Development 129, 2419-2433 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV5986

The *bric à brac* locus consists of two paralogous genes encoding BTB/POZ domain proteins and acts as a homeotic and morphogenetic regulator of imaginal development in *Drosophila*

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Accepted 15 February 2002

SUMMARY

The *bric à brac* (*bab*) locus acts as a homeotic and morphogenetic regulator in the development of ovaries, appendages and the abdomen. It consists of two structurally and functionally related genes, *bab1* and *bab2*, each of which encodes a single nuclear protein. Bab1 and Bab2 have two conserved domains in common, a BTB/POZ domain and a Psq domain, a motif that characterizes a subfamily of BTB/POZ domain proteins in *Drosophila*. The tissue distribution of Bab1 and Bab2 overlaps, with Bab1 being expressed in a subpattern of Bab2. Analysis of a series of mutations indicates that the two *bab* genes have synergistic, distinct and redundant functions during imaginal development. Interestingly, several reproduction-related traits that are sexually dimorphic or show diversity among *Drosophila* species are highly sensitive to changes in the *bab* gene dose, suggesting that alterations in *bab* activity may contribute to evolutionary modification of sex-related morphology.

Key words: *bric à brac*, Ovaries, Legs, Abdomen, Secondary sexual traits, Anteroposterior and proximodistal patterning, BTB/POZ domain, Psq domain, *Drosophila*

INTRODUCTION

As every newcomer to a fly laboratory learns, the easiest way to distinguish between *Drosophila melanogaster* (*Dm*) females and males is to look for the sex-specific pigmentation pattern of the posterior abdomen, the male-specific sex combs on the forelegs and/or the genital structures. These morphological features not only constitute sexually dimorphic traits but show considerable interspecific variation among members of the *Dm* species group and subgroup, and some of these features also show intraspecific variation. Concerning sex combs, variation is observed in the number of tarsal segments that display sex combs, their number per segment, and the number of sex comb bristles (Bock and Wheeler, 1972; True et al., 1997; Nuzhdin and Reiwitch, 2000; Macdonald and Goldstein, 1999). In *Dm* males, a sex comb is only found on the most proximal or first tarsal segment (TS1), and consists of a single row of approximately ten sex comb bristles.

Another sexually dimorphic trait in *Dm* is the pigmentation of the tergites of the posterior abdomen. Whereas an alternating dark-light stripe pattern is characteristic of all abdominal tergites in females, the posterior-most two tergites in males are darkly pigmented throughout. Sexually dimorphic abdominal pigmentation is not characteristic for all *Drosophila* species, however (Kopp et al., 2000), and a large diversity in abdominal pigmentation is seen within the genus *Drosophila* (Ashburner, 1989; Hollocher et al., 2000; Eisses and Santos, 1997). Even within the *Dm* species subgroup, in which the abdominal pigmentation pattern is rather homogeneous, a newly discovered species, *D. santomea* lacks any abdominal pigmentation (Lachaise et al., 2000). In addition, considerable intraspecific variation in the pigmentation of posterior tergites in females has been described for different wild-type populations of *Dm* (Robertson et al., 1977; Eisses and Santos, 1997). Together, these findings indicate a rapid phylogenetic divergence of sex comb and abdominal pigmentation patterns.

The genetic and developmental mechanisms that allow for fast evolutionary modifications of characteristics that are connected to reproduction/mating (secondary sexual traits) and may be involved in speciation are largely unknown. A strong modifier locus of abdominal pigmentation in wild-type populations of *Dm* was mapped to the distal arm of the third chromosome, and is called *fap* (female abdominal pattern) (Robertson and Riviera, 1972; Robertson et al., 1977). *fap* is

very likely to correspond to the *bab* locus that maps to the same chromosomal position, and was recently shown to be involved in the regulation of abdominal pigmentation and morphology (Kopp et al., 2000). The correlation between the expression pattern of *bab* and the abdominal pigmentation pattern in species of the *Dm* species group suggests that divergence in the regulation of *bab* expression may have contributed to the variation of abdominal pigmentation in this species group (Kopp et al., 2000).

The *bab* locus is also a homeotic regulator of the sex comb pattern (Godt et al., 1993). Mutations in *bab* cause a transformation of the bristle pattern in the tarsal segments, leading to the appearance of ectopic sex combs in distal tarsal segments. The graded requirement and graded expression pattern of *bab* along the proximodistal axis of the tarsus suggest that the concentration of Bab specifies the identity of the bristle pattern in tarsal segments in a dose-dependent manner. Moreover, *bab* plays a second role in leg development, which appears to be independent of its function in the specification of the bristle pattern. *bab* mutations cause a failure in joint formation, resulting in the fusion of tarsal and antennal segments (Godt et al., 1993). The phenotype of *bab* mutants shows that this locus is also an important morphogenetic regulator of ovarian development. During ovarian development, *bab* mediates the formation of the terminal filaments, stacks of cells that are required for the formation of ovarioles (King, 1970; Godt and Laski, 1995; Sahut-Barnola et al., 1995) and contribute to the regulation of germline and follicle stem cell divisions during oogenesis (Lin and Spradling, 1993; Forbes et al., 1996).

We show that the *bab* locus is composed of two paralogous genes, *bab1* and *bab2*. The corresponding proteins Bab1 and Bab2 belong to a subfamily of BTB/POZ domain (BTB domain)-containing nuclear proteins that have a Psq-domain but no Zn-finger motif. Although partially redundant in function, our data indicate that both genes are required for normal development. *bab2* appears to play a predominant role in ovarian and particularly in leg development, which points to a functional divergence of the two Bab genes.

MATERIALS AND METHODS

Flies

The *bab* alleles bab^{E1}, bab^{P}, bab^{PR72}, bab^{PRDS}, bab^{PR24}, bab^{A128} and $Df(3L)bab^{PG}$ are described elsewhere (Godt et al., 1993). bab^{AR07} was isolated in a P-element-mediated excision mutagenesis that was conducted for bab^{A128} (=*P[ry*$^{+}$*, lacZ]A128*) similar to that described for bab^{P} (Godt et al., 1993). The mutations bab^{E3}, bab^{E4}, bab^{E5} and bab^{E6} were isolated in an EMS mutagenesis using an *ri e* mutant background similar to that described by Godt et al. (Godt et al., 1993). Lethal hits on the chromosomes that carried the *bab* mutations were removed using an *ecd st red e ca* chromosome for recombination. The deficiencies *Df(3L)Fpa1* (61D1;61F1-2) and *Df(3L)Fpa2* (61F1;61F4) are γ-ray induced. The allele bab^{D1} was a gift from K. Burtis. *TM6B, Tb* and genetic markers are described elsewhere (Lindsley and Zimm, 1992). Oregon-R was used as a wild-type stock.

Flies used for phenotypic analysis of the abdomen were free of genetic markers that affect the body color, such as *y* or *e*. In flies that carry the bab^{E1}, bab^{E5} and bab^{P} allele, respectively, the *e* marker was replaced by an e^{+} allele using an Oregon-R chromosome for recombination. Cuticles of abdomina and legs were mounted in Hoyer's medium (Ashburner, 1989). Flies were raised at 25°C if not indicated otherwise.

Molecular analysis of the *bab* locus

Genomic DNA from the bab^{P} line was used to clone a *Taq*I fragment adjacent of the *PlacZ* insertion site by inverse PCR (Godt et al., 1993). This fragment was used to establish a genomic walk of 140 kb of DNA that covers both *bab* genes, using genomic lambda phage (Maniatis et al., 1978) and cosmid libraries (Tamkun et al., 1992). The orientation of the *bab* walk and the molecular breakpoints of deficiencies were defined by chromosome in situ hybridization using genomic DNA probes. The structures of the *bab1* gene and transcript were deduced from genomic sequence in combination with an RT-PCR analysis. The 5′ and 3′ ends of the terminal exons were identified by RACE-PCR analysis using polyA^{+} RNA from third instar larvae. Overlapping RT-PCR fragments were used to reconstruct a complete *bab1* cDNA (AJ252082). The BTB-II domain identified previously (Zollman et al., 1994) was shown to be part of *bab2*. Sequence and Southern blot analysis, using *bab2* genomic DNA and cDNAs led to the determination of the structure of the *bab2* gene and transcript (AJ252173). Close to full-length *bab2* cDNAs were isolated from an 8- to 12-hour-old embryo library (provided by N. Brown). The longest *bab2* cDNA isolated, 5.1 kb in size and named cDNA2, corresponds to the predicted *bab2* transcript, except that it retains the 70 bp intron 3. This intron is not present in a second cDNA (cDNA4) analyzed, nor was it found when mRNA from embryos and adult ovaries was analyzed by RT-PCR using primers that flank the intron, suggesting that the retention of intron 3 in cDNA2 is an anomaly, and the 70 bp intron is normally removed by splicing. For Northern blot analysis, total RNA was prepared from third instar larvae with the SDS-hot phenol-chloroform technique (Palmiter, 1974), and PolyA^{+}-RNA was selected via oligo-dT.

New cDNAs of the *tkr* gene were isolated from a 0- to 24-hour-old embryonic cDNA library (Palazzolo et al., 1990).

Generation of *UAS-bab2* and *UAS-bab1* transgenic flies

The *bab2* cDNA2 and cDNA4, both of which contain the entire open reading frame of *bab2* (5′ and 3′ untranslated regions are incomplete in cDNA4) were subcloned into the pUAST vector (Brand and Perrimon, 1993). More than 20 independent transgenic lines were established for each construct. *UAS-bab2* cDNA2 and cDNA4 transgenic lines produced comparable mutant phenotypes. A *Hsp70-Gal4* driver line, carrying the *P[Gal4-Hsp70.PB]* construct on the second chromosome (Brand et al., 1994), was used for ubiquitous expression of *bab2*.

To generate a *UAS-bab1* transgene, a *bab1* minigene was constructed as follows. A 2.7 kb *Pst*I-*Sal*I genomic fragment, starting at a *Pst*I site 6 bp upstream of the ATG and ending at a *Sal*I site 422 bp after the 5′ splice site of intron 1, and a 4.5 kb *Bam*HI genomic fragment that contains all of the other exons from *bab1*, starting 146 bp upstream the 3′ splice site of exon 1 and ending 273 bp after the end of the *bab1* cDNA, were fused via blunt-ended *Sal*I and *Bam*HI sites and cloned into the pUAST vector. Five independent transgenic lines were established.

Production of polyclonal anti-Bab2 antibodies

A 1.3 kb *Bam*HI fragment (called 1.3B), which encodes amino acids 126 to 558 of Bab2, and a 1.6 kb *Xma*I fragment (called 1.6X; amino acids 470 to 1014 of Bab2) were subcloned into the pGEX-1N and pGEX-3X protein expression vectors (Pharmacia-Amersham), respectively. Fusion protein 1.3B-GST was soluble and extracted as described by Godt et al. (Godt et al., 1993). Fusion protein 1.6X-GST was insoluble, and was purified from inclusion bodies using urea (Grisshammer and Nagai, 1995). The fusion proteins were used for immunization of rats at Pocono Rabbit Farm and Laboratories.

The Bab2 antibodies R6 and R7 are directed against the 1.3B-peptide that includes the BTB domain of Bab2. To test whether these antibodies are specific for Bab2, their ability to recognize the BTB

domain of Bab2 and Bab1 was tested by immunoblot analysis (data not shown). Both antibodies, diluted 1:4000 recognized the BTB domain of Bab2 (0.1 μg of protein; a gift of G. Privé) but did not recognize the BTB domain of Bab1 (0.5 μg of protein). As the BTB domain is the only conserved domain between Bab1 and Bab2 in this peptide, we believe that the R6 and R7 antibodies are Bab2-specific. The Bab2 antibody R10 is directed against the 1.6X peptide that includes the BabCD with the Psq motif (Fig. 2C) The Bab2 specificity of this antibody was demonstrated indirectly by showing that the R10 antibody does not recognize a protein in *bab2* mutant tissues that are homozygous for bab^{E1} (data not shown).

Immunoblotting and tissue staining

Imaginal discs and brains were dissected from white prepupae in phosphate-buffered saline (PBS), pH7, transferred to 2× Laemmli buffer (Sambrook et al., 1989) on ice and homogenized. Protein samples containing tissues from two prepupae were boiled for 5 minutes and loaded onto a 6% polyacrylamide gel (20 μl/lane). To identify the Bab proteins on western blots, α-Bab2-R6 (1:20,000) and R7 (1:30,000) were used as primary antibodies, and α-rat HRP-conjugated antibodies (1:1000; Jackson Laboratories) and the ECL-solution (Pharmacia-Amersham) were used for signal detection. β-Tubulin was detected with mAB-E7 (1:250; DSHB).

Dissection of larval tissues, tissue in situ hybridization and immunostaining were done as described by Godt et al. (Godt et al., 1993), with some modifications. For in situ hybridization, the first fixation was in 10% formaldehyde in PBS for 20 minutes, and hybridization took place at 50°C for 48 hours. For immunostaining, larval tissues were fixed in 5% formaldehyde in PBS for 10 minutes. Primary antibodies were α-Bab1-r2 (1:1000) (Godt et al., 1993) and α-Bab2-R7 or R10 (1:4000). α-rat secondary antibodies were Biotin-conjugated (1:400; Jackson Laboratories).

RESULTS

The *bab* locus consists of two structurally related genes

bab was originally identified by the bab^{P} allele, a P-element insertion mutation that maps to polytene band 61F1-2 (Godt et al., 1993). An open reading frame (ORF) adjacent to the bab^{P} insertion identified a BTB domain, a domain found primarily but not exclusively in Zn-finger containing transcription factors (Collins et al., 2001). In addition to *bab*, a second gene encoding a BTB domain (called *BTB-II*) was identified at 61F1-2 and found to have an expression pattern similar to *bab* (Zollman et al., 1994). We show that *bab* and *BTB-II* constitute a gene complex. For clarity, the gene previously called *bab* will be renamed *bab1*, and *BTB-II* will be renamed *bab2*. The *bab* locus or *bab* will refer to both genes together. The structure of the *bab* locus is shown in Fig. 1, and the structure of the Bab1 and Bab2 proteins is shown in Fig. 2.

The *bab1* and *bab2* genes have the same orientation and show several structural similarities (Fig. 1), suggesting that they are the result of a chromosomal duplication. *bab1* and *bab2* span approximately 60 kb and 25 kb of genomic DNA, respectively. The insertion point of bab^{P} is located in intron 1 of *bab1*, 236 bp downstream of the 5′ splice site. Both genes have four introns, three of which are at homologous positions in the coding region. Sequence analysis of the *bab1* and *bab2* transcript predicts proteins of 967 and 1067 amino acids, respectively (Fig. 2A). Bab1 and Bab2 have two evolutionarily conserved domains in common. Outside of these domains

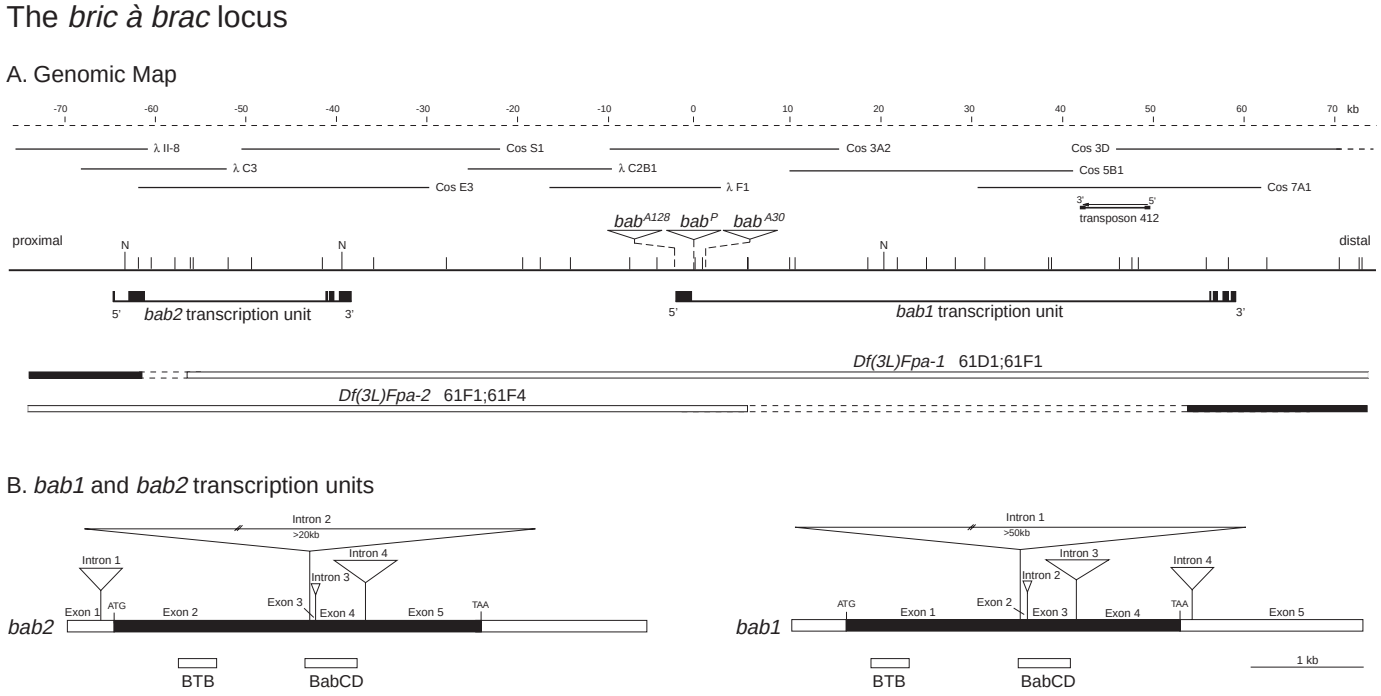
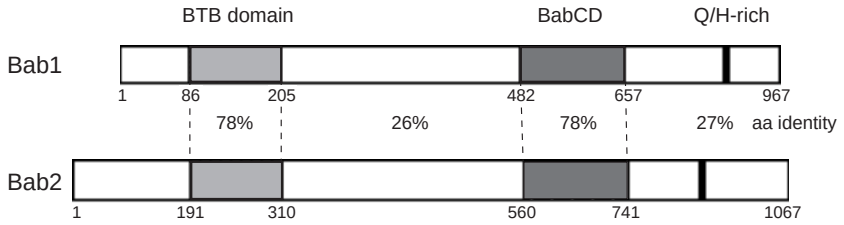
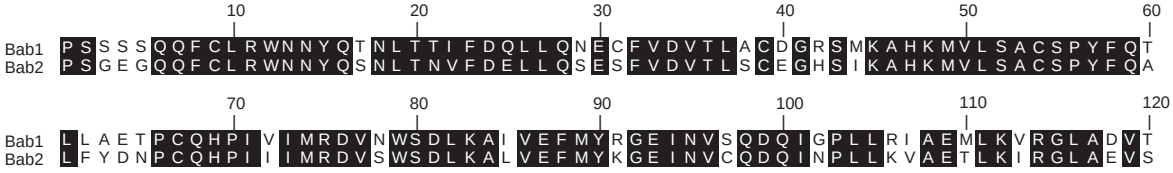


Fig. 1. The *bab* locus consists of two genes. (A) Molecular map with the center line representing the genomic DNA. *Not*I (N) and *Eco*RI sites (vertical lines) and the P-element insertion sites of bab^{A128}, bab^{P} and bab^{A30} are indicated. The bab^{P} insertion point is defined as 0 on the map, the entire locus covers approximately 130 kb of DNA. The positions of the cosmid and lambda fragments of the genomic walk are shown at the top. A *412* transposable element is located in intron 1 of *bab1*. At the bottom are two overlapping deficiencies that each have one breakpoint within the *bab* locus. White regions represent deleted sequences, black regions represent DNA still present, and the deletion breakpoints are within the dashed regions. (B) Structure of the *bab1* and *bab2* transcripts. Untranslated regions (open boxes), exons (black boxes) and positions and sizes of introns are shown. The BTB domain and BabCD-encoding regions are also indicated.

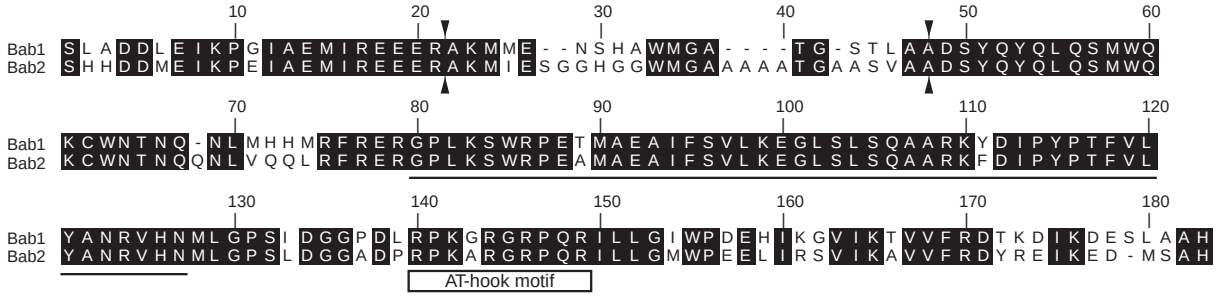
A. Protein structure



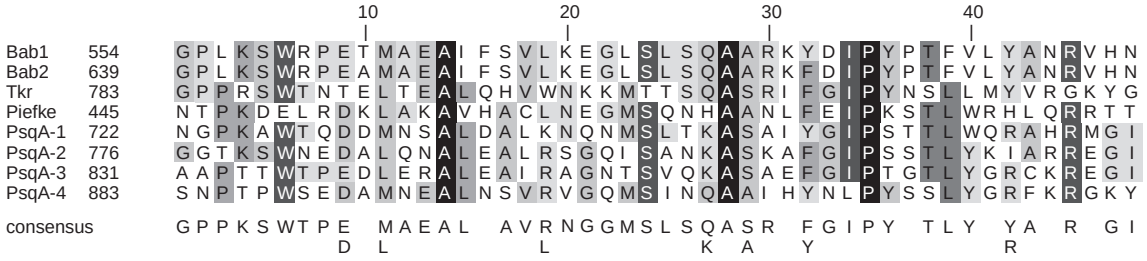
B. BTB domain



C. Bab Conserved Domain (BabCD)



D. Psq domain



E. Gln and His rich domain

Bab1 [aa 800-840] **HHQQQQQAAHHHMQQLHLQQQQAHLHHHQQQQQQQQQQHHQ** Bab2 [aa 800-829] **QQQQHQQHHQQQAHHQQQPSHHQQQSPHAQ**

Fig. 2. Bab1 and Bab2 have a BTB domain, a Psq-domain and an AT-hook-like motif. (A) Sequence similarity between Bab1 and Bab2 is restricted to the N-terminal BTB domain and the C-terminal BabCD. Sequence identity is indicated in percent. (B) Alignment of the BTB domains of Bab1 and Bab2. (C) Alignment of the BabCDs of Bab1 and Bab2. A Psq domain (underlined) and an AT-hook-like motif are contained within the BabCD. The BabCDs of Bab1 and Bab2 are encoded by three exons (black triangles indicate exon boundaries). (D) Comparison of the Psq domains of Bab1, Bab2, Tkr and Piefke, and the four Psq domains of the Psq protein. Residues conserved in all the domains are highlighted in black, less conserved residues are highlighted in gray. (E) Glutamine- (Q, gln) and histidine- (H, his) rich domains of Bab1 and Bab2.

Bab1 and Bab2 show only low sequence similarity to each other and no significant similarity to other proteins. A BTB domain, a conserved domain of 115 amino acids, is found in the N-terminal region of both proteins (Fig. 2A). In contrast to most known BTB domain-containing proteins that are transcriptional regulators, the BTB domains of Bab1 and Bab2

do not start within the first few amino acids of the predicted proteins but at amino acids 90 and 195, respectively. The Bab1 and Bab2 BTB domains are the most closely conserved domains within the BTB family (Fig. 2B) (Zollman et al., 1994).

In contrast to many other known BTB domain-containing proteins that are transcription factors, neither Bab1 nor Bab2 contains a Zn-finger motif. However, these proteins have a second domain in common that we call BabCD for Bab-conserved domain (Fig. 2C). In both proteins, this domain is encoded by three exons with the splice sites at homologous positions. The BabCD contains two known motifs, a Psq domain and an AT-hook like motif. The Psq domain can mediate DNA binding, and is named after *pipsqueak* (*psq*), another BTB domain-encoding gene (Weber et al., 1995; Horowitz and Berg, 1996; Lehmann et al., 1998). The Psq domain, which is 48 amino acids in length, is 97% identical between Bab1 and Bab2, and is the region of highest similarity within the BabCD (Fig. 2C). A Psq domain has also been identified in the Tkr protein (Haller et al., 1987) and in Piefke (CG15812) (Schwendemann et al., 2001). Piefke also has a BTB domain. The previously published sequence of Tkr lacks a BTB domain, however newly isolated cDNAs for the *tkr* gene show that it contains the BTB-III domain (Accession Number AJ252174), which we previously found to map to the same chromosomal location and to display the same expression pattern as *tkr* (Zollman et al., 1994). Therefore, all five genes known to encode proteins with a Psq domain in *Drosophila* also have a BTB domain (Fig. 2D).

The C-terminal region of the BabCD contains an AT-hook-like motif (Fig. 2C). It has the invariant peptide core motif R-G-R-P flanked on either side by other positively charged amino acids but does not correspond to any known AT-hook variant identified so far. AT-hook motifs have been shown to mediate binding to the minor groove of stretches of AT-rich DNA (Reeves and Nissen, 1990). Whether the AT-hook-like motif in Bab1 and Bab2 is involved in DNA-binding remains to be investigated. Both Bab proteins also have a short motif that is very rich in glutamine and histidine residues (Fig. 2E), which could aid transactivation (Courey and Tjian, 1988; Gerber et al., 1994). The domain architecture of Bab1 and Bab2, together with their nuclear localization (Godt et al., 1993; Godt and Laski, 1995), suggest that these proteins function as transcriptional regulators.

bab1 and *bab2* have overlapping expression patterns

During embryogenesis, *bab2* is zygotically expressed in a complex pattern that has been described previously (Zollman et al., 1994), whereas *bab1* is not expressed at a detectable level. *bab* seems to have no essential function during embryonic development as even mutants that lack both *bab* genes are not embryonic lethal.

During post-embryonic stages, *bab2* is expressed in a broader range of tissues than *bab1* and generally shows a higher level of expression. In larval and prepupal ovaries, *bab1* transcript and protein are only detected in cells that form the terminal filaments (Fig. 3B,C, Fig. 4D). The expression of *bab2* is more complex (Fig. 3A, Fig. 4A-C). At early to mid third larval instar, prominent *bab2* expression is seen in the developing terminal filaments and in a cell population that we named 'swarm cells'. Swarm cells migrate from anterior to posterior past the cluster of germ cells during third larval instar (Fig. 4A,B). They produce the basal stalks, a pupal-specific tissue, and may also contribute to tissues of the adult ovary (M. L. and D. G., unpublished). The highest level of *bab2* expression in the swarm cells is seen during their migration. *bab2* is also expressed in the apical cells of the larval ovary. After terminal filaments have formed, apical cells migrate between the terminal filaments posteriorly and form the outer sheaths of the egg tubes. The level of expression in these cells increases during the third larval instar and is highest at the time the cells begin their posterior migration (Fig. 4C). *bab2* expression is also seen in the interstitial cells that intermingle with the germ cells. *bab* mutant ovaries not only display defects in terminal filament formation but also in other cell populations of the ovary, such as the apical cells and the basal stalk primordium (Godt and Laski, 1995). If the development of these cell populations depends on the presence of terminal filaments, then the observed defects in these cell populations could be a secondary effect of *bab* mutations. Alternatively, *bab* may be directly required for the development of these cell populations as the apical cells and swarm cells express *bab2*.

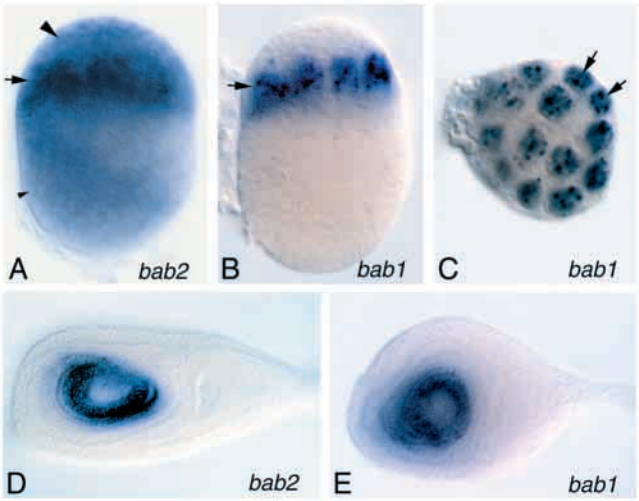


Fig. 3. Comparison of the *bab1* and *bab2* mRNA distribution patterns in ovaries of white prepupae (A-C) and leg imaginal discs of late 3rd instar larvae (D,E). (A) A lateral view (anterior is upwards) showing high levels of *bab2* expression in terminal filaments (arrow) and lower levels in the apical cells (large arrowhead) and the swarm cells (small arrowhead). (B) Lateral view, and (C) top view showing the specific expression of *bab1* in the terminal filaments. (D) *bab2* and (E) *bab1* show a similar expression in the primordium of tarsal segments TS1-TS4 in leg imaginal discs. A 4.2 kb genomic fragment of *bab1* that includes the first exon and a full-length cDNA of *bab2* were used as probes for tissue in situ hybridization.

bab1 and *bab2* transcripts are expressed in a similar pattern in the tarsal primordium of leg imaginal discs (Fig. 3D,E) and in a subdistal region of the antennal disc (data not shown) (Godt et al., 1993). Similar to the protein distribution of Bab1 (Fig. 4J) (Godt et al., 1993), Bab2 protein is expressed in a graded manner in the tarsal primordium, with the concentration of Bab2 highest in tarsal segments TS3 and TS4, lower in TS2 and even lower in TS1 (Fig. 4H,I). However, the differences in the level of expression between the tarsal segments are not as pronounced as with Bab1. Both Bab proteins are enriched in

the ridges compared to the furrows of the tarsal folds. In contrast to Bab1, Bab2 expression is not restricted to TS1-4 but is also found in the proximal region of TS5 (Fig. 4H), in the peripodial membrane, and in the periphery of the leg imaginal disc that gives rise to thorax structures (Fig. 4I). No morphological defects have been observed in derivatives of leg imaginal discs outside the tarsus.

Both *bab* genes are expressed in the female and male genital discs. The genital discs give rise to the internal and external structures of the genitalia, the A8 and A9 tergites and the anal plates (Bryant, 1978; Casares et al., 1997). The strongest expression of Bab proteins in the female genital disc is found in the primordium of the vaginal plates and the A8 tergite (Fig. 4F), structures that are affected in *bab* mutants. In the male genital disc, *bab* expression is mainly seen in a region of the male genital primordium (Fig. 4G). In addition, in the central nervous system (CNS), Bab1 and Bab2 are distributed in a similar pattern. *bab*-expressing cells are found in the central brain hemispheres and the thoracic ganglia of late 3rd instar larvae and prepupae (Fig. 4E). In *bab* mutants, no gross morphological defects were observed in histological sections of the prepupal CNS, and it remains uncertain whether *bab* has a function in the brain.

In summary, the expression pattern of *bab1* during imaginal development can be described as a subpattern of the *bab2* expression pattern. In tissues that require *bab* function for development, *bab1* and *bab2* are usually co-expressed.

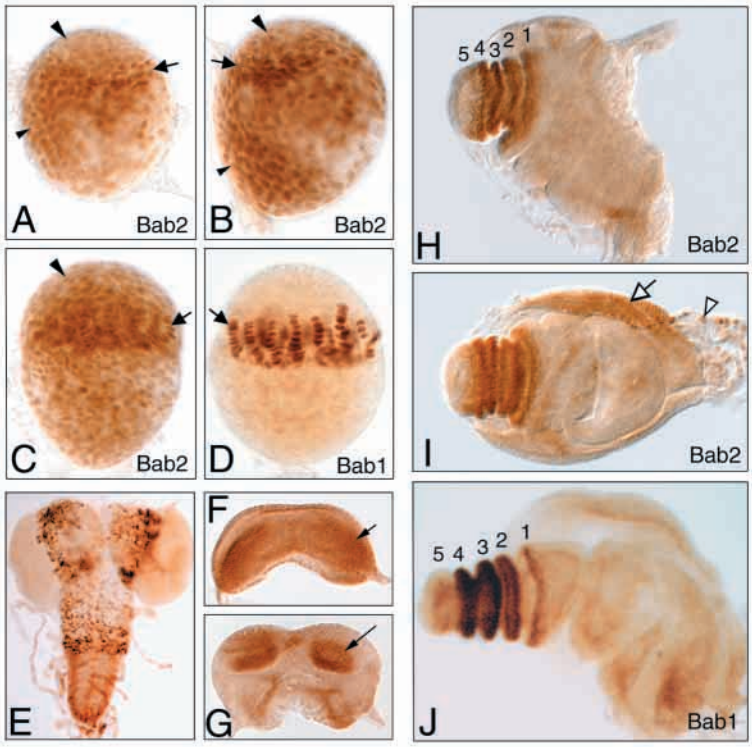


Fig. 4. Comparison of the Bab1 and Bab2 expression patterns in imaginal primordia. (A) Early-mid and (B) mid-late instar larval ovary (lateral view), showing high levels of Bab2 expression in the developing terminal filaments (arrow) and migrating swarm cells (small arrowhead), and low levels of expression in apical cells (large arrowhead) and other somatic cells. (C) Ovary at puparium formation (frontal view): the level of Bab2 expression remains high in the terminal filaments (arrow), and has increased in the apical cells that migrate posteriorly between the terminal filaments (arrowhead) and decreased in the posteriorly located swarm cells. (D) Ovary at puparium formation: Bab1 is seen only in the nuclei of terminal filament cells. (E) In the prepupal CNS, Bab1-expressing cells are found in a peripheral layer of the central brain hemispheres and the thoracic ganglia. (F) In a late 3rd instar female genital disc, and (G) in a male disc, strongest Bab2 staining is seen in the female genital primordium and the male genital primordium (arrows). (H-J) In leg imaginal discs, both Bab proteins are found in a graded distribution in the tarsal primordium with the highest level of expression seen in TS4 and 3, lower in TS2 and lowest in the distal region of TS1 (numbers indicate tarsal segment primordia). In contrast to Bab1 (J), Bab2 is also found in the proximal region of TS5 (H,I), in the peripodial membrane (arrowhead in I), and in the disc periphery (arrow in I). Anterior is upwards in A-D,F,G; distal is towards the left in H-J. Bab2 is detected with Bab2-R7 (A-C) or Bab2-R10 antibodies (F-I), and Bab1 with the Bab1-r2 antibody (D,E,J).

Molecular analysis of *bab* mutations reveals the functional importance of both *bab* genes

To determine the roles of *bab1* and *bab2* in mediating bab function, we conducted a molecular and phenotypic analysis of mutations in the *bab* locus. The P-element insertion of bab^P maps close to the 5′ end of the first intron of *bab1* (Fig. 1). bab^P does not affect the transcription of *bab2* but results in the loss of the *bab1* 5.4 kb transcript and the appearance of an abundant 2.6 kb transcript (Fig. 5A). This shorter *bab1* transcript is detected in bab^P heterozygotes and homozygotes, and is detected only with probes located upstream of the insertion. Characterization of the 3′ end of this 2.6 kb mRNA by 3′ RACE-PCR showed that this transcript is a hybrid of the first *bab1* exon and a region of the *P[ry*$^+$*, lacZ]* construct. The 5′ splice site of the *bab1* transcript that normally functions to splice out the large first intron of *bab1* and the 3′ splice site of the *l(3)S12* gene, contained in the *P[ry*$^+$*, lacZ]* construct just upstream of the *rosy* gene (Dutton and Chovnick, 1991), are spliced together. This demonstrates that the *P[ry*$^+$*, lacZ]* insertion causes aberrant splicing and transcription termination of the *bab1* transcript. A similar event has been reported for a *PlacZ* allele of the *psq* gene (Horowitz and Berg, 1995). The 2.6 kb truncated *bab1* transcript is more abundant than the 5.4 kb RNA (Fig. 5A) suggesting that it might be more stable than the wild-type transcript. Translation of this transcript would produce a protein that contains the BTB domain of Bab1 but not the BabCD. A Bab1-specific antibody directed to a domain of the Bab1 protein that is encoded by the truncated transcript, however, did not detect any protein in bab^P homozygous flies (data not shown). These results suggest that the bab^P P-element insertion severely disrupts or abolishes the function of the *bab1* gene. Nevertheless, bab^P homozygous flies display ovary defects of only intermediate strength and reveal no leg defects (Table 1) (Godt et al., 1993; Sahut-Barnola et al., 1995). This indicates that a loss of *bab1* does not produce a *bab* null mutant phenotype and suggests that a second gene is involved in *bab* function.

This hypothesis is corroborated by the analysis of another P-element insertion, bab^{A128} (Godt et al., 1993) that maps 57

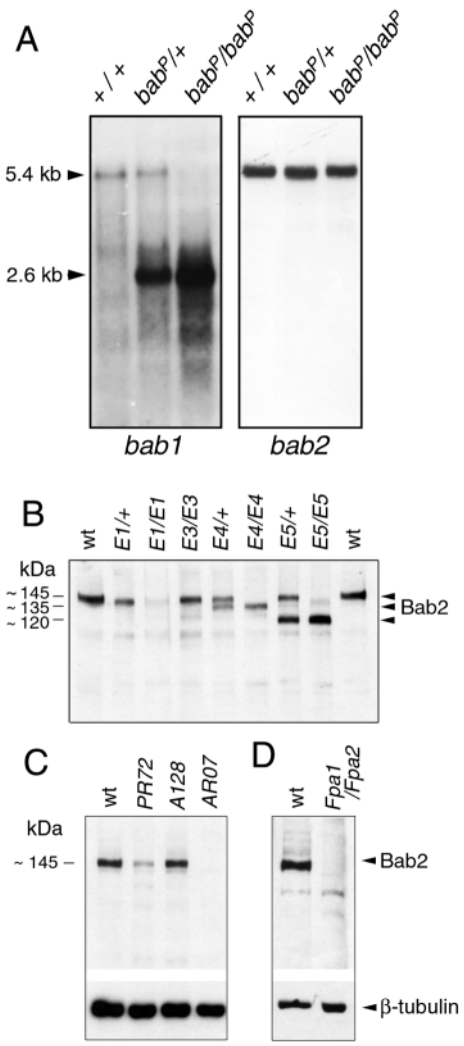


Fig. 5. Molecular analysis of *bab* mutations. (A) PolyA$^+$ RNA (10 μg) from 3rd instar larvae that were wild type (+/+), bab^P/+ or bab^P/bab^P were each hybridized with *bab1*- and *bab2*-specific riboprobes. Both, *bab1* and *bab2* encode a 5.4 kb transcript. Detection of the *bab1* wild-type signal required 4 days of film exposure, whereas the much stronger *bab2* signal was detected after only 12 hours. The bab^P insertion causes a truncation of the *bab1* mRNA, yielding a 2.6 kb transcript, but does not affect the *bab2* transcript. (B) Bab2 has a molecular weight of approximately 145 kDa. bab^{E1} causes a strong reduction of Bab2, and bab^{E4} and bab^{E5} cause a truncation of Bab2. (C,D) Bab2 is not detected in *Df(3)Fpa1/Df(3)Fpa2* or bab^{AR07}/bab^{AR07} mutants, and reduced levels of Bab2 are found in bab^{PR72} homozygotes. bab^{A128} (C) and bab^{E3} mutants (B) produce a Bab2 protein of normal size. Bab2 protein was detected using Bab2-R6 (B,C) or Bab2-R7 antibodies (D), which recognize the N-terminal region of Bab2.

bp from the 5′ end of the *bab1* transcript. Only one phenotypic trait of *bab* mutants, an abdominal pigmentation defect in females is associated with this insertion. Because homozygous bab^{A128} flies have no ovary or leg defects, it was surprising to find that Bab1 protein is not detectable in bab^{A128} mutant tissues (data not shown). A strong reduction in the amount of *bab1* transcript, as seen by in situ hybridization and RNA blot analysis (data not shown), indicates that the bab^{A128} insertion interferes with the transcription of *bab1*, reducing Bab1 to undetectable levels. By contrast, flies that are heterozygous for strong *bab* mutations, such as bab^{PR72}, or deletions of the *bab* locus, such as *Df(3L)Fpa1*, have reduced but clearly detectable levels of Bab1 (data not shown), and nevertheless show leg defects in addition to defects in abdominal pigmentation (Table 1). Taken together, analysis of the mutations bab^P and bab^{A128} strongly suggests that *bab1* is not the only gene involved in *bab* function.

That *bab2* is involved in *bab* function was confirmed by a protein analysis of *bab* mutants. The wild-type Bab2 protein is detected in immunoblots as a band of approximately 145 kDa using different anti-Bab2 antibodies that recognize either the N- or C-terminal region of Bab2 (Fig. 5B-D, data not shown). An analysis of *bab* mutants revealed that the alleles bab^{E1}, bab^{E4} and bab^{E5} affect the Bab2 protein (Fig. 5B). In a homozygous bab^{E1} mutant, Bab2 protein is reduced to barely detectable levels. bab^{E4} and bab^{E5} mutants produce truncated Bab2 proteins. By contrast, Bab1 expression appears normal in these three mutants, shown by tissue immunostaining as the anti-Bab1 antibody does not produce a signal in immunoblots (Table 1). No change in the size of Bab2 and the expression level of either Bab1 or Bab2 were detected in bab^{E3} and bab^{E6} mutants. bab^{E1}, bab^{E4}, and bab^{E5} mutants display developmental defects in ovaries, legs and the abdomen (Table 1), demonstrating that the *bab2* gene plays an essential role in development and that it is functionally related to *bab1*.

The *bab* null phenotype requires a lack of both *bab1* and *bab2* activity

The strongest *bab* mutations previously published have both a strong ovary and leg phenotype (Godt et al., 1993; Godt and Laski, 1995). Further analysis of two of these mutations, bab^{PRDS} and bab^{PR72}, revealed that they affect the expression of both *bab1* and *bab2*. They each lack detectable amounts of Bab1 and have reduced levels of Bab2 (Fig. 5C). The mutant phenotypes caused by bab^{PRDS} or bab^{PR72} are slightly enhanced in *trans* to a large deletion ($Df(3L)bab^{PG}$), indicating that these mutations are not null for the *bab* locus (Godt et al., 1993).

Additional *bab* alleles were isolated and studied to find one that completely lacks *bab* activity. Two deletions, *Df(3L)Fpa1* and *Df(3L)Fpa2*, that were isolated based on a dominant female pigmentation defect (Ken Burtis, personal communication), extend into the *bab* locus from opposite sides, each having a breakpoint in the *bab* locus (Fig. 1). *Df(3L)Fpa2* deletes *bab2* completely, and deletes the 5′ region of *bab1*, including the BTB domain. *Df(3L)Fpa1* deletes *bab1*, and has a breakpoint in the second intron of *bab2*, deleting everything downstream of the BTB domain. In *Df(3L)Fpa1/Df(3L)Fpa2* transheterozygotes and in flies homozygous for the mutation bab^{AR07}, neither Bab1 nor Bab2 are detected (Fig. 5C,D). The phenotype of these genotypes is stronger than that of previously described mutations (Table 1). As these flies lack both *bab1* and *bab2* function, this phenotype corresponds to the null phenotype of the *bab* locus.

The *bab* locus regulates the development of ovaries, limbs and the abdomen

To further analyze the function of the two *bab* genes, we studied the phenotypic series of *bab* mutations and the *bab* null

Table 1. Characterization of *bab* mutations

bab alleles	Mutagen	*bab* gene affected by the mutation	Bab1 protein: Tissue immunostaining	Bab2 protein: Tissue immunostaining	Bab2 protein: Western blot analysis	Recessive ovary phenotype	Recessive leg phenotype
bab^{P}	*P[ry⁺,lacZ]* insert*	*bab1*	–	+	+	I	WT
bab^{PRDS}	bab^{P} P-excision*	*bab1, bab2*	–	+	Reduced	S	S
bab^{PR72}	bab^{P} P-excision*	*bab1, bab2*	–	+	Reduced	S	S
bab^{A128}	*P[lArB]* insertion*	*bab1*	–	+	+	WT	WT
bab^{AR07}	bab^{A128} P-excision	*bab1, bab2*	–	–	–	VS (null)	VS (null)
bab^{E1}	EMS*	*bab2*	+	–	Very reduced	S	S‡
bab^{E3}	EMS	n.d.	+	+	+	VW	W
bab^{E4}	EMS	*bab2*	+	+	Truncated	I	I
bab^{E5}	EMS	*bab2*	+	+	Truncated	I	I
bab^{E6}	EMS	n.d.	+	+	+	W	W
Fpa1/Fpa2†	γ-ray	*bab1, bab2*	–	–	–	VS (null)	VS (null)

Tissue immunostainings: +, protein present; –, protein not detectable in larval and prepupal ovaries, imaginal discs and CNS.

Western blot analysis: +, protein of normal size at a concentration similar to wild type; Reduced, reduced concentration of a normal-sized protein; –, not detectable; Truncated, change in protein size.

Phenotypes: WT, phenotypically wild type; VW, very weak; W, weak; I, intermediate; S, strong; VS, very strong *bab* mutant phenotype; n.d., not determined at the molecular level.

†*Fpa1/Fpa2 = Df(3L)Fpa1/Df(3L)Fpa2*; ‡the phenotype of bab^{E1} homozygotes has changed slightly in our stock and is now stronger than reported by *Godt et al. (1993).

mutant phenotype. *bab* null mutants are semi-viable. They develop into pharate adults but often have difficulties in eclosing from the pupal case, which may be a result of their leg defects. *bab* null mutants display defects in ovaries, tarsal segments, antennae, abdominal segments and female genital disc derivatives.

Ovarian defects

Based on the phenotypic series of *bab* alleles of varying strength, we define four phenotypic classes of *bab* mutant adult ovaries (Fig. 6).

(1) Females with a weak *bab* mutant ovary phenotype are fertile but have ovaries that are somewhat smaller than wild-type ovaries, slightly irregular, and rounded at the anterior end, owing to defects in terminal filament formation (Fig. 6B). The ovarioles contain normal-looking follicles and mature oocytes.

(2) Female flies with an intermediate *bab* mutant ovary phenotype are semi-sterile to sterile. The ovaries have a very irregular shape and are substantially smaller than wild-type ovaries (Fig. 6C,D). They contain a reduced number of ovarioles that are abnormally oriented with the germaria often not located at the anterior end but inside the ovary (Sahut-Barnola et al., 1995).

(3) Females with a strong *bab* mutant ovary phenotype do not lay eggs. The ovaries are very small and contain only one to two ovarioles of very abnormal structure and orientation (Fig. 6E,F) (Godt and Laski, 1995). Only very few and defective follicles are found in these ovarioles.

(4) In *bab* null mutants, the ovaries are even smaller and no developing follicles have been observed.

Leg defects

The *bab* mutant leg phenotype that involves all three leg pairs in both females and males has two characteristics: (1) a fusion of tarsal segments, characterized by a shortening of tarsal segments and a loss of tarsal joints; and (2) a transformation of the bristle pattern of distal tarsal segments toward the bristle pattern of the first tarsal segment (Fig. 7) (Godt et al., 1993). Most sensitive to a fusion are tarsal segments TS5 and TS4 (Fig. 7C,D). The stronger the *bab* mutation, the further proximal the fusion extends. In *bab* null mutants, TS5 to TS2 are frequently fused into a single segment (Fig. 7E-H). Sensitivity to a transformation of the bristle pattern of tarsal segments decreases from proximal to distal, involving only TS2 in weak *bab* mutants and TS2-4 in strong *bab* mutants (Fig. 7) (Godt et al., 1993). This can best be seen using the prominent sex comb bristles of the prothoracic legs of males as a marker (Fig. 7C,E), and the transverse bristle rows of the pro- and metathoracic legs of both sexes (Fig. 7C-H). In a *bab* null mutant, the bristle pattern of TS2-4 is transformed; however, the sex combs are often eliminated, owing to the shortening and fusing of the tarsal segments (Fig. 7G). A thickening of the distal tarsal segments seen in *bab* mutant legs

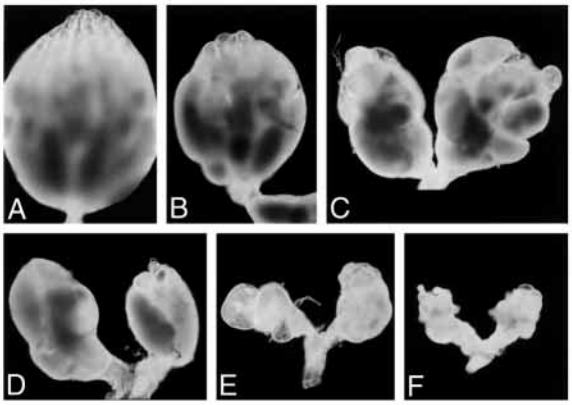


Fig. 6. Phenotypic series of *bab* mutant adult ovaries. Morphology of (A) a wild-type ovary, and (B-F) *bab* mutant ovaries of increasing phenotypic strength, showing changes in size and shape of the female reproductive organ because of a decreasing number of ovarioles and developing follicles. Genotypes: (B) bab^{E5}/bab^{E6}, (C) bab^{E5}/bab^{PR72}, (D) bab^{E1}/bab^{E4}, (E) bab^{PR72}/bab^{E1} and (F) bab^{PR72}/bab^{PR72}. (A,B) A single ovary, (C-F) a pair of ovaries of 2- to 3-day-old females. All panels are at the same magnification, and anterior is upwards.

is an additional indication that the distal tarsal segments are transformed towards the identity of the first tarsal segment, which in wild type is much thicker than the distal tarsal segments (Fig. 7A,B,E-H).

bab loss-of-function defects in the abdomen

Wild-type females have eight tergites formed by abdominal segments A1-A8 (Fig. 8A), whereas wild-type males have seven tergites corresponding to A1-A6 + A9 (Fig. 8G) (Jürgens and Hartenstein, 1993). In females, the tergites of A1-A6 each show a darkly pigmented posterior and lightly pigmented anterior band. The two tergite plates of A7 are variably pigmented and A8 has a light coloration. The tergites of A1-A4 in males are similarly pigmented as in females, whereas the tergites of A5-A6 + A9 are darkly pigmented throughout. *bab* null mutants display a change in the pigmentation pattern of both sexes. A thorough dark pigmentation is found in A3-A8/A9 and is seen with low penetrance also in A2 (Fig. 8D,F,H). Ectopic dark pigmentation in A2 and A3 is usually patchy and restricted to the anterior margin (Fig. 8D,H). The phenotypic series of *bab* mutants shows that the sensitivity towards a change in pigmentation decreases from posterior to anterior, with A6 being more sensitive than A5, and A5 more sensitive than more anterior segments (Fig. 8B-D). In weak *bab* mutants, a change in pigmentation is therefore seen only in females. In summary, this indicates that loss of *bab* function leads to a transformation of the pigmentation from a female to a male-like pattern as well as from an anterior to a posterior-like pattern. The *bab* locus regulates the pattern and amount of pigmentation in all abdominal segments (except for A1), and suppresses dark pigmentation in the anterior region of abdominal segments in both sexes with the exception of A5 + A6 in males.

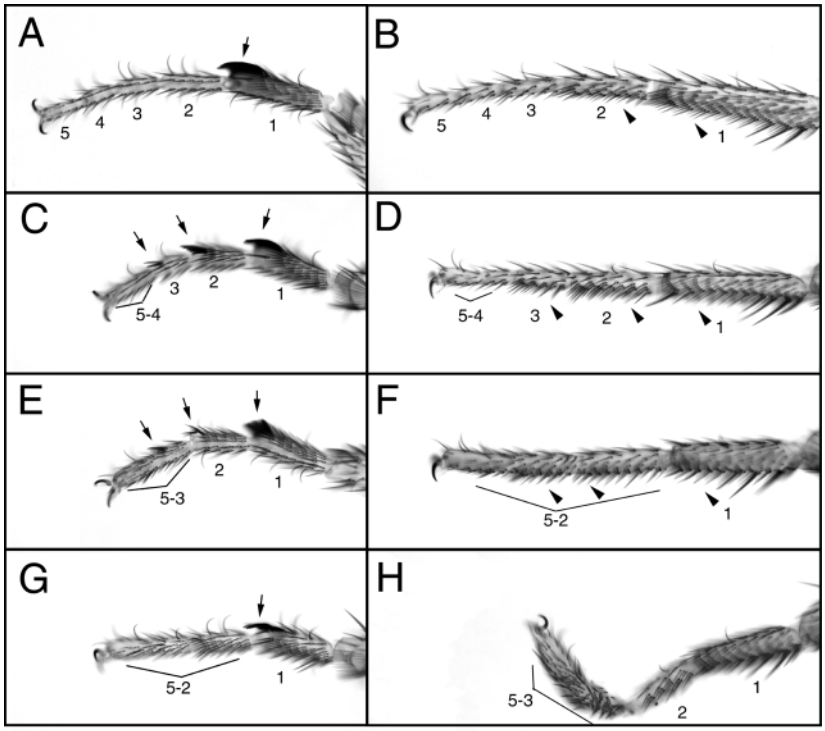


Fig. 7. *bab* specifies tarsal segment morphology. The tarsal region of the prothoracic leg of a male fly is shown in A,C,E,G, and the metathoracic leg in B,D,F,H. (A) Wild-type prothoracic leg showing a sex comb and transverse bristle rows only on TS1. (B) Wild-type metathoracic leg showing transverse bristle rows on TS1 and TS2. (C,D) bab^{PR72}/bab^{PR72} mutants (strong hypomorphic) show sex comb bristles and transverse bristle rows on TS1-TS3, and a fusion between TS5 and TS4. (E-H) *Df(3L)Fpa1/Df(3L)Fpa2* mutants show the *bab* null phenotype. Tarsal segments are strongly shortened and thickened. Although the bristle pattern of TS2-4 is transformed, the ectopic sex combs on TS2 and TS3 are smaller than in a hypomorphic mutant (E) or are missing altogether (G), and the TS1 sex comb is smaller than in wild type (E). Tarsal fusion extends further proximally, often leading to a fusion of TS5 to TS2 (F,G). Kinks and abnormal bristle arrangements (H) or loss of distal segments are frequently seen in metathoracic tarsi. In all panels, distal is towards the left; numbers 1-5 mark TS1-TS5; arrows indicate sex combs; arrowheads indicate transverse bristle rows; an angled line points to tarsal segments that are fused rather than connected by joints.

Except for a change in the pigmentation pattern, the morphology of A2-A5 appears to be normal in both sexes of *bab* null mutants. However, the posterior segments A6-A8 show additional morphological abnormalities, most of which are restricted to females. The trichome pattern of A6 is affected in both sexes. In *bab* null mutants, trichomes are not restricted to the anterior and lateral margin of the A6 tergite as in wild type, but are found at a low density throughout the tergite, similar to the normal trichome pattern of A5 (data not shown). This suggests a posterior-to-anterior transformation of the trichome pattern. Furthermore, the A6 tergite of *bab* mutant females is broader (anteroposterior) than the more anterior tergites (Fig. 8F, double-arrow) in contrast to wild type (Fig. 8E), which together with the heavy pigmentation gives this tergite a male-like appearance.

In contrast to A1-A6, in which the two primordia of each tergite fuse into a single plate, the A7 tergite consists of two loosely connected triangular plates in wild-type females that show small slightly twisted bristles and often two to three larger bristles (Fig. 8E). In *bab* null mutants, the A7 plates are fused into a continuous plate, are considerably broader (anteroposterior) than in wild type, and display an increased number of large bristles (Fig. 8F). These morphological changes suggest a transformation of A7 towards a more anterior segment fate. Furthermore, instead of the small pale bristles, which are characteristic of an A8 tergite in wild-type females, larger pigmented and slightly twisted bristles are found in a *bab* mutant A8 tergite (data not shown). Such bristles are similar to those normally found in A7 of females or in A9 of males, again suggesting homeotic transformations. In addition, the two rows of thorn bristles seen on the vaginal plates of wild-type females (Fig. 8I) are replaced in *bab* mutants by a different type of bristles, which are longer and twisted (Fig. 8J,K).

bab mutants also display defects in the sternites of the abdominal segments. Shape, pigmentation, and bristle pattern of the A6 and A7 sternites in females are different from wild type and show similarities to the A6 sternite in males (Fig. 8I-L). Both sternites are more strongly pigmented, and the number

of bristles is considerably decreased compared with wild type. Taken together, the alterations in the shape and the bristle and trichome patterns of posterior segments indicate that loss of *bab* function causes posterior-to-anterior transformations of some abdominal features (opposite to the change of the pigmentation pattern), and also transformations from a female to a male-like morphology.

bab gain-of-function defects in the abdomen

Ubiquitous overexpression of a *UAS-bab2* transgene under control of *Hsp70-Gal4* causes reduced viability, a general reduction in the pigmentation of the cuticle and bristles, and defective macrochaetae when flies are raised at a constant temperature of 25°C. In the abdomen of both sexes, the posterior dark pigmentation of A6 is reduced or missing, and little of the dark pigmentation is left in A5 and A4 (Fig. 8M-P). Tergites anterior to A4 are less affected than the posterior tergites. In both, *bab* loss- and gain-of-function experiments, pigmentation in posterior segments is more strongly affected than in anterior segments, indicating a graded requirement for *bab* along the anteroposterior axis. Similar phenotypic effects were observed in *bab1* overexpression experiments (data not shown). Together, our loss- and gain-of-function studies show that the *bab* locus is a suppressor of dark cuticle pigmentation in the fly.

When flies, carrying *UAS-bab2* under the control of *Hsp70-Gal4*, were shifted to 32°C during the late 3rd instar/pupal stages, they showed a 'split-tergite'-phenotype in addition to the loss of pigmentation (Fig. 8N). Here, the tergite primordia of all abdominal segments do not fuse, a trait normally only found in A7. The split-tergite phenotype is also seen when *UAS-bab2* expression is driven by a *bab1-Gal4* transgene (F. L., unpublished transgenic line), and is therefore likely not an artifact of the heat shock but a consequence of *bab2* overexpression. These data suggest that *bab* plays a role in tergite morphogenesis and is required to prevent a fusion of the A7 tergite primordia.

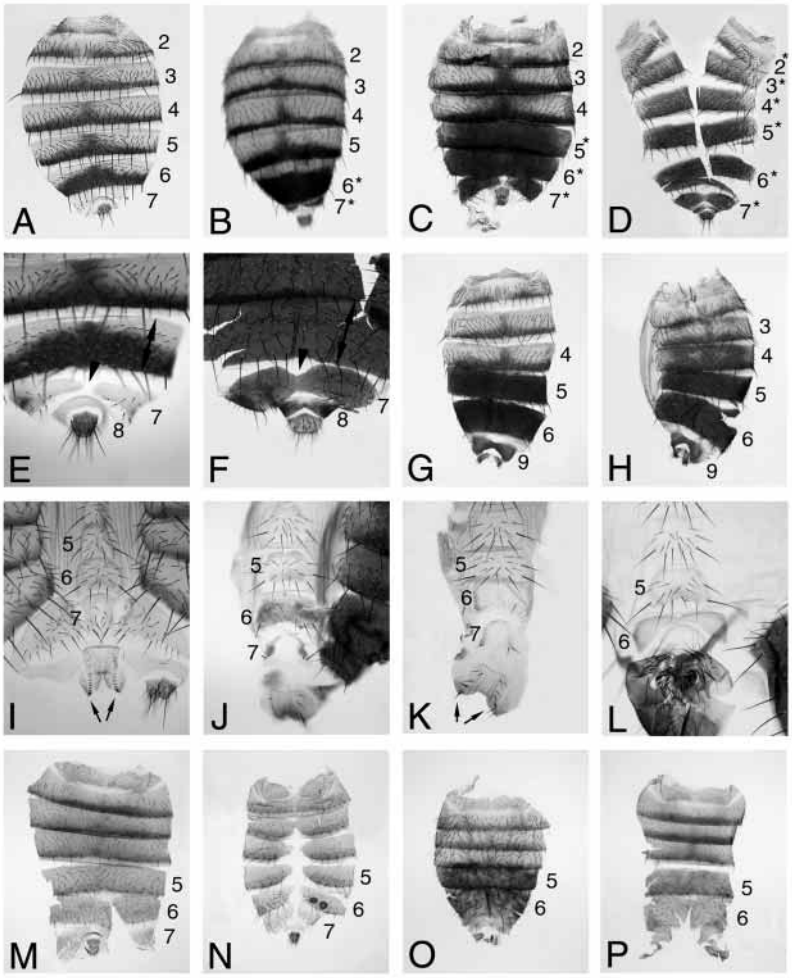


Fig. 8. *bab* controls the morphology of abdominal segments. Female tergite pigmentation in wild type (A) and in loss-of-function *bab* mutants of increasing phenotypic strength: (B) bab^{A128}/ bab^{A128} (C) bab^{Dl}/ bab^{Dl} and (D) bab^{AR07}/*Df(3L)Fpa2*, a null mutant. Ectopic dark pigmentation in the anterior portion of the tergites is indicated by asterisks. (F) In a *Df(3L)Fpa1/Df(3L)Fpa2* female, the two tergite plates of A7 are fused (arrowhead) and enlarged, and contain more bristles in comparison with wild type (E). In addition, the A6 tergite is enlarged (double arrow in E,F). (G) Wild-type and (H) *Df(3L)Fpa1/Df(3L)Fpa2* male abdomina, showing ectopic pigmentation in *bab* mutant tergites. Ventral view of the abdomen of (I) wild-type, (J) *Df(3L)Fpa1/Df(3L)Fpa2* and (K) bab^{AR07}/*Df(3L)Fpa2* females, and a (L) wild-type male showing features of the A6 and A7 sternites, and the female vaginal plates (arrows in I,K). (M-P) Ubiquitous overexpression of *bab2* causes a loss of pigmentation. A *Hsp70-Gal4/+;UAS-bab2*$^{66/3}$/+ female (M) and male fly (P), raised at 25°C, lack dark pigmentation in A6. The loss of pigmentation is stronger in posterior than more anterior abdominal segments. (N) In a *Hsp70-Gal4/+;UAS-bab2*$^{66/3}$/+ female fly, raised at 18°C until late 3rd instar and at 32°C during the pupal stage, the tergite primordia have not fused. (O) A *Hsp70-Gal4/+;UAS-bab2*^{51A3}/+ male fly, raised at 25°C, shows reduced pigmentation in A5 and A6. In all panels, anterior is upwards and segment identity is indicated by numbers.

The functional relationship between Bab1 and Bab2: synergistic and distinct effects

To analyze the relative functions of *bab1* and *bab2*, and to look for possible interactions, we compared the phenotypic effects of *bab* mutations in ovaries and legs, and studied their complementation behavior. This study involved mutations that affect *bab1* (bab^{P} and bab^{A128}), *bab2* (bab^{E1}, bab^{E4}, and bab^{E5}), *bab1* and *bab2* (bab^{PRDS} and bab^{PR72}), or are null for *bab1* and *bab2* (bab^{AR07} and deletions of the *bab* locus), and some molecularly uncharacterized *bab* alleles.

All five EMS alleles (bab^{E} series of alleles) were isolated based on dominant leg defects. bab^{E1} is the strongest EMS allele, causing a strong recessive phenotype in ovaries and legs; bab^{E4} and bab^{E5} produce an intermediate, and bab^{E6} and bab^{E3} a weak recessive phenotype in those organs (Tables 1-3, Fig. 6). The phenotype seen in flies transheterozygous for any two of the EMS alleles is intermediate in strength to the phenotypes displayed by the homozygotes, indicating that the phenotypic effect of the EMS alleles is additive (Tables 1-3). The EMS alleles in *trans* to the strongest allele bab^{E1} produce a mutant phenotypic series that is comparable with, but less severe than, each EMS allele in *trans* to a deletion of the *bab* locus (Tables

Table 2. Complementation analysis of *bab* mutations based on ovary morphology

	Df(3L)bab^{PG}	*bab^{E1}*	*bab^{P}*	*bab^{PRDS}*
Wild type	+	+	+	+
bab^{A128}	(+)	+	+	+
bab^{E3}	<W	(+)	+	(+)
bab^{E6}	W	W	+	<W
bab^{E4}	I	I	<W	W
bab^{E5}	I	I	<W	W
bab^{E1}	S	S	<I	<I
bab^{PRDS}	S	<I	<S	S
bab^{PR72}	S	I	<S	S
bab^{AR07}	>S	n.d.	>I	n.d.
Df(3L)bab^{PG}	L	S	n.d.	S

A chromosomal aberration, *Df(3L)bab^{PG}*, that deletes *bab1* and *bab2*, a mutation in *bab2* (*bab^{E1}*), a mutation in *bab1* (*bab^{P}*), and a mutation that affects *bab1* and *bab2* (*bab^{PRDS}*) were tested in *trans* to several mutations of the *bab* locus.

Ovary phenotypes: +, phenotypically wild type; (+), very subtle structural defects; W, weak; I, intermediate; S, strong (see text for the definition of these phenotypes); < and > indicate a weaker or stronger expression, respectively, of these phenotypic classes; L, lethal; n.d., not determined.

2, 3). As *bab^{E3}* and *bab^{E6}* do not complement *bab^{E1}* but complement *bab^{P}* they should represent *bab2* mutations like the other EMS alleles (Table 2). Therefore, all *bab* alleles isolated on the basis of a dominant mutant leg phenotype are mutations in *bab2*.

All *bab* alleles that were isolated as excision derivatives of *bab^{P}* (the *bab^{PR}* alleles) show non-complementation in *trans* to each other or in *trans* to a deletion of the *bab* locus, and display a normal phenotypic series (Tables 2, 3). The *bab^{PR}* alleles that cause a strong mutant phenotype, such as *bab^{PR72}* and *bab^{PRDS}*, not only reduce the expression level of *bab1*, but also of *bab2*. Mutations in *bab* that affect *bab1* but have no detectable effect on the expression of *bab2*, such as *bab^{P}* and *bab^{A128}*, have a considerably weaker mutant phenotype. No *bab* mutation has been identified that affects only *bab1* and causes strong mutant defects in ovaries and/or legs. Therefore, we propose that *bab2* plays a predominant role in exerting *bab* function in ovarian and particularly in leg development.

We also compared the effects of *bab1* and *bab2* mutations on abdominal pigmentation. Mutations in either *bab* gene cause dominant and more pronounced recessive pigmentation defects. Females homozygous for the strong *bab2* allele *bab^{E1}* display an uniformly dark pigmentation of tergites in A5 and A6, and females homozygous for the intermediate allele *bab^{E5}* show uniformly dark pigmentation in A6 and partial ectopic pigmentation in A5 (data not shown). By contrast, *bab1* mutant females that are homozygous for *bab^{P}* or *bab^{A128}* show ectopic dark pigmentation in the tergites of A6 and A7 (Fig. 8B). These observations suggest that there is an overlapping and differential requirement for *bab1* and *bab2* in abdominal segments.

To gain a better understanding of the relationship of the two *bab* genes, mutations in *bab1* and *bab2* were tested for complementation. Partial non-complementation is observed between *bab1* and *bab2* alleles in ovaries (Table 2) and legs (Table 3). First, flies carrying a *bab1* and a *bab2* mutation in *trans* display a mutant ovary phenotype, although it is weaker than the one observed in flies homozygous for either the *bab1* or the *bab2* mutation. This may be caused by an interaction of these alleles with the wild-type copy of *bab1* and/or *bab2*, as flies heterozygous for a deletion of the *bab* locus do not have ovary defects. Second, although *bab1* mutations produce neither dominant nor recessive leg defects in a background that is wild-type for *bab2*, flies carrying a *bab1* mutation in trans to a *bab2* mutation show leg defects that are stronger than the dominant leg defects caused by the *bab2* mutation in *trans* to a wild-type chromosome. This suggest that *bab1* is functionally active although not essential in leg development. Our complementation data point to functional dependency between mutations in *bab1* and *bab2*, the nature of which remains to be explored.

In summary, our genetic analysis of the bab locus indicates that *bab1* and *bab2* are partially but not fully redundant in function, and that both *bab* genes are required for normal *bab* function.

Table 3. Complementation analysis of *bab* mutations based on leg morphology

	Wild type	*A128*	*P*	*E3*	*E6*	*E4*	*E5*	*E1*	*PRDS*	*PR72*	*AR07*	*Fpa2*	*Fpa1*
Wild type	0	0	0	0	0	2	2	3	2	2	3	3	4
A128		0	0	1	2	2	1	3	3	4	3	3	7
P			0	0	2	3	2	4	–	4	4	4	5
E3				1	–	–	3	3	2	–	–	3	5
E6					3	–	4	4	5	–	–	4	5
E4						4	5	6	5	–	–	5	8
E5							6	6	5	5	–	7	7
E1								8	7	8	–	8	8
PRDS									8	7	–	8	9
PR72										9	–	9	9
AR07											10	10	10
Fpa2												L	10
Fpa1													L

Classes 0-10 designate the severity of the *bab* mutant leg phenotype, with 0 being phenotypically wild type and 10 corresponding to the *bab* null phenotype. Classes 1-6 correspond to the maximal number of sex comb bristles on TS2 found on ≥10% of the legs. Class 7: sex comb on TS2 and TS3 and a partial fusion of segments TS5 and TS4 (TS5^4) or no sex comb on TS3 but a complete TS5^4 fusion. Class 8: sex combs on TS2-TS3 and a TS4^5 fusion. Class 9: sex combs on TS2-TS4 and a TS5^4^3 fusion. Class 10: most severe leg phenotype, showing a TS5^TS2 fusion, and a severe shortening of tarsal segments. The bristle pattern of TS2-TS4 is transformed towards the bristle pattern of TS1; however, ectopic sex combs are often missing from TS2-TS4 and reduced in TS1. *Df(3L)Fpa1* causes generally a stronger mutant phenotype in combination with hypomorphic mutations of the *bab* locus than other *bab* null mutations. This could be due to a modifier of *bab* function that is uncovered by the deletion, or alternatively could be due to a dominant negative effect caused by an undetectable level of expression of the *bab2* BTB domain (compare Fig.1A). *Fpa1*, *Df(3L)Fpa1*; *Fpa2*, *Df(3L)Fpa2*. Other designations indicate *bab* alleles. L, lethal, –, not determined.

DISCUSSION

Bab1 and Bab2 belong to a distinct subfamily of BTB domain proteins in *Drosophila*

The superfamily of BTB domain proteins, to which Bab1 and Bab2 belong, constitutes one of the largest protein families in *C. elegans*, *Dm* and humans, with 58 representatives found in the *Dm* genome (Zollman et al., 1994; Lander et al., 2001) (Pointud and J.-L. C., unpublished). The majority of these proteins in *Dm* and humans also contain Zn-finger domains and are thought to be transcriptional regulators. The BTB domain is likely to contribute to this role by mediating protein oligomerization (Bardwell and Treisman, 1994). BTB domains can form homo-oligomers and hetero-oligomers (Dhordain et al., 1995; Dong et al., 1996; Kobayashi et al., 2000). Crystallographic data suggest that the BTB domain of PLZF (promyelocytic leukemia Zn-finger) can form homodimers that show an extended hydrophobic interaction between the BTB domains, indicating a high degree of stability (Ahmad et al., 1998; Li et al., 1997). We have shown that the BTB domain of Bab1 can interact with itself and with the BTB domain of Bab2 in a yeast two-hybrid system suggesting that the Bab proteins can homomerize and/or heteromerize via this domain (Pointud et al., 2001).

It has also been shown that BTB domains can interact with non-BTB domain-containing proteins. For example, the transcriptional repressors PLZF or BCL6 interact via their BTB domains with the co-repressors SMRT, N-CoR and mSin3A (Hong et al., 1997; Dhordain et al., 1997; Guidez et al., 1998; Huynh and Bardwell, 1998; Lin et al., 1998). These co-repressors bind in turn to a histone deacetylase that can modify the local chromatin and inhibit transcription (David et al., 1998; Dhordain et al., 1998; Lin et al., 1998). This mechanism seems not to be universal for all BTB domain-containing transcriptional repressors, however, because the HIC-1 protein is not able to bind to N-CoR, SMRT or mSin3, and its repression activity is not affected by inhibitors of histone deacetylases (Deltour et al., 1999). We have recently shown that the BTB domain of the Bab proteins binds to the two nuclear proteins BIP-1 and BIP-2 (Bab Interacting Protein) (Pointud et al., 2001). BIP-2/$TAF_{II}155$ is a TFIID component, and is homologous to $yTAF_{II}47$, a histone fold containing TAF_{II} (Gangloff et al., 2001). This suggests that the Bab proteins may regulate transcription of their target genes by directly interacting with a component of the general transcription machinery via their BTB domains.

The Bab proteins belong to a subfamily of nuclear BTB domain proteins that have no Zn fingers for DNA binding. However, the BabCD in Bab1 and Bab2 contains a Psq domain and a motif that may correspond to an AT-hook. As both Psq domains and AT-hooks are known DNA-binding domains (Lehmann et al., 1998; Reeves and Nissen, 1990), we propose that the BabCD mediates DNA binding. It is noteworthy that the protein di/oligomerization domain is always found in the N-terminal region and widely separated from the DNA-binding domain in BTB domain proteins, including Bab1 and Bab2. Such an organization, unlike many other transcription factors, should allow for high flexibility of these proteins when bound to DNA. This property and the capacity of the BTB domain to form oligomers suggest a model for how BTB domain proteins can regulate gene expression at the chromatin level by binding to distant DNA-binding sites and forming a large complex that brings together regions that are far apart on the DNA, moving enhancers or silencers closer to the basal transcriptional machinery.

The functional relationship of the paralogues *bab1* and *bab2*

Gene duplications are thought to play a crucial role in the evolution of new morphologies and the generation of new species. Increase of gene copies through duplication can lead directly to morphological changes if the gene function is dose sensitive. Furthermore, duplications provide the raw material for the development of new gene functions (for reviews, see Sidow, 1996; Wagner, 1998), as seen, for example, in the Hox gene cluster (for reviews, see Duncan, 1987; Carroll, 1995; Gellon and McGinnis, 1998). It has been also proposed that the inactivation or loss of duplicated genes can efficiently induce reproductive isolation, a basis for divergence and speciation (Lynch and Conery, 2000). The functional study of duplicated genes can therefore illuminate evolutionary processes and can help to determine how molecular divergence translates into divergence of gene function. Our characterization of the paralogous genes *bab1* and *bab2* suggests (1) that both genes are required for normal development, (2) that their functions show redundant and divergent aspects, and (3) that *bab2* plays a predominant role in exerting *bab* function.

Divergence in function of two genes that arose by tandem duplication might be due to qualitative or quantitative differences in *cis*-regulation of gene expression or due to differences in protein structure. In case of the *bab* paralogues, we document evidence for both as discussed below. *bab1* is expressed in a subpattern of *bab2* and shows a generally lower expression level than *bab2* at the RNA and protein level. In most tissues that have been found to display morphological defects in *bab* mutants, *bab1* and *bab2* are co-expressed, raising the possibility that *bab1* and *bab2* are co-required for normal *bab* function. They could function as heterodimers that are formed through the interaction of their BTB domains. However, if Bab1/Bab2 heterodimers would be the only functional unit of *bab* action, we would expect that a knock out of either *bab1* or *bab2* would yield a *bab* null phenotype, which is not the case. In addition, ectopic expression of either *bab1* or *bab2* alone has a phenotypic effect suggesting that each *bab* gene is able to exert a function on its own. Whether Bab1 and Bab2 interact molecularly in the regulation of downstream genes awaits further analysis.

The two *bab* genes seem partially redundant as the strongest developmental defects in ovaries, legs and the abdomen associated with the *bab* locus have been observed only in mutants that are null for *bab1* and *bab2*. The two *bab* genes are not functionally equivalent, however. First, there is an overlapping but also differential requirement for *bab1* and *bab2* in the pigmentation of different abdominal segments, with A7 being more dependent on *bab1* and A5 on *bab2* activity. Second, ovarian defects are seen with mutations affecting either *bab1* or *bab2*, but loss-of-function of *bab2* causes a more severe phenotype. As the function of the *bab* locus is strongly dose/concentration dependent, the predominance of *bab2* in regulating ovarian development may be a result of the higher expression level of *bab2*. Furthermore, the differences in the ovarian expression patterns may have functional significance. *Cis*-regulatory differences, however,

cannot sufficiently explain the differential requirement of *bab1* and *bab2* in leg development. Although both genes are similarly expressed in leg imaginal discs, a *bab1* knockout does not cause a mutant leg phenotype, whereas even weak *bab2* mutants display dominant leg defects. This indicates that only *bab2* plays an essential role in leg development and suggests a qualitative divergence in the function of Bab1 and Bab2 proteins. Taken together, we propose that *bab1* and *bab2* have not only developed differences in transcriptional regulation but also differences in protein function that could be responsible for changes in the interaction with other transcription factors and/or DNA-binding sites.

Function of *bab* as a homeotic and morphogenetic regulator of imaginal development

bab acts as a homeotic regulator, as *bab* mutations cause homeotic transformations in the legs and the abdomen (this work) (Godt et al., 1993; Kopp et al., 2000). Here, we show that the homeotic transformations in the abdomen of *bab* mutants are complex. *bab* loss-of-function mutants display a combination of anterior-to-posterior transformations (pigmentation), posterior-to-anterior transformations (bristles, trichomes and segment shape and size), and female to male transformations (pigmentation, bristles and segment shape and size). *bab* seems to be mainly required in the posterior segments A5-A8. This domain that is mostly controlled by the Hox gene *Abdominal-B* (*Abd-B*), whose loss-of-function causes posterior to anterior transformations of segment identity (Karch et al., 1985; Sánchez-Herrero et al., 1985). It has been demonstrated that *bab* expression is repressed by *Abd-B*, either directly or indirectly, in posterior abdominal segments at the late pupal stage (Kopp et al., 2000). As *bab* acts as a suppressor of pigmentation, the repression of *bab* expression by *Abd-B* function leads to the complete pigmentation of the A5 and A6 tergites in wild-type males. In females, the repression of *bab* by *Abd-B* is counteracted by the female specific *doublesex* (*dsxF*) gene product (Kopp et al., 2000). It is unlikely, however, that *Abd-B* is a general repressor of *bab* activity, as *bab* mutants show not only anterior-to-posterior but also posterior-to-anterior transformations in the abdomen. This indicates that the regulation of *bab* activity is complex. *Abd-B* in conjunction with co-regulators might repress or activate *bab* function, dependent on the cell type and on the developmental time at which specific morphological features are specified. We propose that the differential, fine-tuned spatial and temporal regulation of *bab* expression plays a crucial role in providing morphological diversity between the abdominal segments along the anteroposterior axis and between the sexes. Similar to the abdomen, *bab* plays a role in the generation of morphological diversity between distal segments in the leg. *bab* is part of a network of transcription factors that divide the proximodistal axis into successively smaller domains, leading to the formation and specification of the different leg segments (Godt et al., 1993; Duncan et al., 1998; Campbell and Tomlinson, 1998; Couso and Bishop, 1998; Galindo and Couso, 2000).

bab also plays a role as a morphogenetic regulator of development. Previous studies have indicated that *bab* controls cell rearrangements during terminal filament formation in the ovary (Godt and Laski, 1995; Sahut-Barnola et al., 1995; Sahut-Barnola et al., 1996). *bab* is also required for the proper folding of leg imaginal discs, which may be important for tarsus segmentation (D. G., unpublished). Furthermore, *bab* negatively regulates the fusion of the tergite primordia in the abdomen, a process that is also controlled by the Hox genes (Karch et al., 1985). This suggests that the Bab transcription factors control the morphogenetic behavior of cells in different developmental processes. It will be a future challenge to determine whether *bab* directly regulates expression of proteins that mediate cell shape changes and cell movements.

Does *bab* play a role in variation and divergence of reproduction-related traits in the *Drosophila* family?

Flies of the *Drosophila* family show substantial intraspecific and interspecific variation in sex-related traits, including sex combs and abdominal pigmentation as described before, as well as male genital structures (Bock and Wheeler, 1972; True et al., 1997) and number of ovarioles (Mahowald and Kambysellis, 1980; Hodin and Riddiford, 2000). Variation in these traits can affect mate choice and fertility, and thus reproductive success (Spieth, 1952; Cook, 1977; Coyne, 1985; Markow et al., 1996; Arnqvist, 1998; Kopp et al., 2000; Hodin and Riddiford, 2000). Furthermore, there is evidence that divergence of phenotypic traits related to reproduction in combination with ecologically adaptive divergence in sexual selection can lead to reproductive isolation and speciation (Boughman, 2001; Schneider, 2000).

Interestingly, *bab* controls the morphology of several traits that are involved in reproduction and show rapid evolutionary divergence. *bab* regulates the formation of the reproductive organ in females, as it is required for terminal filament formation and consequently for the development of ovarioles in the ovary (Godt and Laski, 1995; Sahut-Barnola et al., 1995). *bab* mutations of increasing strength cause a decrease in the number of ovarioles, raising the possibility that *bab* might be involved in determining ovariole number in *Dm*. Moreover, *bab* controls several secondary sexual traits. *bab* activity suppresses sex combs on tarsal segments distal to TS1 (this work) (Godt et al., 1993). *bab* may also be involved in determining the number of sex comb bristles in TS1, as overexpression of Bab2 in TS1 causes a reduction in the number of sex comb bristles compared with wild type (D. Godt, unpublished). Furthermore, *bab* regulates sexually dimorphic bristle and trichome patterns and the pigmentation of posterior abdominal segments (this work) (Kopp et al., 2000). A comparison of abdominal pigmentation and *bab* expression pattern between the two sexes of different members of the *Drosophila* species group demonstrates a striking correlation between phenotypic differences and *bab* expression patterns, suggesting a causal relationship (Kopp et al., 2000). That changes in *cis*-regulatory elements can indeed lead to variation in morphology between different *Drosophila* species was documented for the *ovo/shavenbaby* locus that controls the segmental trichome pattern in larvae (Sucena and Stern, 2000). As *bab* loss- and gain-of-function mutations of *bab* have pleiotropic effects on the development of reproduction-related characteristics, evolutionary alterations in *bab* function could lead to a diversification of multiple sex traits.

The *bab* locus appears to have two important properties that make it suitable to cause variation in the development of morphological traits. First, because the *bab* locus represents a tandem duplication, redundancy between *bab1* and *bab2* may have facilitated fast molecular modifications, resulting in the

observed alterations of the expression level and pattern of *bab1* and *bab2* and their functional diversification. One potential consequence, for example, would be that abdominal pigmentation could change independently of the leg pattern through mutations in *bab1*, as this gene is no longer essential for leg development. In fact, many alleles of the *fap* locus, which affects female abdominal pigmentation and is presumably identical to *bab*, have been proposed to occur in wild-type populations of *Dm* (Robertson et al., 1977).

Second, *bab* function is highly dose dependent. *bab* is haploinsufficient, and *bab* mutations cause dominant homeotic transformations of adult characteristics that do not interfere with viability in laboratory cultures. The expression profile of *bab* in imaginal discs and the abdomen is graded, and differences in *bab* concentration determine morphology of legs and abdomina (Godt et al., 1993; Kopp et al., 2000). As concentration matters, small variations in the expression level or shape of the *bab* gradient could lead to morphological diversification. Such a mechanism has also been proposed for *Ubx* in establishing the trichome pattern in one of the leg segments (Stern, 1998). Differences in *Ubx* expression between *Drosophila* species are responsible for the difference in their trichome morphology as shown by interspecies crosses involving *Ubx* mutations of these species (Stern, 1998). Taken together, we propose that *bab* is an important regulator of reproduction-related characteristics in *Dm*, and therefore may play an active role in the variation, divergence and speciation in the genus *Drosophila*.

We thank Ken Burtis for sharing unpublished information and bab^{D1} flies; Gail Flores and Silvia Yu for help with mutagenesis experiments; Gil Privé, Andrea Brand, L. Kalfayan, Carl Thummel, the DSHB and Bloomington stock center for flies and reagents; and Ulrich Tepass, Joel Atallah and Corinne Lours for helpful comments on the manuscript. Funding was provided by the INSERM and grant GREG 59/95 (to J. L. C.), by the NSERC (to D. G.), and by the NIH (#GM40451 to F. A. L.).

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