bag-of-marbles: a Drosophila gene required to initiate both male and female gametogenesis

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In *Drosophila*, male and female gametes begin development when a stem cell divides to produce a cyst precursor. Subsequently, four special divisions give rise to a cluster of 16 interconnected cystocytes that develop into a single egg or 64 sperm. We identified and characterized a gene, *bag-of-marbles* (*bam*), that disrupts cyst formation in both sexes. An apparent null mutation causes abnormal cysts to form containing an excess number of cells that cannot differentiate into gametes. *bam* function resides within a simple 2.2-kb transcription unit encoding a single 442-amino-acid protein that shows similarity to the product of the *ovarian tumor* gene. The specific expression of *bam* RNA within female cystoblasts suggested that it might be involved in the specific cell-cycle alterations that occur during cystocyte divisions.

Stereotyped patterns of cell division often accompany differentiation (see Horvitz 1988). Recently, considerable progress has been made in understanding how the basic events of the eukaryotic cell cycle are regulated and particularly how cells are driven into mitosis (for review, see Murray and Kirschner 1989). However, relatively little has been learned about the programming of specialized cell cycles, nor is their developmental significance well understood. The production of 16-cell cysts during early gametogenesis in *Drosophila melanogaster* provides a favorable system for identifying genes that control specialized cell cycles.

The early stages of *Drosophila* gametogenesis are remarkably similar in the two sexes (for review, see Lindsley and Tokuyasu 1980; Mahowald and Kambysellis 1980). In both ovaries and testes, germ-line stem cells are sequestered at the anterior end of the organ. When a germ-line stem cell divides, one of the daughters remains attached and continues to function as a stem cell, whereas the other daughter becomes the "founder" of a 16-cell syncytial cyst. Four successive divisions of this cystoblast or spermatoblast (within the ovary or testis, respectively) produce a clone of 16 sister germ line cells that remain interconnected due to an unusual process of incomplete cytokinesis that generates interconnecting ring canals. In the ovary, a complement of somatic cells encloses each 16-cell cyst sometime after it is formed as it passes through a specific region of the germarium. In contrast, during spermatogenesis, the founding spermatoblast is enclosed by two somatic cells prior to the four spermatoblast divisions. Nonetheless, both processes generate cysts of 16 germ-line-derived cells surrounded by a layer of somatic cells. Shortly thereafter, the pathways diverge; female cysts go on to produce a single egg, whereas male cysts produce 64 spermatids.

These morphological parallels suggest that oogenesis and spermatogenesis may share genetic functions utilized in cyst production. Such genes would be needed to produce germ line founder cells, to program the four blast cell divisions, to limit cytokinesis and generate ring canals, and to mediate enclosure of cysts within an appropriate somatic cell layer. Genes controlling cyst production might be identified in two ways. Mutations should simultaneously produce both male and female sterility. Furthermore, abnormalities in gametogenesis should begin very early, corresponding to the time of cyst production. Extensive genetic searches for mutations disrupting male or female fertility have been carried out in *Drosophila* (see Lindsley and Zimm 1985, 1990). Although some candidate mutations that might define early cyst functions have been generated, including *benign gonial cell neoplasm* (*bgcn*; Gateff 1982), none have been characterized in detail. In contrast, a class of "tumorous egg chamber" genes required solely in the female for cyst formation, particularly the *ovarian tumor* gene (*otu*), have been analyzed more thoroughly (King and Storto 1988; Steinhauer et al. 1989). These mutations produce ovarioles with irregular proliferating

cells and abnormal cysts. Recently, studies of several loci that produce "tumorous" ovarian defects, including *Sex-lethal* (*Sxl*) (Schüpbach 1985; Steinmann-Zwicky et al. 1989), *sans fille* (*snf*) (Oliver et al. 1988; Steinmann-Zwicky 1988), and *ovo* (Oliver et al. 1990) have suggested that some of these genes may interfere with the process of germ line sex determination (see Nöthiger et al. 1989).

We used single P-element insertional mutagenesis (Cooley et al. 1988) to generate a collection of mutations disrupting *Drosophila* gametogenesis. In this report we describe a new gene, *bag-of-marbles* (*bam*), that encodes a function common to both male and female cyst production. Our experiments argue that *bam* may regulate cystoblast cell divisions and suggest a new hypothesis concerning the origin of tumorous egg chambers.

Results

Isolation of a new mutation, bam

Among a collection of 1317 lines bearing single P-element insertions (Cooley et al. 1988), we identified a recessive mutation that sterilized both males and females. This stock carried a single P transposon in polytene band 96C on the right arm of chromosome 3 (data not shown). *Df(3R)L16*, a deletion of polytene bands 96A–96E (Gonzalez et al. 1989), failed to complement the sterility in both sexes, suggesting that the mutant phenotype was caused by the insertion. Consequently, we examined the phenotype in gonads from adult homozygotes to determine whether this mutation might disrupt an early process of gamete development in both sexes.

Homozygous *bam* females were fully viable but did not lay eggs. Microscopic examination of the ovaries from such females revealed that oogenesis was disrupted at very early stages of development. Each of the tubular ovarioles within a wild-type ovary contains, at its anterior end, a germarium, where egg chambers are first assembled, and seven to eight egg chambers in successively older stages of oogenesis. *bam* ovaries remained small, because they contained ovarioles with abnormal germaria and only one to three small abnormal egg chambers. In a wild-type germarium, 16-cell cysts are produced and enveloped with follicle cells (Fig. 1A, bracket). *bam* germaria, like those of previously described tumorous mutants, contained a disorganized mass of cells and assembled cysts containing 50 to several hundred cells (Fig. 1B, bracket). Cysts assembled in *bam* ovaries usually contain 50–100 cells and often contain many hundreds. Very rarely, a few cells within a *bam* chamber attained a large size reminiscent of pseudonurse cells, a second phenotype observed in several tumorous ovary mutants (see King 1970). There was little difference between the phenotype of *bam* homozygotes and *bam/Df(3R)L16* females (Table 1), suggesting that the insertion mutation was a strong hypomorph or null allele.

Examination of *bam* testes suggested that gametogenesis was also disrupted at an early stage in males. These testes contained abnormal cysts populated with
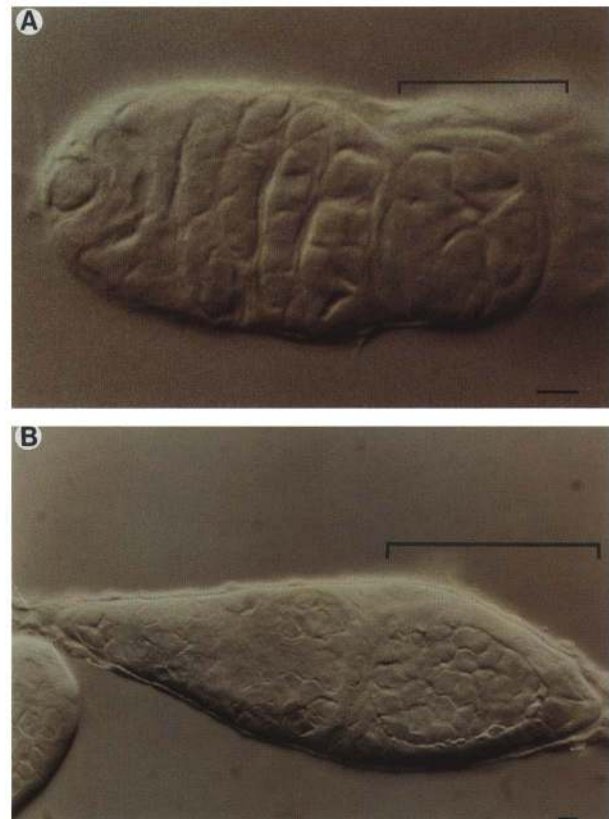
Figure 1. *bam* ovaries contain tumorous egg chambers. In each plate, anterior is to *left*. (*A*) Micrograph of a wild-type germarium. The bracket indicates a fully formed egg chamber with 16 cystocytes enclosed by a layer of follicle cells. (*B*) Representative example of a *bam* ovariole showing the tumorous germarium and egg chamber. Bar, 10 μm. Note that the magnification scales of *bam* and wild type are different by a factor of 2.4.

an excessive number of small cells the size of primary spermatocytes. Normally, the 16 primary spermatocytes within a cyst increase 25-fold in size prior to the onset of meiosis (cf. Fig. 2A,B). However, *bam* spermatocytes remained about the size of early spermatocytes (Fig. 2C). They never underwent subsequent morphological changes characteristic of meiosis and spermiogenesis. Consistent with an early developmental arrest, expression of two genes normally induced during primary spermatocyte growth, β2-tubulin (Kemphues et al. 1982; Michiels et al. 1989) and mst325 (DiBenedetto et al. 1988), was not detected on Northern blots of RNA from mutant testes (data not shown). A final unusual feature of the *bam* spermatocyte cysts was their progression into a highly refractile state (Fig. 2D). Much of the lumen of the testis became filled with these refractile cysts in the mutant.

Cloning the bam locus

The production of cysts with an increased number of cells in both male and female gonads was consistent with a role for the *bam* gene in controlling cystoblast

Table 1. *Deficiency testing* bam *phenotype*

Genotype	Number of females	Chambers		
		TC	PNC	15 + 1
bam/bam	16	420	0	0
bam/Df	17	446	1	0

These data test *Df(3R)L16* for complementation of *bam* and test whether the insertion *bam* allele behaves genetically as a null mutation by comparing the effect of one copy versus two copies of the mutant *bam* allele. Animals were kept at 23–25°C, and their ovaries were dissected 3–6 days after eclosion. The ovarioles of dissected ovaries were teased apart, and the chambers within were scored at 40× in Normarski optics on an Optiphot microscope. The number of discrete chambers that contained 15 nurse cells + 1 oocyte (15 + 1), pseudonurse cells (PNC), or only tumorous cells (TC) was tallied as a measure of the severity of the effects of the two genotypes. The number of females that produced the ovaries from which these egg chambers were recovered is given.

proliferation. We therefore undertook molecular studies. P-element sequences were used as a molecular tag to recover a genomic fragment flanking the insertion site. A 60-kb region was subsequently recovered from a cosmid library of wild-type genomic DNA. Figure 3 presents the structure of a 5.5-kb *Bam*HI fragment from this region that surrounds the site of transposon insertion.

To identify RNAs that might correspond to *bam* tran-scripts, a series of genomic DNA fragments derived from the 5.5-kb fragment were used to probe Northern blots of poly(A)$^+$ RNA from wild-type male and female flies (data not shown). Four transcripts derived from the region adjacent to the site of insertion were mapped at low resolution (Fig. 3). Three overlapping RNAs, γ, δ, and ε, were transcribed toward the left of the insertion site, whereas the fourth, α, was transcribed from the opposite strand.

To associate the *bam* gene with one or more of these transcripts, transformation rescue experiments with two constructs were carried out. *P[C-ry-a]* encompassed the entire rightward transcript, but truncated γ, δ, and ε. The *P[C-ry-;δε]* transposon truncated the rightward RNA but included all fragments that had labeled γ, δ, and ε. A germ-line transformant of each construct was tested for the ability to complement *bam* male and female sterility. *P[C-ry-a]* rescued gamete morphology and sterility in both sexes, whereas *P[C-ry-;δε]* had no effects on fertility or gonadal morphology. We concluded that the 2.9-kb genomic region within *P[C-ry-a]* contained the fully functional *bam* gene and that the α transcript was a strong candidate to encode *bam* function.

To examine the expression of the putative *bam* transcript in more detail, RNA was prepared from gonads and gonadectomized carcasses of wild-type and mutant adults and analyzed on Northern blots (Fig. 4A). Under the conditions of hybridization used, the 2.0-kb putative
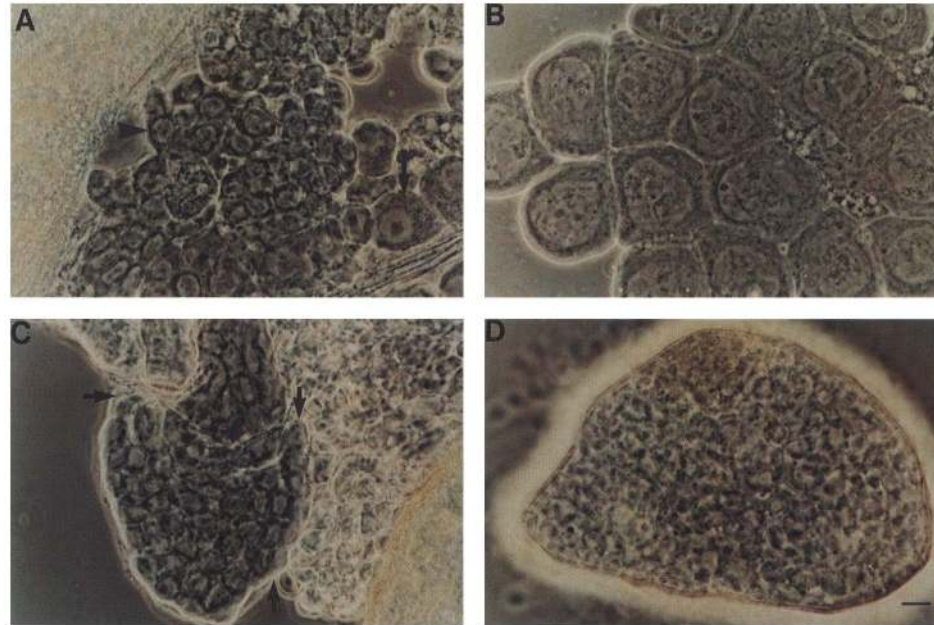

Figure 2. *bam* spermatocytes arrest during early spermatogenesis. (*A* and *B*) Spermatocytes from wild-type testes; (*C* and *D*) spermatocytes from *bam* testes. (*A*) Examples of early (arrowhead) and mid-stage (arrow) primary spermatocytes that have been released by manual disruption from wild-type testes. (*B*) A 16-cell cluster of late stage primary spermatocytes. Note the difference in size between early (*A*) and late stage (*B*) spermatocytes reflecting the accumulation of transcribed and translated gene products during the growth phase of spermatocyte development. (*C* and *D*) Examples of spermatocyte cysts found in *bam* testes. The boundary of an intact cyst is indicated by arrows in *C* for clarity. Note that the cells contained within this cyst are approximately equal in size to early primary spermatocytes. The highly refractile cells that characterize end-stage *bam* cysts are shown at *right* of the outlined cyst. A dramatic example of such a cyst is presented in *D*. In both *C* and *D*, *bam* cysts contain > 16 spermatocytes. All micrographs are taken in phase contrast at the same magnification (330×). Bar in *D*, 10 μm.
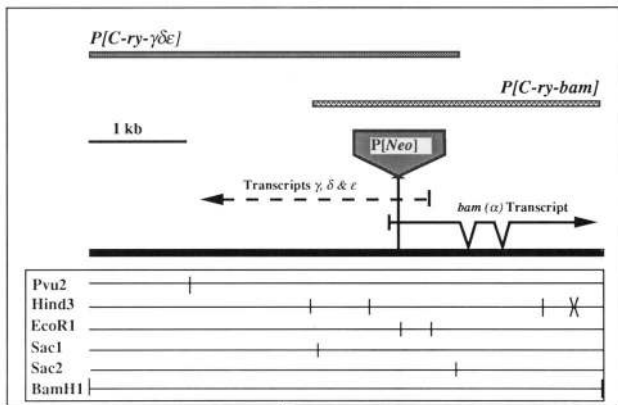
Figure 3. Chromosomal region of the *bam* gene. A restriction map for the wild-type *bam* locus is shown together with the position of the 5.2-kb *P[pUC-hs-Neo]* transposon in the *bam* mutant. The relative positions and orientations of the *bam* transcript and the family of overlapping mRNAs, γ, δ, and ε, are also shown; positions of introns are known for *bam* and are shown. The γ–ε transcripts, which do not rescue *bam*, have not been mapped as accurately and are therefore represented as a broken line, which indicates the genomic DNA that they span; hybridization experiments reveal that much of this DNA encodes introns. (*Top*) A representation of the DNA fragments used to construct the transposons that test for transformation rescue of *bam* phenotype.

bam RNA, as well as a smaller 0.7-kb species, was observed in wild type. The smaller RNA is due to limited homology between the probe and an RNA encoded elsewhere because it was never observed when more stringent hybridization conditions were employed. The 2.0-kb transcript was present in both wild-type ovaries and testes but could not be detected on even very long exposures of RNA from adult carcasses. The 2.0-kb RNA was expressed throughout oogenesis and in 0- to 2-hr embryos but was absent by 4 hr of embryonic development (Fig. 4B). Because the 2.0-kb transcript was specifically expressed in the tissues affected by the mutation, and because no other transcripts encoded within the 2.9-kb rescuing fragment were detected, we concluded that the 2.0-kb transcript encodes *bam* function. In the *bam* mutants, both ovaries and testes contained an RNA that appeared to be slightly larger than the normal 2.0-kb transcript; the molecular basis for the altered mobility of the mutant RNA has not been determined.

Structure and sequence of the bam gene

Once the authentic *bam* gene had been identified, we analyzed its structure in detail (Fig. 5). A 1571-bp cDNA was recovered from an ovary cDNA library and its sequence determined, including an 8-bp poly(A) tail not encoded in the genome. In addition, genomic sequence beginning at a *Hin*dIII site ~400 bp upstream from the site of insertion and continuing into the region defined by the cDNA was determined. The start site for the *bam* gene and the location of introns throughout the gene were identified by RNase protection experiments that covered the entire region encoding the 2.0-kb RNA (not shown). The *bam* transcript spanned ~2160 bp; it initiated 51 bp upstream from the site of transposon insertion and contained two small introns, yielding an mRNA of 1990 nucleotides, close to the 2.0-kb size observed on Northern blots. The first AUG codon is followed by only five amino acid codons prior to a terminator. However, the second AUG codon at position +180, which, unlike the first site, corresponds closely to the nucleotide consensus sequence for *Drosophila* initiation
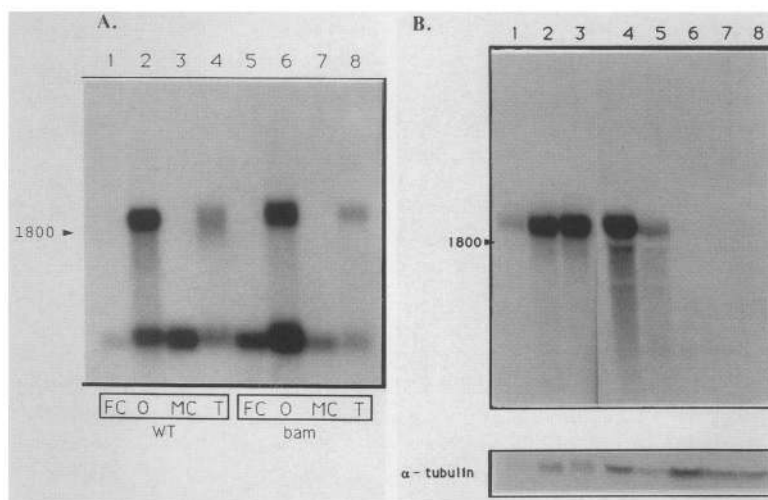

Figure 4. Distribution of *bam* transcript. (*A*) Hybridization of antisense *bam* probe to poly(A)+ RNA from the dissected tissues of wild-type (WT) or *bam* animals. The mRNA detected at ~2000 nucleotides corresponds to *bam* mRNA; the lower molecular weight transcript is a cross-hybridizing species whose identity is unknown. Higher stringency hybridization conditions eliminate this cross-hybridization (e.g., see Fig. 4B). (F) Female animal RNA; (M) male animal RNA; (FC) ovarectomized female carcass; (O) ovary; (MC) gonadectomized male carcass; (T) testis. (*B*) Egg chambers isolated from wild-type females that had been fed an enriched diet for 24–48 hr prior to dissection were staged according to the stages of King (1970). Egg chambers of germarium stage through stage 8 were collected from females <16 hr old because these animals had not yet begun to produce vitellogenic stages. Embryo RNA was isolated as described in Materials and methods. Following hybridization with *bam* probe, the probe was removed and the membranes reprobed with a radioactively labeled DNA for the ubiquitously expressed α1-tubulin (kind gift of L. Kalfayan) to verify mRNA integrity in each lane (shown at *bottom*). The order of the RNA loaded in a lane is germaria, stage 8 (lane *1*); egg chamber, stages 9–11 (lane *2*); egg chamber, stages 12–14 (lane *3*); embryonic development, 0–2 hr (lane *4*); embryonic development, 2–4 hr (lane *5*); embryonic development, 4–8 hr (lane *6*); embryonic development, 8–16 hr (lane *7*); embryonic development, 16–24 hr (lane *8*).

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-379 AGCTTATCGTCGTCAGAATTACAAAAACAAATTGAATATGAAAATGGGTTATTGCTACTTCATTATTATTGTCAC
-304 GATATATGATAATTTATCAAAATGTGATAAATCCCAAATTGTTAAATAATGCTTTGGCTTGCTTTATACAAAAC
-229 CACTAGATAATTAAAATATAGGTGGCCTAAATTGTTGCATGTTGTTTTATAATTAATCAGCAATTTGATTTGGTT
-154 GTGATCGACCAAATCAGTGTGTATAATTGTAGTTAAAATGTAAAGCTTCGTAATGGATTATTGAATCGCATTTCA
-79  AATTTCTTAAAATGCGCCCGGGTCAATGACCTTTTGAGGTGAC_CATAAAT_TGAAACTTATTTGTGCGACGGCAAC
     +1                                                       ⇓
     CCTGTTCTGGGTACTCGACATGATATCGATACGTTAACAACAAAGAGTCTGGACGCCATCATTCTTCCTCTTTCT
71   CCTGAATTCGCAGACAGCGTGGCGTCAGCGATTTCAAACGGTAAAAAGAACCTGGCGATAAGGAAAGATTTAAAA
            M  L  N  A  R  D  V  C  P  E  G  N                                    12
146  GGCAAAAATCGAGTGATTTGTGTGATTTAACTTAAGAATAATGCTTAATGCACGTGACGTGTGTCCTGAGGGCAA
      D  D  Q  Q  L  D  H  N  F  K  Q  M  E  E  H  L  A  L  M  V  E  G  N  E  N   37
221  CGACGACCAGCAGTTGGACCACAATTTTAAGCAGATGGAGGAGCATTTGGCCTTAATGGTGGAAGGCAATGAAAA
      E  D  P  R  K  A  T  C  E  Y  E  D  T  N  E  D  G  A  T  C  T  S  G  V  L   62
296  CGAAGATCCGAGGAAAGCCACTTGTGAGTACGAGGATACGAACGAAGATGGTGCAACCTGCACATCGGGGGTTTT
      S  E  I  Q  E  N  F  G  R  L  R  L  C  D  V  T  A  P  L  L  E  F  H  G  L   87
371  ATCCGAAATCCAGGAGAACTTCGGTAGACTCCGGTTGTGTGACGTTACTGCACCACTCCTCGAATTCCACGGTTT
      D  C  L  Q  Q  I  Q  K  R  S  R  I  L  H  S  T  V  L  R  P  R  S  R  D  P   112
446  GGATTGCTTGCAACAGATTCAAAAGCGCTCGCGCATTTTGCATTCGACGGTTCTCCGGCCAAGAAGTCGCGATCC
      E  G  V  L  V  T  G  P  K  Q  K  Q  L  Q  K  E  N  V  W  N  R  K  S  K  G   137
521  GGAGGGCGTGTTGGTCACCGGGCCAAAGCAGAAGCAACTGCAGAAGGAAAATGTGTGGAACCGGAAGAGTAAAGG
      S  A  S  A  D  N  I  E  K  L  P  I  T  I  E  K  L  H  M  I  G  L  H  G  D   162
596  CTCTGCGTCCGCGGATAATATTGAGAAACTGCCCATAACTATTGAGAAACTGCATATGATTGGTCTGCACGGCGA
      C                                                         L  E            165
671  TTGgtgagtcttctggagtatattcccaaatatatcacataataaaaagctccttatctaaacaatagCTTAGAG
      H  N  A  V  L  R  I  M  N  L  F  R  S  L  H  D  H  L  T  A  D  L  G  F  S   190
681  CACAACGCCGTGCTGCGTTTGATGAATCTGTTCAGATCCCTGCATGATCACCTGACCGCCGATTTGGGCTTCTCG
      R  Q  N  S  M  P  S  D  Y  L  F  D  M  P  V  K  S  T  M  P  K  S  L  N  V   215
756  CGCCAAAACTCAATGCCCTCGGACTATCTGTTCGGATATGCCGGTGAAGAGACGATGCCTAAGAGCTTGAATGTG
      R  Y  Q  L  Q  V  L  C  T  K  V  E  R  F  L  V  G  Q  R  R  T  L  E  S  N   240
831  CGCTACCAACTGCAGGTGCTGTGCACCAAAGTAGAGCGCTTCCTTGTCCAGCAGCGCCGCACCTTGGAGTCGAAT
      R  H  F  D  F  E  K  Y  D  E  C  D  K  L  L  K  G  F  A  S  Y  L  D  N  F   265
906  CGCCACTTCGATTTCGAGAAATACGACGAGTGTGACAAGTTGCTTAAGGGTTTCGCATCCTATTTGGACAACTTC
      K  L  L  K  P  K  M  R  N  R  N  G  N  S  G  S  N  A  D  K               286
981  AAACTGCTTTTAAAGCCCAAAATGCGCAATCGAAACGGAAACTCGGGGAGCAATGCGGACAAGTgtaagctgtag
                                                            F  H  T  Q  R  M  E  R  L  295
1044 atttgcaagcaaccattcagctattcctgcaacgattttattatttacagTCCATACTCAGCGCATGGAGAGATT
      L  I  G  L  R  D  W  I  K  A  A  H  L  S  V  H  V  F  N  W  E  M  D  L  E   320
1069 GCTAATTGGTCTGCGCGATTGGATCAAGGCTGCGCATCTCAGTGTGCACGTATTTAACTGGGAAATGGATCTGGA
      H  R  Y  S  G  A  M  T  E  S  H  K  S  L  N  E  R  A  I  L  L  S  G  A  E   345
1144 GCACCGCTACTCCGGGGCCATGACCGAAAGCCCACAAGTCGTTGAACGAGCGGGCCATCCTTTTGTCCGGTGCCGA
      L  R  A  A  E  A  R  G  I  S  A  E  D  L  F  I  A  Q  R  Y  K  L  G  G  P   370
1219 GCTAAGGGCGGCCGAAGCGCGTGGAATCAGTGCGGAGGATCTGTTCATCGGCCAGAGATACAAACTGGGAGGTCC
      I  Y  C  V  L  E  Q  H  E  F  L  S  A  L  I  A  N  P  E  T  Y  F  P  P  S   395
1294 GATCTATTGCGTTCTGGAGCAGCATGAGTTCCTCTCCGCTCTGATCGCCAATCCAGAGACCTATTTCCCGCCCAG
      V  V  A  I  C  G  P  Q  K  L  G  A  V  S  M  E  Q  P  S  A  S  E  E  F   420
1369 TGTTGTCGCCATTTGCGGGCCACAGAAGCTTGGCGCAGTGAGCATGGAGCAGCCGTCAGCGTCGGAGGAGGAGTT
      E  E  T  E  E  V  P  S  S  P  P  R  H  T  G  R  V  P  A  F  R  S  *        442
1444 TGAGGAGACCGAGGAAGTGCCATCATCGCCACCTCGTCACACCGGACGTGTACCTGCCTTCAGAAGCTAAACTAA
1519 TGCTGTGCACATCGATAAAAGAATGACAGCAAATATGCAATTTAAAAAAGCTACTCTTCTCATGGGAAGCAATAA
1594 TTTCGTAAAGTAAACATATCTATAGTGTAAGATATATTTGTCCAATAGTGCGGACTCCATATTTGTATTCGTGAA
1669 TAAGCTTATATAAGCTTTTTAAAAATATTTATCAAATCGATACAAACAAAATCAAAATGAAAACGATTTATTACC
1744 CCTGTTTTGAGATTGATAACAAATTTATATAAGTTTAACTGTGTTTACATTTATTTGGCAAAACTACAAATGTGT
1819 TTGCTTTTCACTTTTATAAATCTGTATTTTACTTAAACTTTAGAAATAAGAAATCCTTTAGTGCCCTGAATTTAT
1894 TTTGCAACTACGTTTTATTTGTATGAGGAACTTACCAGTTTTTCTTATTTGCTTTGCATTTGTATTTTGAAAGTC
1969 AAATAAATATTTACGATTTGTGTTTGGACT.poly-A
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Figure 5. Sequence of the bam⁻ gene. The bam gene sequence, together with 379 bp of sequence at the 5′ end of the gene, is shown. Sequences of the two introns are included in lowercase letters. The numbers at left refer to nucleotides; those at right refer to amino acids of the predicted protein. Nucleotide numbers are presented only for bases that appear in the mature bam transcript and therefore do not include the intron nucleotides. The putative start site of transcription, based on RNase protection assays, is indicated as +1. An arrow at +51 indicates the nucleotide that lies to the 5′ side of the site of insertion of the mutagenic transposon in the bam mutant. A putative TATA-box sequence at −31 is indicated in underlined italics. The ATG at position +186 initiates the open reading frame (ORF) for the putative bam protein, the predicted sequence of which appears as a one-letter code over the central nucleotide of the corresponding codon; this ORF terminates with a codon ending at +1514. A poly(A) addition sequence (boldface) lies 23 nucleotides upstream of the end of the transcript.

codons (Cavener 1987), is followed by a 442-amino-acid open reading frame that predicts a conceptual protein of 49 kD. This polypeptide contains an amino acid sequence at positions 404–432 that meets the criteria for a PEST sequence (Rogers et al. 1986). PEST sequences have been correlated with protein instability; the predicted bam protein carries a potential proteolytic cleavage site at residues 234–235. To complete the transcription unit, the mRNA contains a 3′-untranslated region of 480 nucleotides that ends 23 nucleotides downstream from a consensus poly(A) addition signal.

When the predicted polypeptide sequence for bam protein was used to search the Swiss Protein Database, the highest scoring match was to the protein from another Drosophila locus that mutates to produce tumorous egg chambers, the otu gene (Steinhauer et al. 1989). Weak similarity was detected within three regions spanning ~150 amino acids in the central region of the bam protein (Fig. 6). About 20% of the residues were identical and 15% corresponded to conservative replacements within these regions. This degree of similarity does not necessarily indicate shared function, and the biochemical roles played by both the bam and otu gene proteins remain unknown. However, this finding suggests that there may be some relationship between the tumorous egg chamber defects produced by bam and otu mutations.

bam is expressed specifically in early ovarian cysts

If bam functions during cystocyte divisions, we might expect it to be specifically expressed during this stage in the germarium. To test this prediction, we characterized bam transcript expression by hybridization of digoxigenin-labeled antisense bam RNA probes to whole wild-type ovaries and embryos (Tautz and Pfeifle 1989). Figure 7, A and B, shows that bam mRNA is present at high levels in only one or two cells located near the anterior tip of the germarium. Following a stack of ~10 somatic "terminal filament" cells, germaria contain two or three large germ-line stem cells, and then more posteriorly, one or two cystoblasts and two-cell cysts. The hybridization we observed was always located in germ-line cells immediately posterior to the stem cells. Thus, bam is expressed in cystoblasts and/or very early cystocytes. A much lower level of RNA was detected in older cysts within the germarium.

Transcription from the bam gene appeared to be repressed in maturing egg chambers because no labeling could be detected in subsequent stages of oogenesis (Fig.
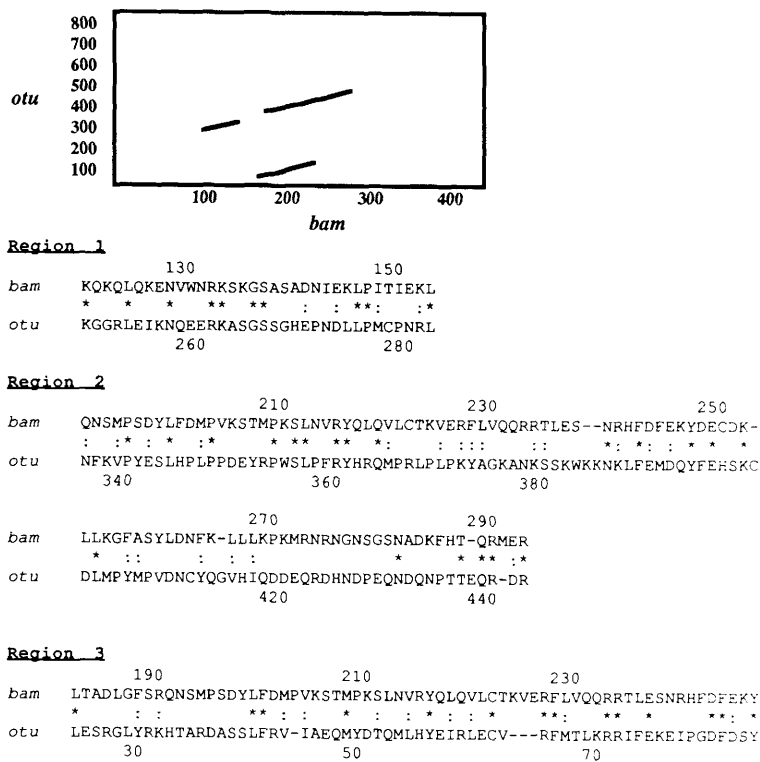
```
Region 1
               130                    150
bam   KQKQLQKENVWNRKSKGSASADNIEKLPITIEKL
      *    *    *   **  **    :  :  **:   :*
otu   KGGRLEIKNQEERKASGSSGHEPNDLLPMCPNRL
               260                    280


Region 2
                   210              230              250
bam   QNSMPSDYLFDMPVKSTMPKSLNVRYQLQVLCTKVERFLVQQRRTLES--NRHFDFEKYDECDK-
      :  :*  :  *  :*    *  **  **  *:   : :::     ::    *: *: : *  *  *
otu   NFKVPYESLHPLPPDEYRPWSLPFRYHRQMPRLPLPKYAGKANKSSKWKKNKLFEMDQYFEHSKC
           340              360              380


                   270              290
bam   LLKGFASYLDNFK-LLLKPKMRNRNGNSGSNADKFHT-QRMER
      *   ::       :  :  :          *       *  **  :*
otu   DLMPYMPVDNCYQGVHIQDDEQRDHNDPEQNDQNPTTEQR-DR
           420              440


Region 3
          190              210              230
bam   LTADLGFSRQNSMPSDYLFDMPVKSTMPKSLNVRYQLQVLCTKVERFLVQQRRTLESNRHFDFEKY
      :  :          **  : :  : * :: *       **:   **  *       **:  *
otu   LESRGLYRKHTARDASSLFRV-IAEQMYDTQMLHYEIRLECV---RFMTLKRRIFEKEIPGDFDSY
          30              50              70
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Figure 6. Comparison of the proteins predicted by the clones for the *otu* and the *bam* gene. When *bam*-predicted protein sequence was used for comparison to other protein sequences deposited in the Swiss Protein Database, the highest scoring match was achieved with comparison to the *otu* protein. The protein sequence for the *otu* protein was recovered from the Swiss Protein Database and was compared to our sequence for the *bam* protein in the Staden program on the JHU VAX in matrix analysis format. The x-axis shows numbers of amino acids of the predicted *bam* protein; the y-axis is the same for the *otu* protein (Steinhauer et al.). Alignments were carried out on the protein sequence alignment program on the JHU VAX computer. The numbers in parentheses indicate the number of identical residues and conservative replacements, respectively, in the stretch of amino acids indicated. Asterisks (*) indicate identities, whereas colons (:) indicate conservative replacements. Conservative replacements were scored as follows: [L-M-A-I-V; K-R; D-E; S-T; Y-W-F; N-Q]. H, G, C, and P were not scored as conservative replacements for any residues.

7C) until midway through stage 10, when high levels of RNA were observed in nurse cells (Fig. 7D). This RNA was transferred to the oocyte at the time of nurse cell breakdown and persisted in the mature egg and the early embryo (Fig. 7E). However, by gastrulation (3.5 hr), *bam* RNA could no longer be detected. The pattern of expression observed in situ thus supported the results of Northern blots (Fig. 4B).

Discussion

bam *is required very early to form both male and female cysts*

The *bam* mutation disrupted both male and female gametogenesis at very early steps. The earliest sign of egg development, the formation of interconnected cysts of germ-line cells, was blocked. Primary spermatocyte differentiation in the testis was also aberrant and appeared to stall prior to meiosis. In both cases, abnormal cysts formed, containing an excess number of cells that did not continue to differentiate. The similarity between the defects in both male and female gonads suggested that *bam* plays a role common to gametogenesis in both sexes. Consistent with this interpretation, the 2.0-kb *bam* mRNA encoding a single 442-amino-acid protein was found to be expressed in both gonads.

The cystoblast divisions in both ovaries and testes differ from the stem cell divisions that precede them. Cystoblasts and spermatoblasts divide exactly four times, whereas stem cells can divide indefinitely. The cystoblast contractile rings produce ring canals that maintain direct cytoplasmic contact between daughter cells. Finally, at least in the female, cystoblast divisions are cleavage-like because individual cells in 16-cell cysts are smaller than the precursor cystoblast. *bam* may be required to bring about these cell-cycle alterations. Because *bam* RNA appeared shortly after the stem cell division that produces each new cystoblast, the character, and possibly the number, of subsequent cystocyte divisions may be programmed at this time by the *bam* product in the cystoblast. The transient expression of *bam* mRNA and the fact that the protein carries a sequence that implies rapid turnover are consistent with the hypothesis that *bam* functions as a titratable counting factor during cystocyte divisions. In the absence of *bam* protein, stem cell daughters would be unable to modify their cell cycles following division, leading to an abnormal proliferation of stem-like cells within the germarium.

Potential cystoblast cells in the *bam* mutant ovaries apparently did not simply remain as stem cells. The small size of the ovary indicated that their ability to proliferate became very limited. Furthermore, cystoblast differentiation was not completely blocked in the mutant because groups of their daughter cells were surrounded by somatic cells to form abnormal cysts in both the ovary and testis. Although we have not looked for the continued presence of ring canals, some of these structures still form between the proliferating cells of other tumorous mutants (King 1970). The residual differentiation observed in the mutant gonads suggested that *bam* is not required for all the changes associated for cystoblast determination. Genetic tests suggested that the *bam* mutation we studied was a null allele and did not support the alternative view that the observed
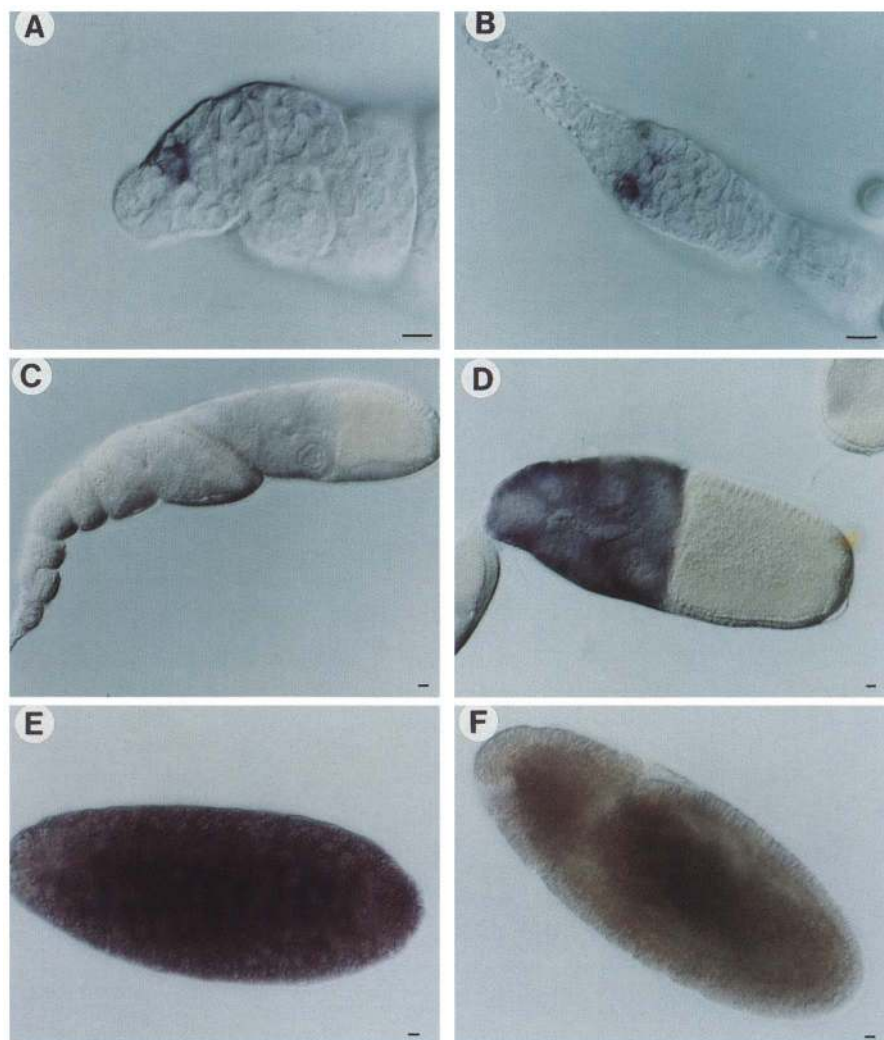
Figure 7. The expression of *bam*⁺ mRNA in ovaries. Ovaries were dissected from wild-type females and prepared for in situ hybridization by modifications to the methods of Tautz et al. Embryos were collected and prepared as described by Tautz and Pfeifle (1989) (see Materials and methods). Single-stranded antisense RNA probes for *bam* mRNA were hybridized to ovaries, and stable hybrids were visualized according to methods described by the manufacturer. Egg chamber stages are defined according to King (1970), and embryo stages are defined according to Campos-Ortega and Hartenstein (1985). Positive reactions for *bam* RNA appear as dark purple in these Nomarski micrographs. (*A* and *B*) Germaria: Cells at the location expected for cystoblasts are labeled; (*C*) ovariole with (unlabeled) egg chambers stages up to stage 10A; (*D*) Stage 10B egg chamber: The nurse cells are heavily labeled; (*E*) embryo, ~1.5 hr: labeling is seen throughout; (*F*) embryo, ~3.5 hr: No labeling is present above background.

differentiation was caused by a low level of residual *bam* activity. Molecular studies were also consistent with a null allele; P-element insertion within the *bam* transcription unit generated an altered mRNA that may no longer be capable of encoding *bam* function. Recently, we isolated a small deficiency allele of the *bam* gene that removes the promoter and the amino-terminal third of the putative protein (A. Hilliard and D. McKearin, unpubl.). The phenotype of this deletion allele is essentially indistinguishable from that described for the insertion allele.

A function for *bam* in establishing the number and character of cystocyte divisions suggests a role for the later expression observed in stage 10 nurse cells. *bam*

RNA was transported to the oocyte and persisted throughout the first 2 hr of embryonic development. The 13 cleavage divisions that occur during this time resemble cystocyte divisions: The number of divisions is precisely counted, divisions take place within a syncytium, and mitosis is induced prior to a doubling of the cell mass. *bam* may play a role in the cleavage cell cycle similar to its function during cystoblast divisions. To test this hypothesis, it will be necessary to genetically disrupt *bam* function after cyst formation, but prior to stage 10. The ability to rescue *bam* mutants with transposons containing genomic sequences opens several approaches to this task.

Cystoblast differentiation and cyst formation may not

occur autonomously. Germ line and somatic cells are known to interact late in oogenesis to pattern the egg chamber and early embryo (Schüpbach 1987; for review, see Manseau and Schüpbach 1989). However, germ-line cells require early signals from gonadal mesoderm cells to undergo sexual differentiation (see Nöthiger et al. 1989). Two observations suggested that *bam* acts only in germ-line cells. *bam* RNA was detected only in the gonad by Northern analysis and only in germ-line cells of the gonad by in situ hybridization. Furthermore, "enhancer-trap" transposons that normally express β-galactosidase only in ovarian germ-line cells were activated in the cells inside *bam* egg chambers (not shown). Construction of mosaic ovaries will be necessary to confirm the suggestion of these experiments that *bam* function is germ line autonomous.

Ovarian tumor genes and germ-line sex determination

The pathway of sexual differentiation in *Drosophila* differs between somatic and germ-line cells (for review, see Pauli and Mahowald 1990). Sex determination in germ-line cells is partially nonautonomous; signals from the somatic gonodal mesoderm are thought to be required to initiate and/or maintain the sexually differentiated state. The master regulatory gene *Sxl* is needed for this signaling (Schüpbach 1985; Steinmann-Zwicky et al. 1989). In the absence of *Sxl*, X/X stem cells produce tumorous cysts in the ovary that contain cells transformed toward primary spermatocytes. These observations suggested that correct sexual identity is required for cyst development and that the tumorous ovary phenotype may indicate a failure of germ-line sex determination. At least two other tumorous ovary genes, *snf* and *ovo^D*, genetically interact with *Sxl* and are proposed to carry out additional steps in germ-line cell sex determination (Oliver et al. 1987; Steinmann-Zwicky 1988; Oliver et al. 1990).

The similarity between the tumorous ovaries that result from *bam* mutation to those caused by *Sxl*, *snf*, or *ovo* mutants suggested an alternative model for *bam* function, in which the gene carries out a role in germ-line sex determination. Because *bam* is expressed and is functionally required, only in the gonads, it would have to encode a function limited to germ-line sex determination, unlike *Sxl* or *snf*. Because *bam* is required for male as well as female differentiation, it might act downstream of the previously characterized genes to execute male-specific or female-specific functions, a role analogous to that of *doublesex* in somatic cells. However, the limitation of *bam* expression to cystocytes and stage 10B egg chambers and the failure to detect different male and female transcripts favor the view that *bam* controls cell divisions directly, rather than indirectly through effects on sexual differentiation.

If *bam* does function to control cystoblast divisions, why is the phenotype in the ovary so similar to tumorous ovary genes that disrupt sex determination? We propose that this phenotype is a general sign that cellular interactions organizing the germarium have been

disrupted. These interactions may be required to maintain as well as to establish the normal differentiation of cells within the gonads, including their sexual differentiation. Any mutation disrupting the relative number and position of cells might therefore have extensive pleiotropic consequences. When cystoblast differentiation fails due to *bam* mutation, germ-line cells in the germarium may be plunged into sexual limbo, producing transformed and intersexual cells. Tumorous ovarioles would therefore represent germaria in which communication between germ line and soma has broken down, or where it occurs sporadically and to a limited degree. Only a fraction of the genes that have been shown to mutate to this phenotype would represent sex determination functions, whereas others would carry out roles necessary to maintain normal cell number, position, and communication within the germarium.

Relationship between bam *and* otu

The middle half of the *bam* protein contained regions with weak but significant similarity to the product of the *otu* locus. This structural similarity and their nearly identical phenotypes in females suggested that the two genes might be functionally related. Like *bam*, *otu* is expressed specifically in developing egg chambers and in the testis (Mulligan et al. 1988). *otu* RNA accumulates in mature eggs (although with different kinetics from *bam*—see Parks et al. 1986; Parks and Spradling 1987) and may encode an embryonic function because *otu^{11}* homozygous females produce embryos that die at an early stage (see Storto and King 1988). At present, there is no evidence that *otu* functions in the testis (Steinhauer et al. 1989), although a redundant function cannot be ruled out. Nonetheless, our results suggest that there may be (at least) two classes of tumorous egg chamber genes—genes required for germ-line sex determination like *Sxl*, *snf*, and *ovo*, and those like *bam* and *otu* that participate in early steps in gametogenesis and embryogenesis.

Materials and methods

Fly stocks

Flies were maintained on yeasted cornmeal/molasses media in bottles or vials as appropriate. Whenever selection for the *Neo* marker was required, as in recombination experiments for stock construction, G418 was added to the media to a final concentration of 600 µg/ml, as described in Cooley et al. (1988). The *bam* mutation was recovered from the original single P-element mutagenesis carried out by Cooley et al. (1988), and the specifics of the chromosomes and methods used to induce the mutation are described in that reference. All previously reported genetic locus designations are described in Lindsley and Zimm (1985, 1990). Transformation was carried out as described (Spradling 1986).

Cytology

Microscopy to evaluate the morphology of tissues of homozygous *bam* and wild-type animals was performed on a Zeiss Axiophot microscope equipped with phase and differential in-

terference contrast objectives at magnification values of $20-100 \times$. In the case of ovaries, tissue was dissected in saline and fixed with 2% formaldehyde or viewed unfixed, and ovarioles were teased apart to reveal the egg chambers. These preparations were mounted under a coverslip in excess saline or in saline/25% glycerol to prevent excessive distortion by compression. When the goal was to release cells from tumorous egg chambers to investigate their morphology under high magnification, individual chambers were manually disrupted or buffer was blotted from under the coverslip to force rupture of chambers by the pressure of the coverslip. These same techniques were used to examine testes.

Isolation of and hybridization to nucleic acids

DNA was isolated from *Drosophila* adults according to Cooley et al. (1988). Isolation of RNA and DNA from ovaries was as described previously (Parks et al. 1986). Embryos were collected on rich molasses plates and harvested at specified times to provide a stage-specific profile of embryonic transcripts. To isolate embryo RNA, dechorionated embryos were ruptured in a Teflon/glass homogenizer in buffer RHB, incubated at 37°C with proteinase K (~100 μg/ml) for 1 hr, and extracted with phenol/chloroform (50 : 50) until the interface was clear [buffer RHB: 25 mM Tris (pH 7.5); 20 mM EDTA (pH 8.0); 1% SDS; 0.25 M NaCl; 5 mg/ml heparin]. Nucleic acids were precipitated by the addition of 2 volumes (DNA) or 2.5 volumes (RNA) of ethanol, precipitates were collected by centrifugation, and pellets were washed with 75% ethanol.

Restriction enzyme and DNA modification enzyme (Boehringer Mannheim, New England Biolabs) incubations were performed according to manufacturer specifications. Poly(A)-containing RNA was purified on oligo(dT)-cellulose resin (Collaborative Research, type 3) by the batch methods described by the manufacturer and in Sambrook et al. (1989). Southern and Northern blotting procedures using GeneScreen nylon membrane, and labeling of DNAs by random hexamer priming were as described in Sambrook et al. (1989).

Hybridizations using DNA probes and subsequent washing protocols were performed at 65°C using the procedure of Church and Gilbert as described in Sambrook et al. (1989). Hybridizations using RNA probes were carried out at 65°C (except where noted in one instance) in 50% formamide, $5 \times$ SSPE; $5 \times$ Denhardt's solution, 1% SDS, 250 μg/ml denatured DNA, and 250 μg/ml yeast RNA. Final washes were carried out in 25 mM sodium phosphate (pH 6.8), 1% SDS, 2 mM EDTA (pH 8.0), and 0.1% nonfat dry milk at 68°C.

RNase protection assays

Uniformly labeled RNA probes were synthesized from cloned genomic DNA by bacteriophage RNA polymerases. Assays were carried out essentially as described by Sambrook et al. (1989). Each assay contained 0.5 ng of probe (5×10^8 to 8×10^8 cpm/μg) and 10 μg of poly(A)$^+$ RNA from the sample to be assayed. Positive controls were known masses of antisense *bam* transcripts derived from a *bam* cDNA clone; these also provide an internal quantification standard. Protection of probe by yeast RNA was included in all sets of assays as a negative control. Protected fragments remaining after digestion were separated on sequencing gels and analyzed by autoradiography of the dried gels.

DNA sequencing and sequence analysis

DNA was sequenced by the methods of dideoxynucleotide-de-

pendent chain termination either from nested deletions of plasmids or by initiation at oligonucleotides complementary to previously determined sequences. Sequences obtained were analyzed on the Staden Nucleic Acid programs on the VAX computer at Johns Hopkins University. Data base searches of the nucleic acid and predicted protein sequences were carried out on the same computer system.

In situ hybridizations to ovaries and embryos

Developmentally staged embryos were prepared and fixed as described in Tautz and Pfeifle (1989). Ovaries were dissected in EBR and fixed according to modifications provided by S. Parks and R. Lehmann. Briefly, tissue was fixed in 4% paraformaldehyde, 10% DMSO, and $1 \times$ PBS with 3 volumes of heptane for 30 min at room temperature. Tissue was then transferred to 4% paraformaldehyde/$1 \times$ PBS for additional fixation for 20 min at room temperature. Subsequent steps were as described in Tautz and Pfeifle (1989) beginning with methanol dehydration.

bam antisense RNA probes were prepared using the digoxigenin RNA labeling kit from Boehringer Mannheim following the manufacturer's instructions (RNA Genius Kit). Before use in the hybridization reactions, the average size of the probe was reduced to ~75 nucleotides following the protocol of Cox et al. (1984). Hybridization was performed in 50% formamide, $5 \times$ SSC, 100 μg/ml denatured DNA, 50 μg/ml heparin, 0.1% Tween, and 160 μg/ml yeast tRNA at 53°C for 14–18 hr. Posthybridization washes and hybrid detection were as described in Tautz and Pfeifle (1989).

Acknowledgments

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Note added in proof

Sequence data described in this paper have been submitted to EMBL/GenBank Data Libraries under accession number X56202.

bag-of-marbles: a Drosophila gene required to initiate both male and female gametogenesis.

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