

## A discrete transcriptional silencer in the *bam* gene determines asymmetric division of the *Drosophila* germline stem cell

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### SUMMARY

The *Drosophila* germline lineage depends on a complex microenvironment of extrinsic and intrinsic factors that regulate the self-renewing and asymmetric divisions of dedicated stem cells. Germline stem cells (GSCs) must express components of the Dpp cassette and the translational repressors Nanos and Pumilio, whereas cystoblasts require the *bam* and *bgn* genes. Bam is especially attractive as a target of GSC differentiation factors because current evidence indicates that *bam* is both necessary and sufficient for cystoblast differentiation. In this paper, we have sought to distinguish between mutually exclusive transcriptional or post-transcriptional mechanisms as the primary regulators of *bam* expression

in GSCs and cystoblasts. We find that *bam* transcription is active in young germ cells but is repressed specifically in GSCs. Activation depends on a 50 bp fragment that carries at least one germ cell-specific enhancer element. A non-overlapping 18 bp sequence carries a transcriptional silencer that prevents *bam* expression in the GSC. Promoters lacking this silencer cause *bam* expression in the GSC and concomitant GSC loss. Thus, asymmetry of the GSC division can be reduced to identifying the mechanism that selectively activates the silencer element in GSCs.

Key words: Stem cell, Asymmetric division, *bam* transcription, *Drosophila melanogaster*

### INTRODUCTION

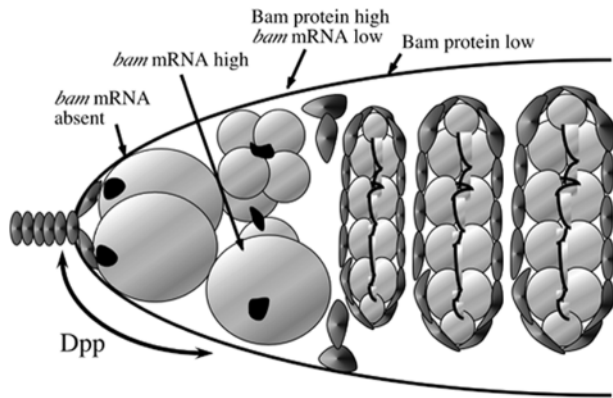
Stem cells in adult tissues share two defining characteristics – cell division is self-renewing and asymmetric. In the *Drosophila* oogenic germline, the self-renewing aspect of the division produces a new stem cell that remains associated with a complement of somatic cells (terminal filament cells, cap cells and inner sheath cells; collectively, somatic tip cells) that maintain a supportive microenvironment (Spradling et al., 2001). Parallel investigations of the fly's spermatogenic lineage have revealed that a morphologically similar but molecularly distinct microenvironment supports spermatogenic stem cells (Tulina and Matunis, 2001; Kiger et al., 2001).

Studies from several laboratories have identified genes required in ovarian somatic tip cells that are necessary for germline stem cell (GSC) function, including *piwi*, *dpp* and *fs(1)Yb* (Cox et al., 2000; King and Lin, 1999; Xie and Spradling, 1998). Genes required within the GSCs include *nanos* (*nos*), *pumilio* (*pum*) and the Dpp-dependent receptors and transcription factors (Forbes and Lehmann, 1998; Lin and Spradling, 1997; Xie and Spradling, 1998). Asymmetry of the GSC division produces a specialized cystoblast that will produce a clone of cells from which the oocyte proper will form. Mutations in such genes as *pum* or *nos* destroy asymmetry because both daughters differentiate as cystoblasts, causing the loss of GSCs (Forbes and Lehmann, 1998; Lin and Spradling, 1997). The opposite is true when cystoblast differentiation factors, such as *bam* or *bgn*, are inactivated. In

these cases, asymmetry is disrupted because both GSC daughters retain GSC characteristics (Gateff, 1982; Lavoie et al., 1999; McKearin and Ohlstein, 1995; McKearin and Spradling, 1990).

The cystoblast is a differentiated cell because, unlike the GSC, it divides precisely four times to produce 16 daughter cystocytes, which eventually produce 15 nurse cells and one oocyte. Also, unlike the GSC, each cystoblast or cystocyte cell division executes only partial cytokinesis and thus the cystocytes remain interconnected as a cyst (de Cuevas et al., 1997).

Fig. 1 presents a schematic view of germline Region 1 (the cyst-forming region) on which the pattern of *bam* mRNA and protein accumulation is superimposed. Low levels of Bam protein are expressed in GSCs, where it accumulates in fusomes but *bam* mRNA is undetectable by *in situ* hybridization. These observations indicate that *bam* is transcribed at a very low level in GSCs and that this small amount of transcript produces the protein seen associated with GSC fusomes. In the cystoblast and young cystocytes, *bam* transcripts become readily detectable, reflecting an upregulation in transcription rate or increased stability of the mRNA. This more abundant pool of mRNA produces higher levels of Bam protein that begin to accumulate in the germ cells' cytoplasm and continues to decorate the growing fusome (McKearin and Ohlstein, 1995). As cysts mature, the abundance of *bam* transcripts declines such that mRNA is again undetectable in eight-cell cysts and Bam protein levels



**Fig. 1.** Dynamics of *bam* expression. Schematic view of the germarium to demonstrate the dynamic pattern of *bam* mRNA and protein distribution. *bam* transcripts are undetectable in germline stem cells (GSCs) by RNA in situ hybridization but a small amount of Bam protein is produced and decorates the GSC fusome. In the cystoblast, *bam* mRNA accumulates to its highest levels. As cysts mature, the concentration of *bam* transcripts declines and are again undetectable in eight-cell cysts. During the period from cystoblasts through eight-cell cysts, Bam protein concentrations increase dramatically in the cystoplasm and expanding fusomes, reaching their highest levels in eight-cell cysts. Bam protein disappears rapidly from cystocyte cytoplasm once the 16-cell cyst forms.

fall precipitously once the 16-celled cyst forms such that Bam is again restricted to the fusome (McKearin and Ohlstein, 1995).

Although loss of *bam* or *bgcn* function produced hyperplasia of undifferentiated germ cells, ectopic Bam expression caused GSC elimination (Ohlstein and McKearin, 1997) similar to that seen when GSC maintenance factors were inactivated (Xie and Spradling, 1998). These data are most readily explained by assigning *bam* the role of a necessary and sufficient cystoblast differentiation factor (Ohlstein and McKearin, 1997). A simple model to account for the experimental observations is that the microenvironment, or niche (Spradling et al., 2001), created by somatic tip cells maintains the most anterior germ cells as GSCs by suppressing *bam*<sup>+</sup> expression, thereby preventing them from differentiating as cystoblasts.

The nature of intrinsic and extrinsic regulatory factors can provide clues about which aspects of gene expression are targeted by the signaling that sustains GSCs. The abrupt appearance of *bam* transcripts in cystoblasts and their persistence in cystocytes for only a few cell divisions is consistent with control of either *bam* transcription or mRNA stability or both (McKearin and Spradling, 1990). The demonstration by Xie and Spradling (Xie and Spradling, 1998) that GSC maintenance depends on the Dpp signaling cassette suggests that Smad-dependent transcriptional control could regulate the choice between GSC or cystoblast fate (Xie and Spradling, 1998; Xie and Spradling, 2000). Alternatively, the asymmetric distribution of *bam* mRNA could arise if *bam* transcripts were more stable in cystoblasts than in GSCs.

Previously, we had found that a reporter gene driven by a partial *bam* promoter could be transcribed in *bgcn*<sup>-</sup> cells that could not become cystoblasts, implying that *bam* transcriptional activation might precede full cystoblast differentiation (Lavoie et al., 1999). On this basis, we set out

to determine the role of transcriptional control using promoter mutagenesis studies in transgenic flies. We did so with the expectation that, if transcriptional regulation was important for the GSC-to-cystoblast switch, identification of the essential promoter elements could provide significant insights into the signaling pathways responsible. Here we confirm that *bam*<sup>+</sup> transcriptional control is necessary for proper GSC and cystoblast fate and we define the regions of the *bam* promoter that are required and sufficient for the GSC-low/cystoblast-high pattern of expression. We find that one transcriptional control element in the 5'-UTR acts as a silencer in the GSC and *bam* transcriptional silencing is required for GSC fate.

## MATERIALS AND METHODS

### *Drosophila* strains

All fly stocks used in this paper were maintained under standard culture conditions. *w*<sup>1118</sup> flies were used as the host for all P-element-mediated transformation. Microinjections were performed as described by Rubin and Spradling (Rubin and Spradling, 1982), except that embryos were injected without chorion removal. *bam*<sup>Δ86</sup> is a null *bam* allele (McKearin and Ohlstein, 1995) and was used for *bam* rescue tests. *w*; P[*w*<sup>+</sup>; UASp-GFP] was a kind gift from A. Ephrussi's laboratory.

### Transformation constructs

Constructs testing promoter activity used either green fluorescent protein (GFP) alone or Bam:GFP recombinant protein. Although Bam:GFP protein could provide full rescuing activity, the distribution of GFP fluorescence from fusion protein did not entirely recapitulate native Bam protein localization because fusomes were GFP-negative even when the Bam:GFP transgene was the only source of functional Bam protein. The basis for this difference from native Bam distribution (McKearin and Ohlstein, 1995) is currently the object of study.

Unless stated otherwise, all constructs were made using the CaSpeR4 transformation vector (Pirrotta, 1988). The P[*bam* promoter-Gal4:VP16] transgene used a derivative of P[*hsp70*-CaSpeR] that contained the promoter and 3'UTR from the *hsp70* gene (gift of D. Smith). The various constructs described in this paper were generated using primers listed in Table 1.

### Constructs for Bam promoter 5' deletion analysis

PCR reactions to recover promoters that were truncated from the 5'-side were anchored on the 3'-end by an antisense primer at position +133 (Table 1). These PCR fragments were joined to Bam:GFP or GFP reporter gene with appropriate restriction enzyme digestions and subsequently linked to the *bam* 3'UTR in pCaSpeR4.

### Constructs for Bam promoter 3' deletion analysis

Fragments truncated from the 3'-end were generated by a set of antisense primers (Table 1) and were anchored at the 5' side with oligonucleotides corresponding to positions -898, -799 or -698 (Table 1). These PCR fragments were cloned upstream of a GFP reporter and linked to *bam* 3'UTR in pCaSpeR4.

### Constructs for silencer element sufficiency test

Fragments for sufficiency tests were recovered by PCR or assembled by annealing synthetic oligonucleotides. Promoters containing small deletions were synthesized directly by PCR using primers that lacked the targeted residues. A two-step strategy was used to construct promoters with internal deletions; separate PCR products were ligated such that the targeted nucleotides were deleted and the ligation products were used as templates for subsequent PCR. We used *nos* and P-element minimal promoter sequences that had been identified

**Table 1. Oligos used in this study**

Oligo sequence: restriction sites are underlined	Comments
Sense primer	
5'-gcgGGTACCagatctaaccattgattaac, <i>KpnI</i>	Bam promoter -898
5'-gcgGGTACCtcaagaattttgtaaccga, <i>KpnI</i>	Bam promoter -799
5'-gcgACGCGTtcaagaattttgtaaccga, <i>MluI</i>	Bam promoter -799
5'-gcgGGTACCaaaattttgctgctcagtc, <i>KpnI</i>	Bam promoter -698
5'-gcgGGTACCacggagctgccagatgttag, <i>KpnI</i>	Bam promoter -498
5'-gcgGGTACCcacaatcagtggtataatt, <i>KpnI</i>	Bam promoter -198
5'-gcgGGTACCtgaagtctgtaattgatt, <i>KpnI</i>	Bam promoter -168
5'-gcgGGTACCaggtgaccataaattgaaac, <i>KpnI</i>	Bam promoter -96
5'-gcgACGCGTtagtgaccataaattgaaac, <i>MluI</i>	Bam promoter -96
5'-gcgCTCGAGtgaatattttgctgagc, <i>XhoI</i>	Bam promoter -82
5'-gcgGGTACCtactgacatgatagata, <i>KpnI</i>	Bam promoter -47
5'-gcgAAGCTTaaacctgttccagctcagta, <i>HindIII</i>	Bam promoter -60
5'-gcgGGTACCaaagagctgagccatca, <i>KpnI</i>	Bam promoter -16
5'-gcgACGCGTtctctctctctcctgaa, <i>MluI</i>	Bam promoter +5
5'-gcgACGCGTtagacagcgtggcgtcagcga, <i>MluI</i>	Bam promoter +30
5'-gcgGGTACCgaccgttttaacctgaaa, <i>KpnI</i>	Nanos promoter -708
5'-gcgACGCGTactacgctgctgatagccg, <i>MluI</i>	P-element basal promoter
Antisense primer	
5'-gcgGATCCtaagttaaacacacaaatc, <i>BamHI</i>	Bam promoter +133
5'-gcgACCGGTcgatttttgccttttaaac, <i>AgeI</i>	Bam promoter +104
5'-gcgACCGGTtattgccaggtctctttta, <i>AgeI</i>	Bam promoter +79
5'-gcgACCGGTtgaatcgtgacgccacgc, <i>AgeI</i>	Bam promoter +55
5'-gcgACCGGTgacgccacgtctctgcaa, <i>AgeI</i>	Bam promoter +45
5'-gcgACCGGTtctctgcaattcaggagaa, <i>AgeI</i>	Bam promoter +34
5'-gcgACCGGTgcaattcaggagaaagagg, <i>AgeI</i>	Bam promoter +30
5'-gcgACCGGTtgaatgctgctcagactatt, <i>AgeI</i>	Bam promoter +4
5'-gcgACCGGTtaactatcagatcatgct, <i>AgeI</i>	Bam promoter -20
5'-gcgACCGGTtaactatcagatcatgct, <i>MluI</i>	Bam promoter -20
5'-gcgACCGGTcccagaacaggtgcccctc, <i>AgeI</i>	Bam promoter -47
5'-gcgAAGCTTcaatttatgctcactcaaaa, <i>HindIII</i>	Bam promoter -81
5'-gcgAAGCTTacaataaagttcaatttatg, <i>HindIII</i>	Bam promoter -69
5'-gcgACCGGTtgaatcgtgacgcgctgctgc, <i>AgeI</i>	Bam promoter +55 with deletion of +37 to +39
5'-gcgACCGGTtctctgattcaggagaaagag, <i>AgeI</i>	Bam promoter +34 with deletion of +20-22
5'-cgccacgctgctgcaataacgtatcagatcat	Bam promoter -38 to -22/+24 to +42
5'-gcgACCGGTcgtgctgcaataacgtatcagatcat, <i>AgeI</i>	Bam promoter -38 to -22/+24 to +37
5'-ctgacgccaaattcaggagaaagaggaag	Bam promoter +7 to +26/+38 to +46
5'-gcgACCGGTtgaatcgtgacgccaaattcaggagaa, <i>AgeI</i>	Bam promoter +55 with deletion +27 to +37
5'-gcgCTCGAGcaattccaggaattttgtgg, <i>XhoI</i>	Nanos promoter -1
5'-gcgACCGGTgtaactaaactcgtttttg, <i>AgeI</i>	Nanos promoter +94
5'-cgccACCGGTattccacgtaagggttaag, <i>AgeI</i>	P-element basal promoter

previously (Van Doren et al., 1998; Rorth, 1998) for constructing heterologous promoters.

#### Inverted silencer element transgene

A two-step PCR was performed to amplify the fragment containing the *bam* promoter and an inverted silencer element. Fly genomic DNA was used as template for first round PCR. After the first PCR reaction for 10 cycles, resulting PCR products were used as template for second round PCR. The final PCR products were subcloned into CaSpeR vector in front of a GFP reporter.

Two-step PCR method:

1. Sense primer: *bam*-898/*KpnI*.
2. Antisense primer 1 for first round PCR: *bam*-as/SE/s (5'-cgccagacagcgtggcgtcagctacgtatcagatcatg-3').
3. Antisense primer 2 for second round PCR: *bam*/SE/s (5'-cgccacggatttcgacagcagcgtggcgtc-3').

#### Cytological methods

Germline clones were produced by inducing the expression of autosomal P(*hsp70*-FLP) transgenes at 37°C for 1 hour. Heat shock

was repeated at least two more times on the first day and the same flies were occasionally heat-shocked again by the same protocol on the next day to achieve higher production of clones. Cells made homozygous for *brk* mutant alleles were recognized as GFP-negative against a background of GFP-positive cells.

GFP fluorescent images from reporter transgenes and germline clones were collected on a Zeiss LSM410 confocal microscope. Samples were evaluated as single optical sections in the plane that included the terminal filament to make certain that GSCs were included in the image. The optical files were processed using Adobe Photoshop 6.0 and Deneba Canvas 7.0.

#### RACE for determining transcriptional start site

Total RNA was isolated from 0-3 h collections of *w*<sup>1118</sup> embryos and digested with RNase-free DNase. cDNA was prepared by reverse transcription using an antisense *bam* primer and tagged with a poly(A) tail using terminal deoxynucleotide transferase according to manufacturer's instructions (Promega). The ends of tailed cDNA were amplified by PCR with oligo-dT and *bam*-specific primers. The resulting PCR products were cloned into the pGEM T-Easy vector (Promega) and sequenced by a UT-Southwestern DNA Sequencing facility.

## RESULTS

### The native *bam* promoter is essential for proper differentiation

RNA in situ hybridization had previously shown that asymmetric *bam* expression between the GSC and cystoblast could be seen at the level of mRNA expression (McKearin and Spradling, 1990). This finding was consistent with transcriptional control of *bam* expression. More recently, we found that a GFP reporter transgene, driven by a *bam* promoter fragment, was efficiently expressed in *bam* mutant cells but not in GSCs (not shown). This finding provided additional evidence that activation of *bam* transcription preceded cystoblast differentiation because *bam* cells cannot progress to cystoblasts (McKearin and Ohlstein, 1995). Both of these observations supported the premise that *bam* transcriptional control was an early and crucial step in the differentiation of stem cell daughters into cystoblasts. This conclusion led us to undertake a detailed analysis of *bam* promoter elements.

To begin our studies, it was necessary to define the precise transcriptional start site. Initial RNase protection assays had defined the start site at a sequence 'TTCTGGG' (McKearin and Spradling, 1990), but more precise RACE assays placed the transcriptional start site at 'gccAtcattctt'. This site will be designated as '+1' in this paper for the purposes of orienting *bam* promoter elements.

Previously we found that a genomic fragment of 1008 bp immediately upstream from the +1-site plus the 5'-UTR could fully rescue *bam* phenotype (McKearin and Spradling, 1990), and subsequently had refined essential promoter sequences to -898 bp plus 5'-UTR (B. Ohlstein and D. M., unpublished). Both promoters recapitulated the native *bam* pattern by driving expression in cystoblasts and young germ cells, but remained quiescent in GSCs. We concluded that all sequences necessary for *bam*<sup>+</sup> transcriptional control were contained within the sequences spanning from -898 bp 5' to the *bam* transcriptional start site through 133 bp of the *bam* 5'-UTR.

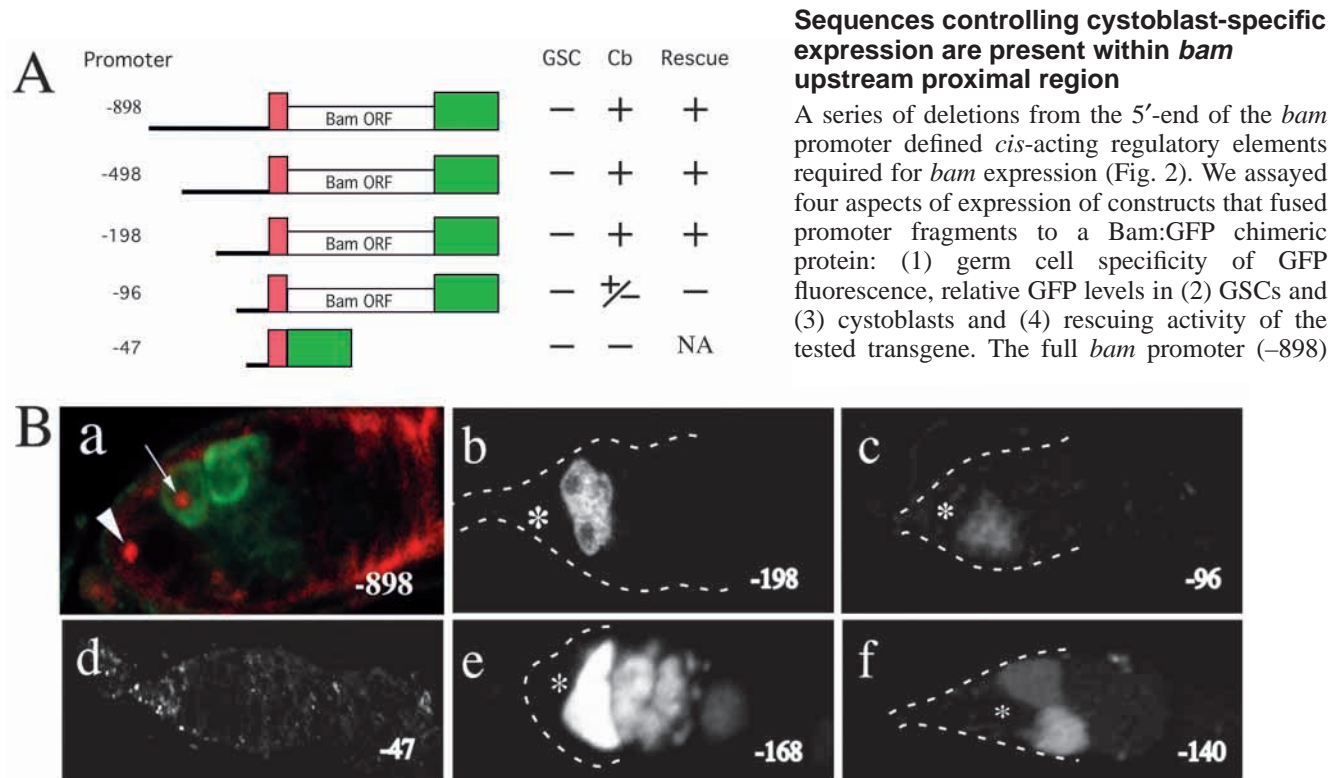
We constructed P[UASp-Bam:GFP] transgenes to test the effects of expressing *bam*<sup>+</sup> from a variety of different

promoters. By itself, the construct produced no detectable GFP expression. When expressed from a *nos* promoter in P[UASp-Bam:GFP]; P[*nosP*-Gal4:VP16-*nos*3'UTR] flies, adult ovaries from young females contained only a few maturing eggs but no other germline cells (not shown). This was the same phenotype obtained when *bam*<sup>+</sup> was expressed from a heat shock promoter (Ohlstein and McKearin, 1997) or when any of several GSC maintenance genes were inactivated by mutation (Forbes and Lehmann, 1998; Lin and Spradling, 1997; Xie and Spradling, 1998; Xie and Spradling, 2000). Thus the *nos* promoter, which directed *bam*<sup>+</sup> expression in all PGCs and adult germ cells including GSCs, caused GSC ablation. We also noted that the testes of [UASp-Bam:GFP]; P[*nosP*-*nos*5'-Gal4:VP16-*nos*3'UTR] males were shriveled but did not determine the basis of the defect. In contrast to heterologous transcriptional control of *bam*<sup>+</sup> expression, oogenesis was normal and GFP was expressed in cystoblasts and young cystocytes but not GSCs when a native *bam* promoter controlled transgene expression in P[*bamP*-*bam*5'-Gal4:VP16-*hsp70*-3'UTR]; P[UASp-Bam:GFP] flies. This combination of transgenes also fully rescued *bam* mutant flies. Because levels of GFP reporters suggested that the relative 'strength' of the *nos* and *bam* promoters was similar in cystoblasts (Van Doren et al., 1998), the different results obtained with *bam* and *nos*

promoters predicted that the timing of *bam* transcription was critical for GSC fate. In addition, as GFP expression in P[UASp-Bam:GFP]; P[*nos*-Gal4:VP16] animals recapitulated the *nos* expression pattern and not the *bam* pattern, we concluded that the Bam ORF and 3'-UTR were not sufficient for the wild-type *bam* expression.

To determine whether the 3'-UTR contained necessary regulatory signals, we examined the GFP expression in P[*bamP*-*bam*5'-Gal4:VP16-*hsp70*-3'UTR]; P[UASp-GFP] flies and found that GFP expression pattern mimicked wild-type *bam* mRNA accumulation (not shown). Thus the *bam* 3'-UTR was not required for the *bam* expression pattern.

Consistent with the conclusion stated above, comparison of the expression of Bam:GFP and GFP reporters using *bam* promoters showed that the Bam ORF did not contain sequences that were necessary or sufficient for the wild-type *bam* expression pattern. Inclusion of the Bam ORF in reporter constructs, however, did affect perdurance of reporter protein in maturing cysts such that GFP fluorescence was much shorter-lived with Bam:GFP than GFP alone (for example, see Fig. 2). This probably reflects instability of Bam protein in maturing cystocytes, as has been postulated previously based on the distribution of native Bam protein (McKearin and Ohlstein, 1995).



**Fig. 2.** The 5' deletion series to refine the minimal *bam* promoter. Schematic representations of the various deletion constructs are shown in A. Each construct's expression in germline stem cells (GSCs) and cystoblasts (Cb) is indicated in columns beside the appropriate construct schematic. The ability of the [Bam:GFP] constructs to rescue *bam* mutant flies was scored and is indicated in an additional column. Red and green rectangles represent the *bam* 5'-UTR and GFP, respectively. (B) Representative confocal images of the GFP expression pattern produced by individual constructs. Each panel contains a single optical section of a germarium in the plane of the terminal filament from animals expressing reporter from the indicated *bam* promoter fragment. The germarium in A was reacted with antibodies against Hts (red) and GFP (green) to show reporter expression in GSCs and cystoblasts. The arrowhead indicates the stem cell fusome located next to the contact point with cap cells, and the arrow points to the fusome located internally in a cystoblast. Asterisks indicate position of GFP-negative GSCs in A-E. (A) -898/+133-Bam:GFP; (B) -198/+133-Bam:GFP; (C) -96/+133-Bam:GFP; (D) -47/+133-GFP; (E) -168/+133-GFP; (F) -140/+133-GFP. gfp, green fluorescent protein.

showed no GFP expression in GSCs and strong expression in cystoblasts and young cystocytes (Fig. 2A). For example, Fig. 2B.a shows an example GFP-negative GSC and a GFP-positive cystoblast within the same germarium. The GSC can be recognized because it is the anteriormost cell in the germarium and carries a spherical fusome (arrowhead) at the most anterior position, whereas the cystoblast is immediately posterior to the GSC and has a fusome (arrow) that is located more centrally within the cell.

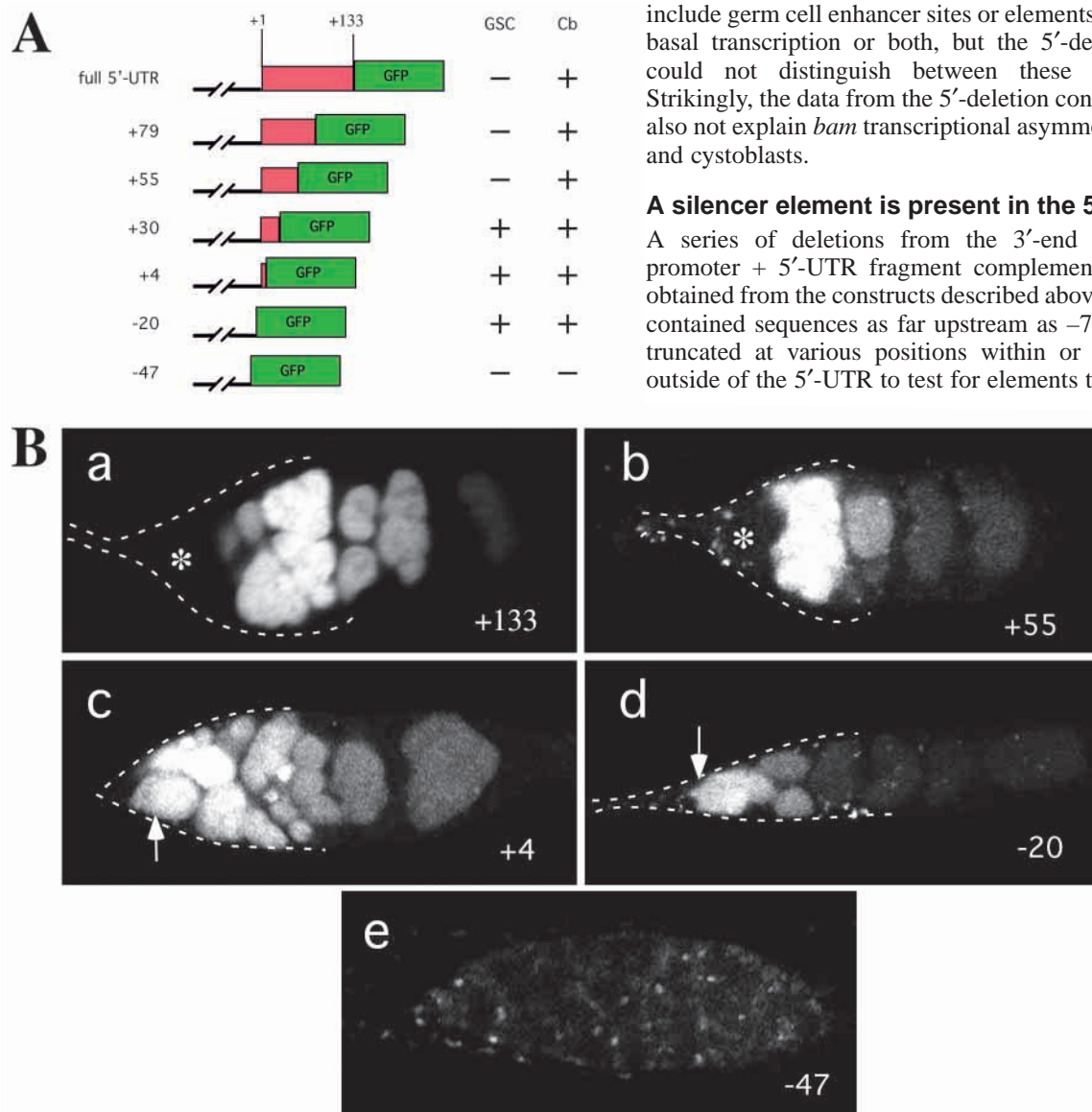
Deletions of sequences between  $-898$  to  $-198$  showed no effect on the timing, germ cell specificity or relative intensity of reporter activity (Fig. 2A,B.b). In addition, *bam/bam* flies expressing Bam:GFP protein from these truncated promoters showed complete rescue because females developed wild-type ovaries and retained an active GSC population (Fig. 2A). Expression from a promoter fragment that contained sequences

starting at  $-96$  was very low or, in approximately half the ovaries assayed, was not detectable at all. Consistent with its apparent weak promoter activity, this construct was also unable to rescue *bam/bam* flies (Fig. 2A). Nevertheless, when Bam:GFP protein was produced at detectable levels, its accumulation was restricted to cystoblasts and the cystocytes of developing cysts (Fig. 2B.c). Thus the sequence between  $-198$  and  $-96$  provided element(s) that enhanced transcriptional output but did not effect the timing or cell specificity of transcription. Additional deletions refined the limits of quantitative element(s) to between  $-168$  and  $-140$  (Fig. 2B.e,f).

When deletions from the 5'-end extended to the  $-47$  position, all GFP expression was eliminated (Fig. 2A,B.d). Thus sequences between  $-96$  and  $-47$  contained elements that could direct germ cell-specific transcription and were essential for *bam* promoter activity. The  $-96$  to  $-47$  region could include germ cell enhancer sites or elements required for basal transcription or both, but the 5'-deletion series could not distinguish between these possibilities. Strikingly, the data from the 5'-deletion constructs could also not explain *bam* transcriptional asymmetry in GSCs and cystoblasts.

#### A silencer element is present in the 5'UTR

A series of deletions from the 3'-end of the *bam* promoter + 5'-UTR fragment complemented the data obtained from the constructs described above. Promoters contained sequences as far upstream as  $-799$  and were truncated at various positions within or immediately outside of the 5'-UTR to test for elements that might be



**Fig. 3.** The 3' deletion series. (A) Deletion constructs in schematic form which indicate if the truncated promoter caused GFP expression in germline stem cells (GSCs) and cystoblasts. (B) Confocal sections of the indicated constructs. Asterisks indicate position of GFP-negative GSCs. Arrows indicate the position of the base of terminal filaments when GSCs are GFP positive. (A)  $-799/+133$ -GFP; (B)  $-799/+55$ -GFP; (C)  $-799/+4$ -GFP; (D)  $-799/-20$ -GFP; (E)  $-799/-47$ -GFP.

included within the UTR region (Fig. 3A). Promoters that started 799 bp upstream of the transcriptional start site and included at least 55 bp of the 5'-UTR reproduced the GSC-negative, cystoblast-positive pattern of *bam* expression (Fig. 3A,B.a and Fig. 3B.b). Note that, because these constructs included only the GFP reporter protein and were therefore not targets of Bam-dependent degradation, cystocytes in fully formed and developing cysts were GFP-positive.

Additional deletions in this series revealed a new feature of *bam* cis-regulatory sequences. When the promoter fragment extended only to +4, reporter activity expanded to include GSCs as well as germ cells in cysts and beyond (Fig. 3A,B.c). Although other deletions from the 3'-side that reached as far as -20 continued to express GFP in all germ cells including GSCs (Fig. 3A,B.d), GFP expression was eliminated when the 3' deletion series reached the -47 position (Fig. 3A,B.e). Thus sequences between -46 and -20 were essential for any transcriptional activity, whereas sequences between +5 and +55 were necessary to silence *bam* expression in GSCs. Combining these observations with the results of deletions from the 5'-end of the *bam* promoter fragment, we concluded that sequences between -96 and -20 provided positively acting elements sufficient for young cystocyte-specific *bam* transcription, and that critical components of a silencer element that blocked *bam* expression specifically in GSCs lay between +5 and +55.

#### Bam promoter without inhibitor element causes GSC loss

Previous studies had shown that *bam* misexpression from a *hsp70* (Ohlstein and McKearin, 1997) or *nos* promoter (Fig. 4A,B) that extended the domain of *bam* expression could cause specific GSC loss. The most attractive model to explain these effects was that inappropriate temporal control of *bam* transcription, specifically misexpression in GSCs, was responsible for GSC ablation. Transgenes that expressed

Bam:GFP from promoter fragments that lacked the silencing element but retained other native promoter elements would provide a powerful test of this hypothesis. In fact, young females that expressed a functional Bam:GFP protein controlled by a promoter fragment from -799 to +4 showed the expected 'few eggs - no germline' phenotype associated with GSC elimination (Fig. 4C,D). The effect was fully penetrant but showed variable expressivity when the transgene was present in one copy; two copies increased expressivity to 100%. These observations indicated that *bam* transcriptional quiescence was necessary for GSC fate and that regulation of the silencer element was the key component of the switch between GSC and cystoblast fate.

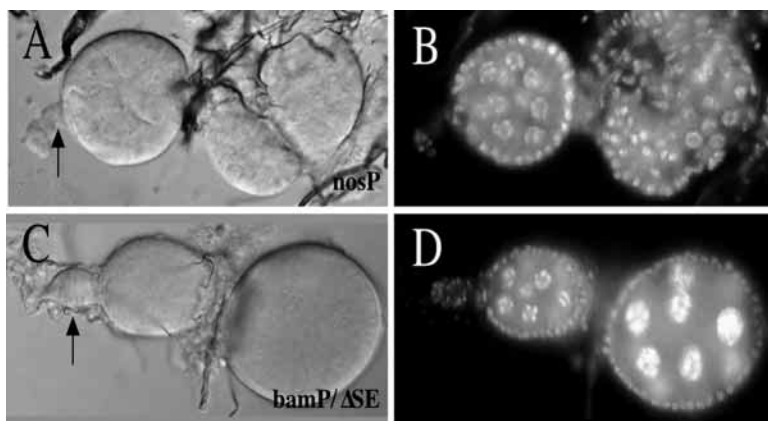
#### Defining the limits of the silencer element

In order to delineate the silencer element more precisely, we tested the ability of various fragments to suppress activity of a promoter containing the germ cell enhancer and basal elements. We chose the (-799 to -20) promoter fragment as the germ cell 'constitutive promoter' because it directed GFP expression in GSCs and remaining germ cells but not ovarian somatic cells (Fig. 3B.d). When an oligonucleotide containing (+5 to +55) was fused to this basal promoter, cystoblasts and cystocytes were GFP-positive but GSCs were negative (not shown), indicating that this fragment carried full silencer activity.

One of the 3'-deletion constructs (-799 to +34) had caused reproducible intermediate silencer activity (Fig. 5B). We suspected that this truncation might leave some of the silencer element intact and noted that the truncation breakpoint fell within an imperfect palindromic sequence (+27-CGCaGaCaGCG to +37; Site A). We probed the structure of the silencer element by constructing (-799 to +55) promoters that precisely lacked the palindromic sequence. The GFP reporter from this construct was active in GSCs as well as cystoblasts and cystocytes (Fig. 5C), indicating that residues

+27 to +37 were essential for full silencer activity. The palindromic element was not sufficient for silencer activity, however, because promoters constructed by fusing an oligonucleotide encoding only Site A to a (-799 to -20) basal promoter permitted GFP expression in both cystoblasts and GSCs (Fig. 5D). These assays demonstrated that the palindromic sequences were an essential part of the silencer element but were insufficient to block *bam* transcription in GSCs.

We discovered that the (+27 to +37) oligonucleotide used in this experiment carried the sequence 'CGCAGACTGCG' instead of the native *bam* sequence 'CGCAGACAGCG'. Although it seemed unlikely, we could not eliminate the possibility that silencer activity of this element might have been destroyed by the mutation that generated an even more symmetrical palindrome. We subsequently constructed a *D. yakuba* *bam* promoter-GFP reporter that allowed us to test the efficiency of this silencer sequence because the *D. yakuba* Site A sequence corresponds to the perfect palindrome. We found that the *yakuba* promoter showed the same GFP expression pattern as wild-type *melanogaster* *bam* and concluded that 'CGCAGACAGCG' and



**Fig. 4.** Silencer activity is necessary for germline stem cell maintenance. (A-D) Ovarioles from females expressing Bam inappropriately in GSCs. A and C are DIC images, whereas B and D are DAPI-stained fluorescent micrographs. The ovariole in A and B was taken from a female expressing Bam:GFP from a *nos* promoter. The ovariole in C and D is from a female carrying 2 copies of a transgene expressing Bam:GFP from a (-799/+4) *bam* promoter fragment that lacks the sequences required for GSC silencer activity. Note that in both examples, the ovariole contains well-developed cysts with nurse cells but the germarium (arrow) is shriveled.

'CGCAGACTGCG' palindromes were both efficient silencers in the context of the *bam* promoter sequences.

Although promoters that included only to +37 permitted GSC expression, promoters that extended to +55 did not, indicating that a fully active silencer must include sequences between +37 and +55. We noted that the eight base pairs directly adjacent to the palindromic element closely matched the consensus binding site (Site B) for a known sequence-specific transcriptional repressor, Brinker (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999; Rushlow et al., 2001; Saller and Bienz, 2001; Kirkpatrick et al., 2001; Zhang et al., 2001). We therefore tested whether Site B was part of the silencer element sequences. A (-799 to +55) construct that contained a deletion of base pairs +37 to +39 expressed GFP in GSCs (Fig. 5E), suggesting that Site B was important for full silencer activity. Note that this deletion altered Site B but reconstituted a complete Site A sequence. To determine whether Sites A and B were sufficient for the silencer element, we joined an oligonucleotide encoding (+25 to +45) (Materials and Methods) to the (-799 to -20) constitutive germ cell promoter. GFP expression from this construct was strong in cystoblast and cystocytes but was undetectable above background levels in GSCs (Fig. 5F), demonstrating that full silencer activity requires only sequences contained in Sites A and B.

Despite the fact that Site B matched a Brinker consensus binding element, the results of subsequent experiments made Brinker a poor candidate for an *in vivo* regulator of *bam* transcription. Ovarian  $\beta$ -galactosidase expression in *brk*[XA]/+ females that carry a P[*lacZ*] enhancer-trap transposon in the *brk* gene (Campbell and Tomlinson, 1999) was restricted to cap, terminal filament and inner sheath cells, but, notably, was

not expressed in any germline cells (not shown). Furthermore, germline clones of *brk*[M68] loss-of-function allele produced wild-type egg chambers and GSCs were stable for at least two weeks after clone induction (Fig. 5G). Thus, germline cells neither express nor require Brinker for GSC maintenance or cyst development.

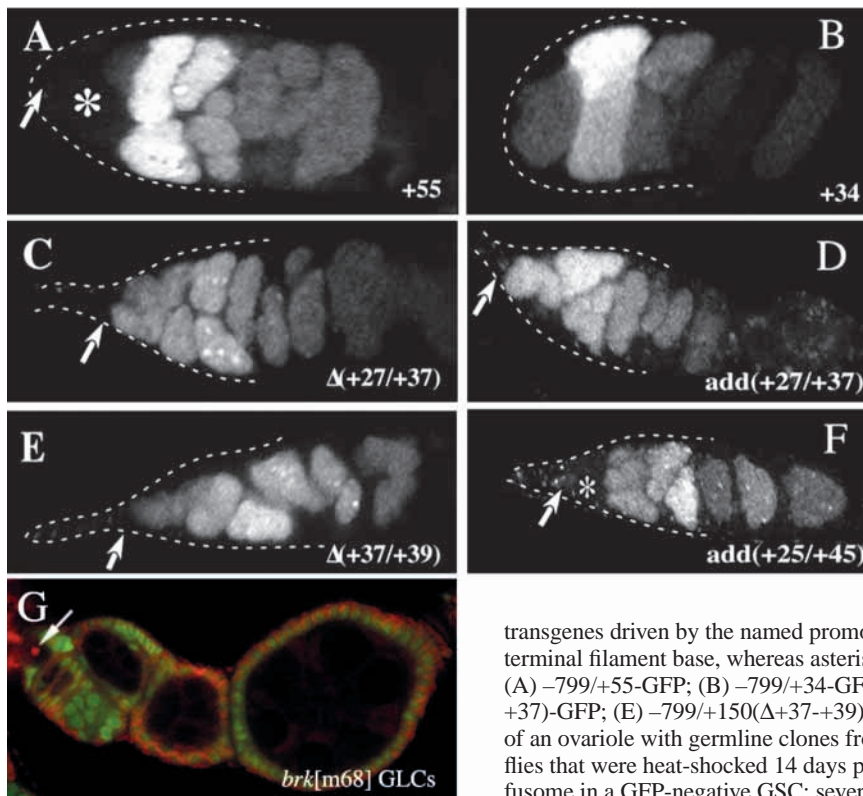
### Is transcription the target of the silencer?

The fact that the silencer was in the 5'-UTR raised the possibility that it acted at a post-transcriptional level. We tested this possibility by making *bam* transcripts that included the silencer element but were transcribed from the *nos* promoter that is active in all germline cells including GSCs (Van Doren et al., 1998). Transgenic flies carrying the construct [*nosP-bam5'-GFP-bam3'*] (Fig. 6A) expressed GFP in GSCs, cystoblasts and other cystocytes at approximately equivalent levels (Fig. 6B). We examined the structure of the [*nosP-bam5'-GFP-bam3'*] mRNA to verify that these transcripts included the silencer element motif using RT-PCR and primers that would bracket potential start sites (Fig. 6A). RT-PCR of RNA from [*nosP-bam5'-GFP-bam3'*] flies showed that the transcript produced from the transgene started very close to or precisely at the native *bam* gene transcription start site (Fig. 6C). Therefore, the *bam* silencer element did not act post-transcriptionally because the reporter mRNA carrying the element was stably expressed in GSCs.

We confirmed the transcriptional role of the silencer element by constructing a [*bamP-SE<sup>rev</sup>-GFP*] transgene that carried an inverted silencer (Materials and Methods). This transgene expressed GFP in the same pattern as a wild-type construct (not shown), demonstrating that the silencer element was active in either orientation. Because the inverted silencer would encode a sequence that was the complement of wild-type in the [*bamP-SE<sup>rev</sup>-GFP*] mRNA, we concluded that the silencer acts as a DNA, not RNA, element.

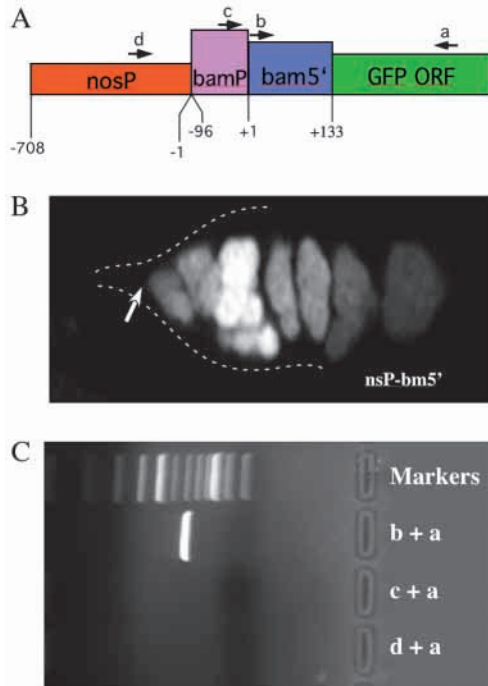
### The silencer element shows specificity for the *bam* promoter

Although transcripts from the [*nosP-bam5'-GFP-bam3'*] transgene showed that the silencer did not act as an RNA *cis*-element, GFP expression in GSCs also revealed that the silencer element did not block transcription from the *nos* promoter. This observation suggested that effective transcriptional silencing by the *bam* element was sensitive to context. As an additional test of silencer properties, a *bam* fragment



**Fig. 5.** Refining the position of silencer sequences. Each panel shows a germarium recovered from females expressing green fluorescent protein (GFP) reporter from

transgenes driven by the named promoter fragments. Arrows indicate the position of the terminal filament base, whereas asterisks mark GSCs when these cells are GFP-negative in (A) -799/+55-GFP; (B) -799/+34-GFP; (C) -799/+55( $\Delta$ +27/+37)-GFP; (D) -799/-20(+27/+37)-GFP; (E) -799/+150( $\Delta$ +37/+39)GFP; (F) -799/-20(+25/+45)-GFP. (G) An example of an ovariole with germline clones from *brk*[M68] P[FRT]18A/P[Ubi-GFP] P[FRT]18A flies that were heat-shocked 14 days prior to ovary dissection. The arrow points to the fusome in a GFP-negative GSC; several cysts with GFP-negative cystocytes are present.



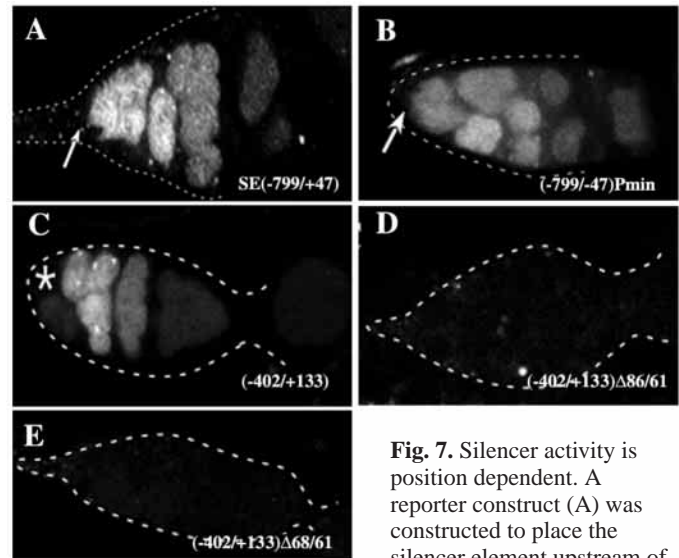
**Fig. 6.** Testing post-transcriptional effects of the *bam* silencer. (A) A schematic of the structure of a transgene in which a fragment of the *bam* region containing putative silencer sequences was joined to the *nos* promoter. The relative positions of oligonucleotides used for PCR primers are also shown above the schematic. (B) An example of the GFP expression pattern from the [*nosP-bam5'-GFP-bam3'*] is shown in a confocal optical section. The cell immediately adjacent to the base of the terminal filament, indicated by the arrow, is GFP-positive. (C) An agarose gel of products generated by RT-PCR of RNA isolated from [*nosP-bam5'-GFP-bam3'*] using oligonucleotides shown in A. The primer pairs used in the reactions are indicated on the right-hand side of the image.

containing a fully active silencer (-16 to +133) was placed upstream of the basal *bam* promoter (-799 to -20). GFP was expressed in GSCs, cystoblasts and cystocytes in flies carrying these constructs, indicating the silencer was inactive in this position (Fig. 7A).

### Refining enhancer elements

Previous experiments had shown that the fragment from (-96 to -20) was sufficient for promoter activity in germline cells and that fragments (-96 to -47) and (-46 to -20) were necessary (Fig. 3). We reasoned that these regions contained enhancer element(s) that directed germ cell-specific transcription and a core promoter element, respectively.

We sought to identify essential enhancer sequences experimentally by joining *bam* fragments to a previously identified core promoter from the *Drosophila* P-element (Rorth, 1998). The transcriptionally inactive (-799 to -47) fragment (Fig. 4E) was joined to the P-element core promoter driving the GFP reporter gene (Fig. 7B). Flies carrying this construct expressed GFP in GSCs, cystoblasts and cystocytes just as had been observed for the (-799 to -20) *bam* promoter fragment (Fig. 4D). Thus the (-47 to -20) region could be replaced by a heterologous minimal promoter element and at



**Fig. 7.** Silencer activity is position dependent. A reporter construct (A) was constructed to place the silencer element upstream of a *bam* promoter that

contained only the positively acting elements. GFP expression from the transgene included GSCs. (B) GFP expression from a construct that replaces the putative basal *bam* promoter element with a P-element minimal promoter element. (C) GFP expressed in the native *bam* pattern from a promoter fragment that includes the region from -402 to +133. (D,E) GFP expression patterns of promoters derived from the same fragment as in C but carrying deletions of -86 to -61 (D) and -68 to -61 (E) are shown. Arrows point to position of terminal filament base in A and B; asterisk marks GSC in C.

least one germ cell-specific enhancer lies in the region between -96 and -47.

An additional deletion series refined the position of the enhancer by revealing that promoters lacking nucleotides from -86 to -61 deletion were inactive (compare Fig. 7C and D). Deletion of a GC-rich block of nucleotides within the required region (-68 to -61) also extinguished germ cell expression of the GFP reporter (Fig. 7E). Thus, 'gcgacggc' block of nucleotides was essential for the *bam* enhancer element. Although the 'gcgacggc' element matches the consensus binding site for the Mad protein (Kim et al., 1997), we note that Xie and Spradling (Xie and Spradling, 1998) have shown previously that *mad*<sup>-/-</sup> cysts develop normally. It is therefore unlikely that this essential *bam* promoter element binds Mad in germ cells.

## DISCUSSION

### *bam* expression is controlled transcriptionally

Although *bam* is expressed throughout cyst formation (McKearin and Ohlstein, 1995), it is first required for asymmetry of the oogenic stem cell division in which it acts as the primary cystoblast differentiation factor (McKearin and Ohlstein, 1995; McKearin and Spradling, 1990). Prior studies suggested that GSC and cystoblast differentiation was sensitive to the levels of Bam and that *bam* expression was tightly regulated (McKearin and Ohlstein, 1995; McKearin and Spradling, 1990). It was not clear, however, whether regulation was pre- or post-transcriptional or a combination



of mechanisms. This is an important issue because GSC-intrinsic factors acting at the level of transcription (Smads) and post-transcriptionally (Pum/Nos) have been identified as critical regulators of GSC fate (Xie and Spradling, 1998; Forbes and Lehmann, 1998; Lin and Spradling, 1997). Accumulation of *bam* mRNA to detectable levels correlates precisely with the choice between the stem cell and cystoblast cell fate, suggesting that mechanisms regulating mRNA abundance could be important factors in the GSC-to-cystoblast switch.

Our collection of transgenes allowed us to address the role of post-transcriptional control in regulating GSC to cystoblast differentiation. Transgenic reporters driven by *bam* promoters but in which GFP replaced the *bam* ORF and *hsp70* 3'-UTR replaced the *bam* 3'-UTR were expressed in cystoblasts and cystocytes but not in GSCs, demonstrating that sequences in the *bam* ORF and 3'-UTR were dispensable for proper regulation. Animals that produced a *bam*5'-GFP-*bam*3' reporter from a *nos* promoter, however, expressed GFP in GSCs as well as other germ cells. RT-PCR showed that the transcript produced from the P[*nos*P-*bam*5'-GFP-*bam*3'] transgene included all of the *bam* 5'-UTR sequences and would therefore contain all elements that could direct post-transcriptional regulation. Because GSCs were GFP-negative when the same reporter was expressed from fully active fragments of the *bam* promoter, we concluded that cell-specific transcriptional activation was the first and most significant level of control for regulating differential *bam* expression in stem cells and cystoblasts.

Alternative explanations for differential *bam* expression are that positively acting elements first become active in cystoblasts, that negatively acting elements function specifically in GSCs to repress *bam* transcription or a combination of these mechanisms. The fact that *bam* promoters that lacked the silencer element could direct reporter expression in germ cells, including GSCs, demonstrated that all germ cells contained the factors necessary to activate the *bam* promoter but that only GSCs contained factors to repress *bam* transcription. Because Bam is necessary and sufficient for cystoblast differentiation (Ohlstein and McKearin, 1997), the choice between GSC or cystoblast fate can be reduced to understanding the *cis*- and *trans*-acting factors that activate and repress *bam* transcription.

### Germ cell specificity

The native pattern of *bam* mRNA expression alerted us to at least two features of *bam* transcription; it was active only in germ cells and it was undetectable in GSCs. The sum of control elements should explain activity in this pattern and we therefore examined putative promoter deletions for their capacity to (1) limit *bam* expression to germ cells and (2) limit *bam* expression to cystoblasts and very early cystocytes.

The 5'-deletion series showed that the minimal *bam* promoter was small because a fragment containing as few as 168 bp upstream of the transcriptional start site was sufficient to reproduce all aspects of native *bam* expression. Additional deletions demonstrated that sequences between -168 and -140 accounted for quantitative effects, but additional functional assays are

necessary to identify the specific elements that regulate transcriptional output.

Even fragments that could not produce wild-type levels of transcription, however, retained cell specificity of the remaining transcriptional activity. At a minimum, these elements (Fig. 8) include (1) an enhancer element between -86 and -61 that directs *bam* transcription in GSCs, cystoblast and germline cyst cells and (2) a silencer between +27 and +44 that blocks enhancer activity in GSCs, (3) sequences between -47 and -20 that are essential for transcriptional activation and can be replaced by a P-element core promoter.

### Promoter elements that act positively

The 3' series of deletions showed that promoters that retained at least to -20 could reproduce the wild-type *bam* expression profile but promoters that retained only to -46 could not. Because fusion of the (-799 to -46) fragment to the P-element minimal promoter could recapitulate *bam*-like expression, we concluded that -46 to -20 contained the 'basal elements' that would interact with the core RNA polymerase subunits (Kadonaga, 1998; Reinberg et al., 1998). We noted that the *bam* promoter did not comply with the rules for a TATA-dependent promoter because none of the sequences within the essential regions matched the TATA element consensus



**Fig. 8.** Transcriptional control elements in the *bam* promoter. The 5'-UTR and proximal upstream sequences are shown with various landmarks indicated. The 5'-end of the smallest promoter with full rescuing activity tested in the studies is indicated as '-198'. Regions containing transcriptional control elements are presented in large, upper case fonts and distinguishing colored backgrounds. The region that contains germ cell (GC)-specific enhancer element(s) (-86 to -61) is highlighted in blue. The GC-rich sequence that is essential for *bam* transcription and matches the consensus binding site for Mad protein is underlined. The sequences outlined in gray could be replaced by basal promoter elements from the P-element promoter, whereas the transcriptional start site (+1) is highlighted with green. The bipartite silencer element in the *bam* 5'-UTR is outlined in two colors, with A in purple and B in yellow. The initiating methionine codon is in red.

(Zenise-Gregory et al., 1992). Residues  $-24$  to  $-17$  (TTAACAA) (Fig. 8) could represent a functional variation on the TATA motif (Singer et al., 1990), but the fact that promoters that included only to  $-20$  were active means that the full motif is not required for normal transcriptional activity. 'TATA'-less promoters that lack the 'TATAA' box and bind core RNA polymerase proteins with Initiator (Inr) *cis*-acting sites have also been characterized (Smale, 1997). The efficiency and accuracy of Inr elements is increased in the presence of two other sequence motifs, the BRE and DPE (Kutach and Kadonaga, 2000). The sequences surrounding the *bam* transcriptional start site, however, do not match the consensus for Inr, BRE or DPE elements. Thus, other elements between  $-46$  and  $-20$  might function as 'TATA-less' RNA polymerase recruitment sites in the *bam* promoter or 'TTAACAA' at  $-24$  to  $-17$  functions as a 'TATAA' box *in vivo* and can be adequately replaced in transgenic constructs.

The same transgenes that identified the core element(s) of the *bam* promoter revealed that a germ cell-specific enhancer(s) was located upstream of  $-47$ . Deletions from the 5'-side showed that the upstream border of this enhancer region was located at  $-96$ . Additional deletions showed that essential enhancer sequences lay between  $-86$  and  $-61$ . The sequence 'gcgacgc' (Fig. 8) matches the binding consensus site for the Dpp-activated transcription factor Mad (Kim et al., 1997) and specific deletion of that eight-base pair element inactivated the *bam* promoter (Fig. 7). Genetic studies, however, have shown that Mad is not required for cystoblast and cyst differentiation (Xie and Spradling, 1998) and is therefore not essential for *bam* transcription. We suspect, therefore, that another activating transcription factor with a Mad-like recognition sequence binds to this site in *bam*. The significance of other sequences within this enhancer region will be determined only by additional transgenic studies with site-directed mutant constructs.

### Elements that act negatively

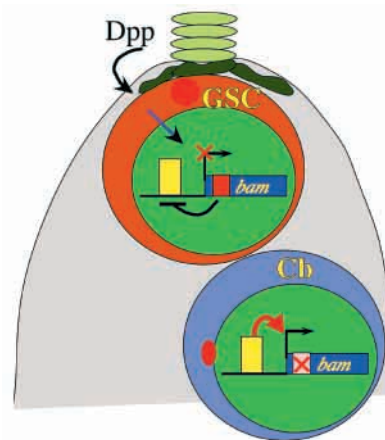
Deletions that caused the spreading of reporter expression into GSCs and constructs that tested the sufficiency of specific *bam* promoter fragments revealed that an 18 bp silencer element (SE; Fig. 8) regulated *bam* expression. Although the SE was located in the 5'UTR, experiments that distinguished between transcriptional and post-transcriptional effects demonstrated that the *bam* SE repressed transcription. Chimeric promoters that joined heterologous sequences to *bam* fragments revealed several other features of the silencer element. The *bam* SE could not block GSC transcription from a *nos* enhancer/promoter fragment nor when the SE was placed upstream of the *bam* enhancer/basal region. Both of these observations can be explained if the SE's range was limited because either construct would place the silencer much further from the relevant enhancer elements. Such short-range repressive effects have been described for the dCtBP family of transcriptional repressors (Chinnadurai, 2002; Mannervik et al., 1999). Other explanations are equally possible, however, and resolution of the silencer's mechanism of action must await biochemical studies with silencer-binding proteins.

The structure of silencer, as revealed by complementary sets of necessity and sufficiency tests, suggests that at least two proteins bind to the site. The palindromic part of the silencer element (Site A) was essential for activity but it does not correspond to any known transcriptional regulatory protein-

binding site. The more 3' part of the silencer (Site B) corresponds to a consensus site for Brinker binding (Sivasankaran et al., 2000; Zhang et al., 2001). Brinker has been identified previously as a transcriptional repressor of Dpp target genes and was therefore a reasonable candidate for a silencer binding protein. Brinker is an antagonist of the Dpp-signaling because it prevents the transcription of a subset of Mad activated genes (Kirkpatrick et al., 2001; Rushlow et al., 2001; Saller and Bienz, 2001), but Brinker is itself a Dpp target (Minami et al., 1999) because Mad and Schnurri heterodimers can block *brk* transcription in *dpp*-responsive cells (Marty et al., 2000). The paradigm established from these studies for regulation of *brk* expression predicted that the gene would be transcriptionally repressed in cells with activated Dpp signaling, such as GSCs. Indeed, previous studies demonstrated that both Mad and Shn are active in GSCs (Xie and Spradling, 1998; Xie and Spradling, 2000) and are therefore in place to repress *brk* expression. In addition, enhancer trap expression and germline clonal analyses did not support a role for Brk in germ cells, making it unlikely that Brk could play a significant role in keeping *bam* transcriptionally quiescent in GSCs. Perhaps another repressor protein that recognizes Brk-like elements binds to *bam* silencer Site B and forms a complex with the palindrome-binding protein to block *bam* transcription in GSCs.

### *bam* transcriptional control and GSC-to-cystoblast differentiation

The discovery of the silencer element permitted refinement of



**Fig. 9.** Regulation of elements controlling *bam* transcription. The transcriptional silencer in the *bam* 5'-UTR is active in the cells in contact with cap cells, preventing *bam* expression. This effectively prevents GSCs from differentiating into cystoblasts and maintains them as a self-renewing population. Dpp signaling, and perhaps other signals delivered by diffusion and gap junction connections, are probably important positive regulators of silencer element-binding proteins (SEBPs). In the stem cell daughter, GSC maintenance signaling would be abrogated, the silencer would be inactive and *bam* would be produced under the control of the enhancer elements that drive transcription in germ cells. During the transition from active-to-inactive silencer, GSC daughters would be 'pre-cystoblasts' until *bam* expression reached critical levels for full cystoblast differentiation. The switch from active-to-inactive silencer would also represent the biochemical mechanism for the asymmetry of the GSC division.

a model explaining the GSC-to-cystoblast switch. Our dissection of the *bam* promoter shows that *bam* can be expressed in GSCs because promoters lacking the silencer element are transcribed in all germ cells. For the same reason, the silencer does not work by blocking an overlapping enhancer site because silencer sequences can be deleted without disrupting enhancer-dependent transcription (Fig. 3C). Our current working model (Fig. 9) is that the silencer is occupied in GSCs and *bam* is transcriptionally quiescent because the germ cell-specific enhancer(s) cannot be engaged. When the presumptive cystoblast is born, silencer element-dependent repression can be relieved, allowing enhancer(s) in *bam* promoter to activate transcription and promote cystoblast differentiation. The clear implication of this model is that *bam* silencer occupancy is the basis of the asymmetric GSC division and that identification of silencer binding proteins can provide a mechanism for the asymmetry.

Previous studies showing that somatic cell signaling maintained GSCs by suppressing cystoblast formation suggested that *bam* might be the target of these signals (King and Lin, 1999; Xie and Spradling, 1998). We propose that occupancy of the *bam* silencer element in the germ cells adjacent to cap cells (i.e. GSCs) is signal dependent and is based on asymmetric activation or distribution of silencer element-binding proteins (SEBPs). For example, with the division of the neuroblast mother cell as a paradigm (Doe, 2001; Jan and Jan, 2001), perhaps asymmetric partitioning of one or multiple SEBPs accounts for cystoblast-specific *bam* transcriptional activation. Alternatively, differences in extrinsic signaling could produce post-transcriptional modifications of SEBPs to account for differential activity in GSCs and cystoblasts. Distinguishing between these and other possibilities to explain the molecular details of the GSC to cystoblast switch requires the identification of SEBPs and understanding the signaling circuits that control the occupancy of the *bam* silencer element.

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