed egg chambers in adult ovaries indicates that these cells also continued their normal developmental program. Detailed analysis of the effects of *bam*$^+$ induction on germ cells over the first 24 hours post-heat shock showed that stem cells were eliminated within 12-16 hours. Yet even after stem cells were undetectable, we could find cystoblasts and all other stages of cyst assembly represented. This suggests that germ cells in incomplete cysts at the time of heat shock continued normal cystocyte differentiation despite transgenic Bam protein expression. We concluded that even follicle cell stem cells were unaffected by ectopic *bam*$^+$ since normal follicular epithelia formed in P[*w*$^+$; HS-*bam*$^+$]; *bam*$^-$ and under heat-shock conditions which eliminated germline stem cells during larval and pupal stages.

Visual examination of heat-shocked P[*w*$^+$; HS-*bam*$^+$]; *bam*$^+$ germaria within the first 12 hours did not reveal necrotic cells in the positions normally occupied by stem cells; apoptosis assays showed that ectopic *bam*$^+$ did not induce programmed cell death in stem cells. Thus, ectopic *bam*$^+$ eliminated germline stem cells specifically and efficiently but what became of those former germline stem cells?

One explanation that we considered was that stem cells simply stopped dividing but remained at the germarium's tip in a quiescent state. If this was true, we expected that mitotically inactive germaria should have retained the quiescent stem cells. Yet staining these germaria with anti-1B1 Ag antibodies revealed that no cells retained fusomes or spectrosomes. Taken together with the fact that ectopic *bam*$^+$ induction did not cause germ cells to simply lose fusomes (immunological double-labeling with Vasa and mAb 1B1), we consider it unlikely that *bam*$^+$ simply induces stem cell quiescence.

We believe that the simplest explanation for P[*w*$^+$; *hsp70-bam*$^+$] transgene-dependent elimination of germline stem cells is that *bam*$^+$ expression caused stem cells to divide as cystoblasts. Some of the processes that we expect will be altered in cystoblasts compared to stem cells are contractile ring formation and closure, fusome growth and composition, and cell-cycle coordination. We anticipate that activation in stem cells of some, or all, of these cystoblast programs interferes with a stem cell's capacity for self-propagating divisions thus eliminating germline stem cells.

It is not possible at this time to demonstrate unambiguously that *bam*$^+$ stem cells became cystoblasts. The only molecular marker that distinguishes between these cell types is the level of *bam* transcription, which is not useful in these experiments since *bam* mRNA is derived from both a transgene and the native chromosomal locus. An unequivocal test for altered cell fate will await the identification of additional distinguishing markers.

Pre-adult *bam*$^+$ activation prevents stem cell formation

Future stem cells were apparently refractory to *bam*$^+$ ablating activity before the third larval instar. At third stage and beyond, however, germline stem cells were sensitive to Bam induction; adults eclosed without germ cells except for a few mature egg chambers. This temperature-sensitive period does not correspond to any previously described event in the differentiation of germline stem cells. According to King (1970), larval germ cells are mitotically quiescent. The fact that the path of germ-cell differentiation can be altered by *bam*$^+$ expression in third instar larvae might reveal that this time marks a key period in the switch from larval germ cells to oogenic stem cells.

The proposal that ectopic *bam*$^+$ can cause a stem cell to divide as a cystoblast could also explain why larval transgene expression ablated the oogenic germ line. Whereas inducing a stem cell to divide as a cystoblast in the adult germarium could produce an egg chamber, pre-adult gonads would not contain functional follicle cells to envelop a developing cyst. The products of premature cystoblast divisions could be lost from the pre-adult gonad and not represented in the eclosed adult. Survival of a few germ cells per developing ovary could explain the presence of one or two egg chambers when ovaries were removed from newly eclosed females.

Implications for the germ-cell lineage

The results of *bam*$^+$ induction in a *bam* null genetic background also shed new light on the developmental status of *bam* germ cells. We noted that transgene expression rescued the tumorous phenotype of *bam* cells, allowing normal and near-normal egg chambers to form. The fact that heat-shocked P[*w*$^+$; *hsp70-bam*$^+$]^{18d}/+; *bam*$^-$ females were fertile indicated complete rescue in many cases (Fig. 2B-D). However, we also found that germline stem cells were eliminated in these rescued ovaries (Fig. 2B,D). The cystoblasts that founded rescued egg chambers therefore arose either from stem cells that were converted to cystoblasts or from non-stem-cell *bam* cells that were rescued from their undifferentiated state by inducing P[*w*$^+$; *hsp70-bam*$^+$] expression. Since clusters of cystocytes that developed from rescued germ cells could become enveloped by an apparently normal follicular epithelium, we concluded that follicle cell stem cells remained active after heat-shock induction of *bam*$^+$.

These observations imply that *bam*$^-$ cells are not irreversibly transformed into a developmental dead-end but are rather arrested in a pre-cystoblast state. Since *bam*$^+$ expression was sufficient to restore germ-cell differentiation, we conclude that *bam*$^+$ can release at least some of the arrested cells to undergo normal cystoblast/cystocyte, nurse cell and oocyte differentiation. A *bam*$^+$-dependent shift from undifferentiated to cystoblast/cystocyte cells also offers a reasonable explanation for the recovery of 'tumorous nurse cell' chambers in heat-shock rescued *bam* ovaries. These could be produced when *bam* germ cells were already enclosed within a follicular epithelium at the time of heat-shock.

Fig. 7 presents a revised model of germ-cell differentiation that we believe accounts for these new observations. We propose that a presumptive cystoblast daughter (pre-cystoblast) of the stem cell division undergoes a maturation process during which *bam*$^+$ activation initiates cystoblast/cystocyte mitoses by

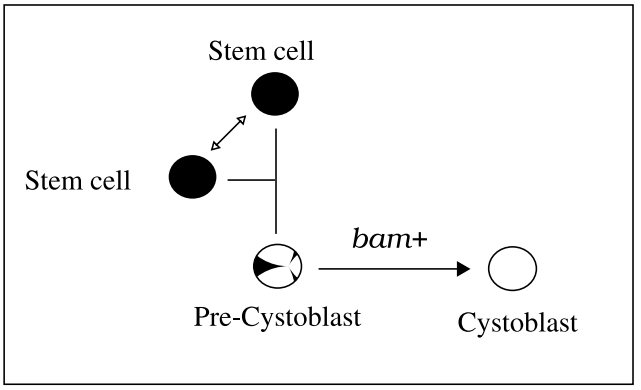

Fig. 7. A proposal for a modified stem cell lineage. This figure presents a modification of the traditional germ-cell lineage scheme, taking into account the data presented in the paper. The principal modification postulates that stem cell divisions produce two nearly equivalent cells, stem cell and pre-cystoblast. The pre-cystoblast daughter must activate *bam* transcription to accumulate sufficient BamC to complete differentiation into a cystoblast.

modifying the germ-cell division cycle. The model accommodates both intrinsic asymmetry that could arise from the partitioning of cytoplasmic factors into the cystoblast or extrinsic asymmetry based on the position of the daughter cells with respect to the apical somatic cells. These mechanisms are not mutually exclusive alternatives.

hs-Bam does not affect other stem cell populations

Immunohistochemical detection of BamC in heat-induced transgenic ovaries showed that Bam protein accumulated in all cell types. We noted however that follicle cells did not appear affected by P[w^+; *hsp70-bam*$^+$] induction; the development of the follicle cell epithelium from germarial region 2 through stage 14 egg chambers appeared normal in most cases. Therefore, P[w^+; *hsp70-bam*$^+$] did not disrupt epithelial follicle cell proliferation nor did it interfere with follicle stem cell activity (Margolis and Spradling, 1995).

Induction of ectopic Bam expression throughout the fly's life cycle did not have deleterious effects on the timing or process of somatic development. This means that Bam did not perturb significantly any somatic cell's function or block the function of any somatic stem cell populations. Therefore Bam is not a disruptor of general stem cell functions or cell proliferation.

The fact that ectopic *bam*$^+$ effects are limited to germline stem cells implies that essential *bam* cofactors are found, in functional form, only in germline stem cells and their precursors. Likely candidates for these factors are components of the fusome and spectrosome. Bam can be found in this organelle at least as early as first instar larvae (A. León, unpublished). Using anti-1B1 Ag antisera, Lin et al. (1994) have determined that a spectrosome-like organelle forms in embryonic germ cells at the time that these cells first associate with somatic cells to form the embryonic gonad. Presumably, germ-cell-specific spectrosomal proteins are induced in these cells and one or several of these could act as Bam cofactors.

Distinct roles in oogenesis and spermatogenesis?

Despite the fact that *bam* is essential for gametogenesis in both sexes and the null phenotypes are very similar (McKearin and Spradling, 1990), *bam*$^+$ transgene activation did not show any deleterious effects for spermatogenesis. This observation suggests that there exist fundamental differences between male and female germline stem cells and that *bam*'s time of action might be distinct in the two types of gametogenesis. Independently, Gönczy et al. 1997) have reached the same conclusion based on their very detailed analysis of *bam* spermatogenic cyst formation. These authors found that *bam* cells proliferate in males as gonial cells, with incomplete cytokinesis and synchronous mitoses, in contrast to proliferation as cells more closely resembling stem cells in *bam* females (McKearin and Ohlstein, 1995).

Can the different reactions of oogenic and spermatogenic stem cells be reconciled with Bam's putative role as an oogenic cystoblast differentiation factor. We propose that Bam acts together with at least one other germ-cell-specific factor to regulate differentiation in oogenic and spermatogenic germ cells. In oogenic stem cells, all the other essential factors are present and only *bam*$^+$ activity is necessary to initiate cystoblast differentiation. However, in spermatogenic stem cells, one or more of the essential factors is missing and ectopically expressed Bam is therefore inactive. This paradigm accommodates either a *bam*$^+$-dependent multistep differentiation pathway or a multicomponent complex that carries out essential steps for germ-cell differentiation.

What biochemical activity might account for Bam's role in differentiation?

An outstanding question for understanding Bam's action on germ cells' differentiation is what biochemical activity might account for its role as a cystoblast differentiation factor. This question has been complicated by the fact that Bam is a novel protein with no significant similarities to biochemically identified molecules. We have noted, however, that *bam* mutant fusomes are deficient in the membranous tubular reticulum that fills the core of fusomes. That observation and finding Bam protein in cystocytes' cytoplasm and the fusome prompted us to suggest that *bam*$^+$, and particularly cytoplasmic Bam protein, might be required to recruit vesicular material into the reticulum (McKearin and Ohlstein, 1995). A role for the fusome reticulum in directing a switch from stem cell to cystoblast-like divisions could explain both the *bam* loss-of-function and ectopic expression phenotypes.

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