Role of *Bar* locus in development of legs and antenna in *Drosophila melanogaster*

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The X-linked *Bar* (*B*) mutation of *Drosophila melanogaster*, responsible for the well-known Bar eye phenotype due to over-expression of the BarH1 homeo-domain protein, is shown to enhance the abnormalities in legs and antennae of flies carrying a viable combination of certain *decapentaplegic* (*dpp*) loss of function mutant alleles. It is also shown that the homeo-domain carrying BarH1/BarH2 protein products of the *B* locus are expressed in a characteristic annular pattern in areas of normal larval leg and antennal discs that correspond to the distal regions of adult fly appendages. *dpp*-mutant background partly disrupts the expression pattern of Bar homeo-proteins in these discs and a combination of *B* and *dpp*-mutant alleles disrupts the Bar expression patterns in these imaginal discs much more severely. This is in agreement with the more severe phenotypes of legs and antennae of such flies. We suggest that the homeo-box containing *B* genes function as new members of the proximal distal sector genes and are important for patterning these appendages along their proximo-distal axes.

THE Bar eye mutant phenotype of *Drosophila melanogaster* is associated with a tandem duplication (*Bar* duplication) of the 16A1-7 region of the X chromosome[1], and is characterized by a drastically reduced number of ommatidia in the compound eyes of adult flies[2]. Organization of the *B* locus is complex since it harbours at least two homeo-box containing genes, the *BarH1* and *BarH2*, of which *BarH1* is reported to be over-expressed due to the *Bar* duplication[3,4]. The *decapentaplegic*, *dpp*, gene product is a member of the TGFβ family[5] and has very important roles in morphogenesis in many developmental pathways in *Drosophila*. The gene *dpp* is expressed in the eye discs of third-instar larvae of *Drosophila* in the anteriorly moving morphogenetic furrow and this is responsible for induction of differentiation of the precursor cells into ommatidia[6]. Over-expression of BarH1 homeoprotein in eye discs of *B* mutant larvae is associated with attenuation of *dpp* gene expression in the morphogenetic furrow[7]. As a result, ommatidial precursor cells fail to differentiate and instead, undergo apoptotic death. Consequently, the number of ommatidia in adult eyes of *B* mutant flies is substantially reduced[7]. All other adult structures are

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normal in the *B* mutant flies. Sato *et al.*[8] recently reported that *BarH1* and *BarH2* genes are expressed in the anterior-most notal region (prescutum) of the third-instar larval wing discs. Other than this, nothing is so far known about the expression and/or role of *B* genes in other larval imaginal discs.

During the course of our studies on interaction of *B* and *dpp* genes in developing eye discs, we screened several *dpp* mutants and found that a heteroallelic combination of *dpp*-recessive lethal alleles[9], viz. dpp^{d6} and dpp^{d12}, was viable. However, the dpp^{d6}/dpp^{d12} flies showed reduced number of ommatidia in their eyes, similar to that in the *B* mutant flies (unpublished). As expected from the wide roles of *dpp* in development and differentiation[10,11], the dpp^{d6}/dpp^{d12} flies were weak, short-lived (2–3 days), and displayed abnormalities in wings, legs, antennae, external genitalia, etc. During these studies, we further found that in the presence of the *B* mutant gene, the dpp^{d6}/dpp^{d12} heteroallelic combination resulted in total absence of ommatidia and, surprisingly, much more severely affected legs, antennae, etc. Since the *Bar* locus has so far not been reported to have any role in development and differentiation of these appendages, the intensification of abnormalities in the appendages in *B*; dpp^{d6}/dpp^{d12} flies was unexpected. Therefore, we examined expression of Bar proteins in developing leg and antennal imaginal discs in larvae of various genotypes, and the results are presented here. Our present work shows that besides the earlier known expression in the eye and the wing discs, the *B* genes are indeed expressed in a characteristic pattern in the leg and the antennal discs. On the basis of our results, we suggest that the homeo-box-containing *B* genes interact with *dpp* and function as new members of the proximal–distal sector genes, which are important for patterning these appendages along their proximo-distal axes.

Figure 1 shows the morphology of the prothoracic leg and antenna in wild-type (+/+), *B*, B^+; dpp^{d6}/dpp^{d12} and *B*; dpp^{d6}/dpp^{d12} flies. All the three pairs of legs in *Bar*-mutant flies (Figure 1 *b*) were indistinguishable from those in wild-type flies (Figure 1 *a*). In B^+; dpp^{d6}/dpp^{d12} flies on the other hand, the tarsal and meta-tarsal segments of all legs were affected due to loss of claws and fusion of the tarsal segments. On some occasions the dorsal parts were ventralized leading to loss of certain structures and duplication of others; the tibia and femur segments were progressively less affected while the trochanter and coxa were almost as in wild-type or *B* mutant flies (Figure 1 *c*). Most interestingly, in *B*; dpp^{d6}/dpp^{d12} flies, the severity of these abnormalities was

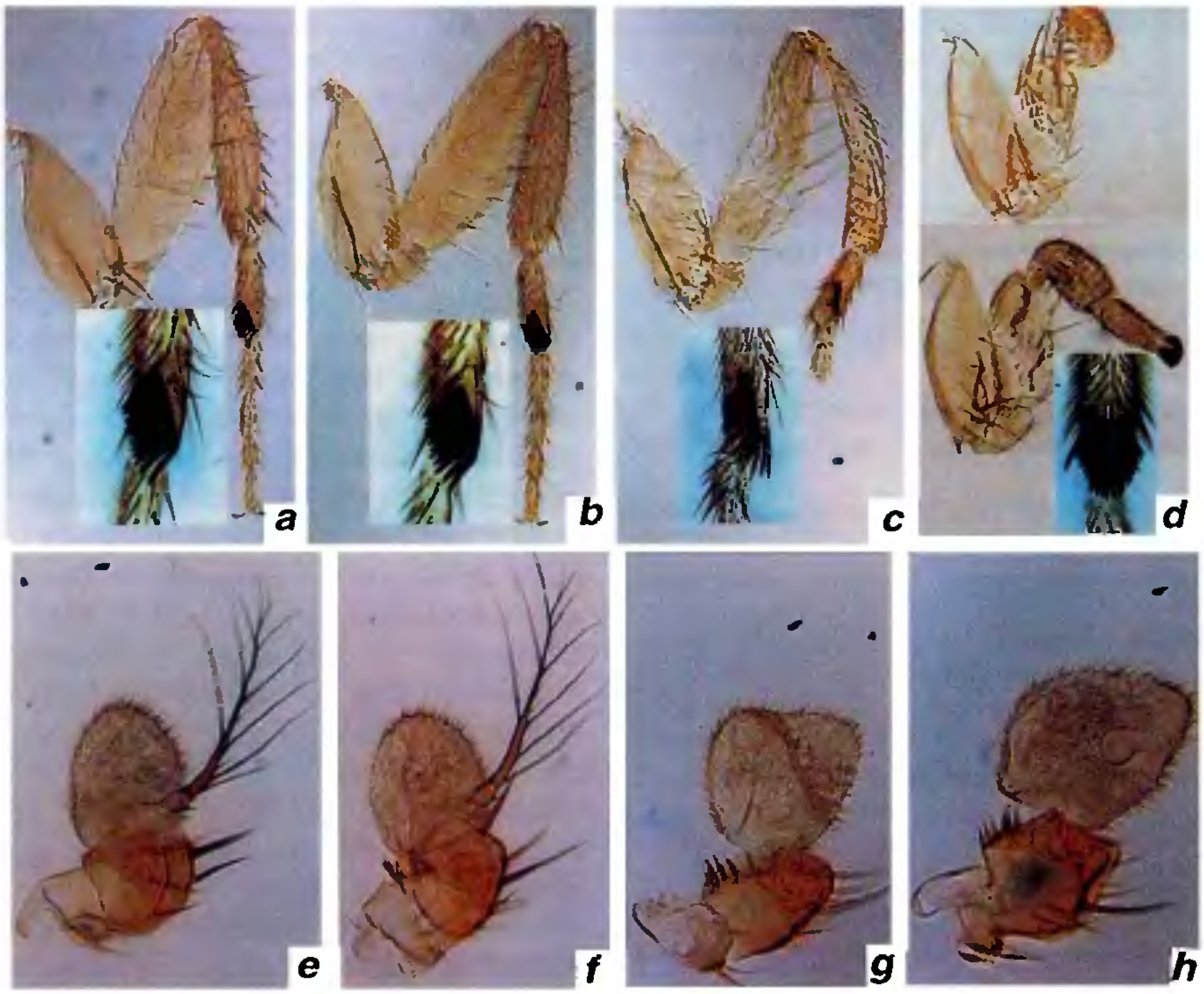

Figure 1. Prothoracic legs (*a–d*) and antennae (*e–h*) of wild-type (*a*, *e*) *B* (*b*, *f*), B^+; dpp^{d6}/dpp^{d12} (*c*, *g*) and *B*; dpp^{d6}/dpp^{d12} (*d*, *h*) male flies. Legs from two different flies are shown in *d*. The inset in each case is an enlarged view of the tarsal segment to show the sex-comb in the corresponding genotype.

further intensified. As evident from the two examples in Figure 1 *d*, there was an overall shortening in length along the proximal-distal axis accompanied by complete or partial fusion or even total loss of the tarsal segments. Moreover, the tibia and femur were also further deformed due to shortening, bulging and disorganized bristle patterns, although the most proximal segments like the coxa and the trochanter were not so much affected. Such extensive abnormalities in distal segments of appendages were never seen in B^+; dpp^{d6}/dpp^{d12} flies. The sex comb on tarsal segment of the prothoracic leg (shown as insets in Figures 1 *a–d*) of male flies was not affected by *B* mutation alone (compare the insets in Figure 1 *a* and *b*). However, in B^+; dpp^{d6}/dpp^{d12} male flies, the number of bristles in sex comb was generally more than in wild-type or *B* male flies (inset in 1 *c*). On the other hand, in *B*; dpp^{d6}/dpp^{d12} male flies, the sex comb, when present, was always duplicated (Figure 1 *d* and inset).

In the antennae also, while the *B* mutation by itself did not result in any abnormality (compare Figure 1 *e* and *f*), B^+; dpp^{d6}/dpp^{d12} flies showed abnormalities in the distal segments. In most cases arista, the distal most antennal segment, was absent and a conical projection on the third segment presumably represented the fused fourth, fifth and the sixth antennal segments (Figure 1 *g*). As in the legs, the structural abnormalities in the antennae of B^+; dpp^{d6}/dpp^{d12} flies were further intensified in *B*; dpp^{d6}/dpp^{d12} flies (Figure 1 *h*) since the arista along with the fourth and the fifth antennal segments were completely absent while the third and the second antennal segments were widened and deformed with disruptions in the bristle pattern.

When compared with *B/B*; dpp^{d6}/dpp^{d12} female or *B/Y*; dpp^{d6}/dpp^{d12} male flies, the severity of abnormalities in legs and antennae was less in *dpp* mutant females heterozygous for *B* mutation (*B/B*$^+$; dpp^{d6}/dpp^{d12}).

The above-noted enhancing effect of *B* mutation on abnormalities in appendages due to *dpp* mutant condition, clearly suggested the possibility of Bar protein expression in the corresponding larval imaginal discs and some interaction between the *Bar* and *dpp* gene products during differentiation of these appendages from the undifferentiated discs. The leg and the antennal discs of larvae are essentially circular flattened, monolayer sac-like structures of columnar epithelial cells which evaginate during the pupal stage to give rise to the respective adult structures. The central part in both types of discs corresponds to the presumptive distal tip while the peripheral regions correspond to the progressively more proximal structures of adult appendages[12,13]. To examine if *B* genes expressed in leg and antennal discs during development, the S12 antibody, which recognizes both BarH1 and BarH2 proteins[4] was used for immunostaining the leg and eye antennal discs for *+/+*, *B*, B^+; dpp^{d6}/dpp^{d12} and *B*; dpp^{d6}/dpp^{d12} late third instar larvae. Immunostaining with the S12 antibody revealed that the BarH1 and/or BarH2 proteins were abundantly present in a group of cells arranged as a 2–4 cell wide asymmetric ring around the central part of all the leg discs of wild-type late third instar larvae (Figures 2 *a*, 3 *a*) and at two small regions in the center within the ring (as indicated by arrows in Figure 3 *a* and *b*). The ring of Bar expressing cells corresponds to the preseumptive tarsal segment as revealed by immunostaining of evaginating leg discs from 9–10 h old pupa: as seen in Figure 3 *c*, the Bar-expressing cells in these discs were essentially restricted to the developing tarsal segment. The two groups of Bar expressing cells in the center of the leg disc (arrows in Figure 3 *a* and *b*) correspond to the presumptive claws. In the wild-type antennal discs also, BarH1 and BarH2 proteins were seen in a ring around the central part of the disc (Figure 4 *b*) but without any additional sites of expression in the central region. The patterns of expression of BarH1 and BarH2 in leg and antennal discs were similar in *B* mutant larvae (Figures 2 *b*, 3 *b*) except that compared to the wild-type discs, the staining was detectably more intense (compare Figure 3 *a* and *b*). The 2–4 cell wide ring of Bar-expressing cells in wild-type as well as the *B* leg, but not the antennal discs was marked by patterned circular areas in which Bar proteins were absent (marked by asterisks in Figure 3 *a* and *b*). It is known that BarH1 and BarH2 are expressed in the thecogen and neuronal cells of embryonic external sensory organs and this plays a key role in determining the sensillum subtype[14]. Futhermore, expression of BarH1 and BarH2 in the notal region of the third instar larval wing disc regulates the formation of microchaetae[8]. Therefore, it is possible that the characteristic pattern of Bar-expressing and Bar-non-expressing cells within the ring in leg discs serves as a pre-pattern for the pattern of the microchaetae and other bristles in adult legs. This needs further detailed analysis.

Compared to the leg and antennal discs from wild-type and *B* larvae, those from B^+; dpp^{d6}/dpp^{d12} showed disruptions in the patterns of Bar expression, in agreement with the earlier noted structural abnormalities in legs and antennae of adult flies. The leg as well as the antennal disc in B^+; dpp^{d6}/dpp^{d12} larvae were somewhat deformed and the ring of *Bar*-expressing cells was distinctly smaller and displaced (see Figure 2 *c*). On closer examination it was seen that the ring of Bar-expressing cells in the leg discs lacked the typical pattern formed by the Bar-expressing and Bar-non-expressing cells in wild-type or the *B* mutant discs. This lack of the 'pre-pattern' within the Bar-expressing ring of cells perhaps correlates with the aberrant bristle and other patterns in adult legs. The two small groups of Bar-expressing cells in the center of the ring were also absent in B^+; dpp^{d6}/dpp^{d12} leg discs and this correlated with the absence of claws in adult legs of these flies.

Most interestingly, the pattern of Bar-expressing cells in the *B*; dpp^{d6}/dpp^{d12} leg discs was completely disrupted

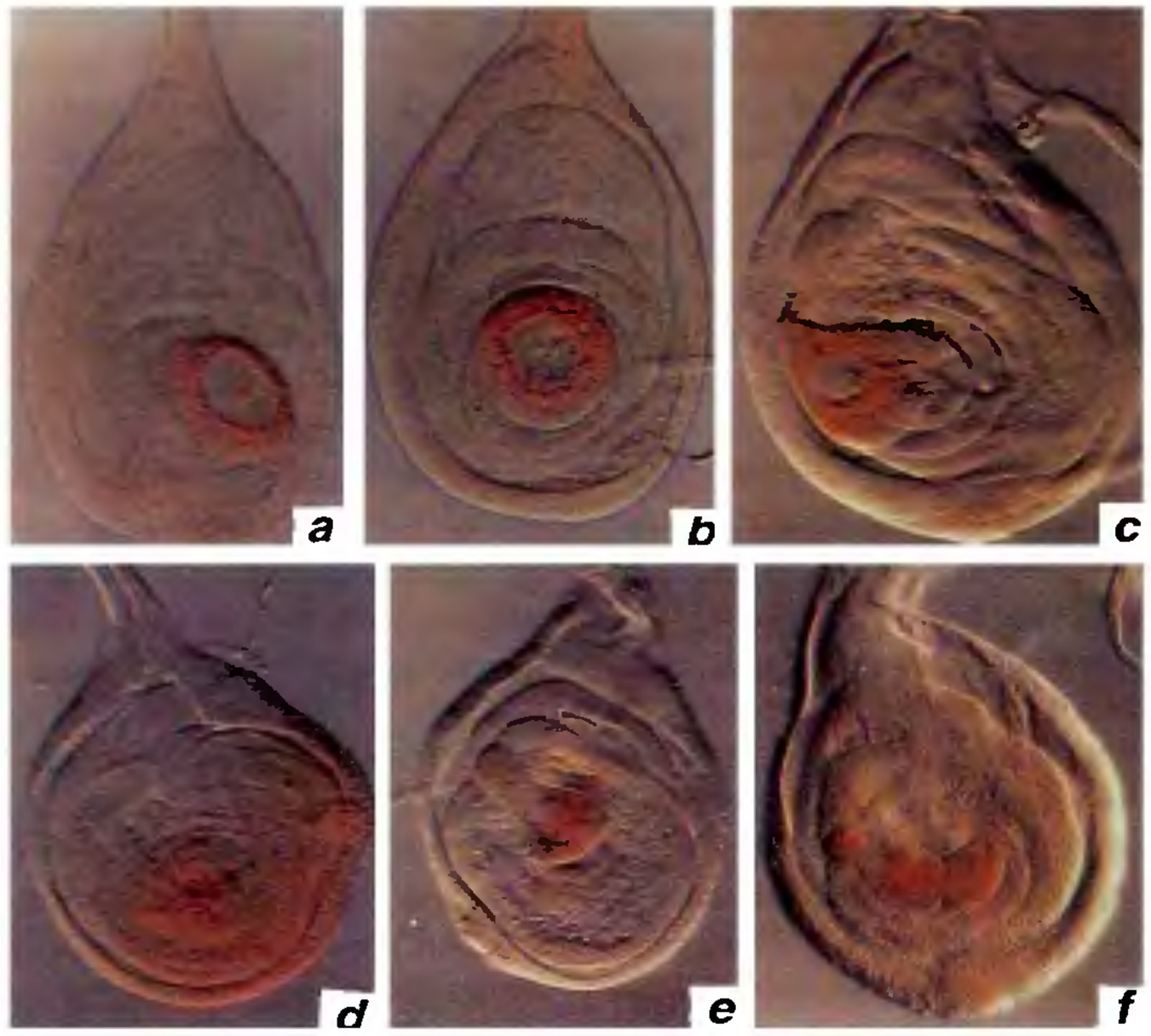

Figure 2. Expression of Bar proteins in mesothoracic leg imaginal discs of wild-type (*a*), *B* (*b*), B^+; dpp^{d6}/dpp^{d12} (*c*) and *B*; dpp^{d6}/dpp^{d12} (*d–f*) late third instar larvae as seen by immunostaining with the rabbit monoclonal antibody S12. Biotinylated anti rabbit IgG antibody and streptavidin conjugated HRP system (Vector) was used to detect the primary antibody following the standard protocol.

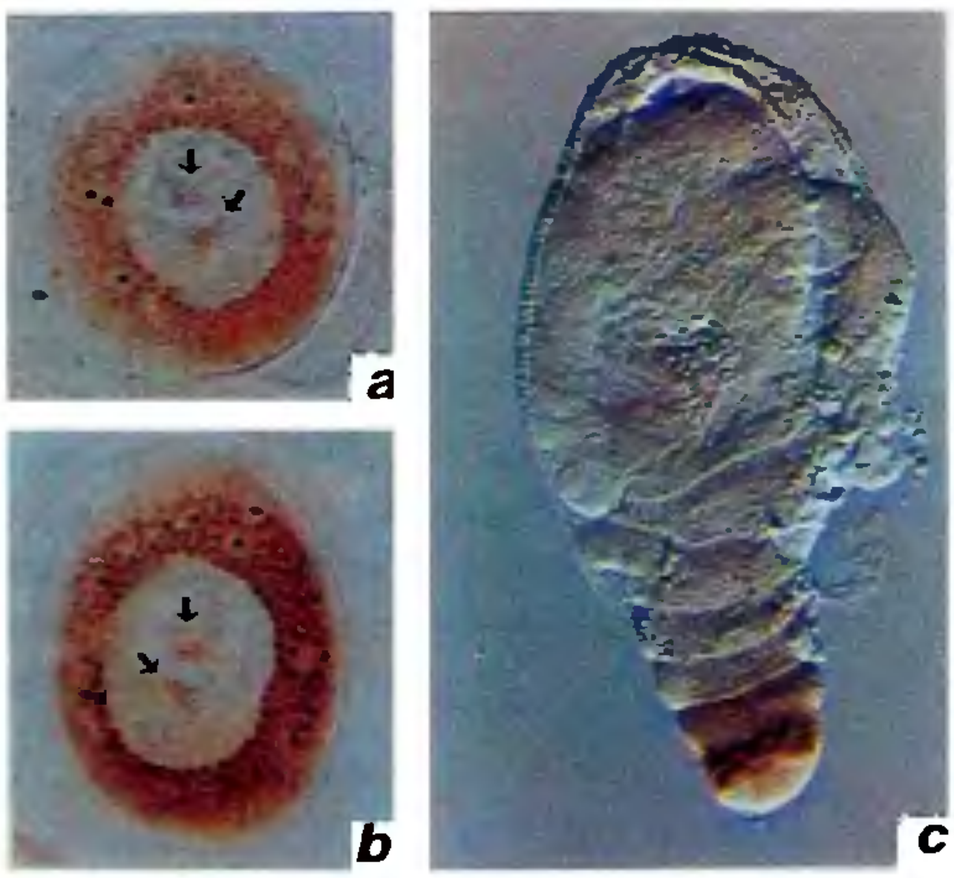

Figure 3. Enlarged view of the ring of Bar-expressing cells in presumptive tarsal region of mesothoracic leg discs of wild-type (*a*), *B* (*b*) late third-instar larvae following immunostaining as in Figure 2. The arrows in *a* and *b* point to the pair of group of Bar expressing cells in the central region of leg disc, corresponding to the distal-most segment of the adult leg. Note the more intense staining in *b* and the patterned absence (some are marked with *) of Bar expression in several areas of the ring in *a* as well as *b*. An evaginating mesothoracic leg disc from 9–10 h old pupa after immunostaining with the S12 antibody is shown in *c*.

(Figure 2 *d–f*). As evident from the examples in Figure 2 *d–f*, the ring was replaced by a group of Bar-expressing cells occupying the entire central region and additional sites of ectopic expression of Bar proteins. These cells were spread over a large area of the disc (Figure 2 *d*) or were limited to a smaller area (Figure 2 *e*, *f*). In some *B*; dpp^{d6}/dpp^{d12} leg discs, the Bar-expressing distal region (central in the disc) appeared to be duplicated (Figure 2 *f*). Change from the annular pattern of Bar-expressing cells to the uniform sheet of Bar-expressing cells in the distal region of *B*; dpp^{d6}/dpp^{d12} imaginal leg discs may perhaps be due to absence or disappearance of the distal-most group of imaginal cells since in the adult legs of these genotypes the meta-tarsal segments were almost completely absent (Figure 1 *d*). Comparable disruptions in the S12 antibody staining patterns were noted (not shown) in the antennal discs of B^+; dpp^{d6}/dpp^{d12} and *B*; dpp^{d6}/dpp^{d12} late third-instar larvae.

Since *dpp* plays a key role in proximo-distal axis differentiation and our above results showed that the *B* and *dpp* genes interact in this process, we examined the spatial relationship between the Bar-expressing and the Dpp-expressing cells in normal late third-instar leg and antennal discs. The *dpp* expression was monitored through X-gal staining in leg and eye-antennal discs of the *dpp-lacZ* transgenic line *BS3.0* (ref. 15) in which *dpp* promoter regulates the *lacZ* reporter gene. The same discs

were subsequently immunostained with the S12 antibody to localize Bar proteins (Figure 4 *a*, *b*). As is already known[16], *dpp* expression in leg and the antennal discs was restricted along the anterior–posterior boundary and accordingly, the X-gal blue staining extended as a narrow stripe along the antero-posterior axis in leg discs and as a wedge sector in antennal discs (Figure 4 *a*, *b*). In leg discs, the stripe of Dpp-expressing cells (bluish-green) crosses the ring of Bar-expressing cells (brown) at the dorsal-anterior diagonal and ends close to the ventral–anterior diagonal of the ring while in antennal disc, the overlap is restricted only to the dorsal side. (Figure 4 *a*, *b*). It may be noted that the spatial distribution of Dpp- and Bar-expressing cells in leg and antennal discs is strikingly different from that in the eye-disc where both are co-expressed all along the anteriorly advancing morphogenetic furrow and the Bar proteins, in addition, are also expressed in specific (R1 and R6) cells of the differentiating ommatidia where Dpp does not express (see Figure 4 *b*).

Growth and pattern formation in the leg and antennal imaginal discs depends mainly on cell–cell interaction rather than cell lineage[17]. The Dpp protein is a secretory protein, which in conjunction with the products of *wingless* (*wg*) and some other genes plays the most important role in specifying the positional information along the proximo–distal and dorso–ventral axes[18–20]. In addition to these, other genes have restricted expression in specific positions along the proximal–distal axis. Expression of these proximal-distal sector genes appears, when viewed from top, as annular rings in third-instar leg discs or as band/s in the everting discs and these genes provide molecular identities to different positions along the proximal–distal axis[21]. Our present study has shown that the *B* gene is also expressed in a distinct ring, corresponding to the presumptive distal region in the third-instar leg and the antennal discs. Therefore, we suggest the *B* gene to be a new member of group of proximal–distal sector genes like *distalless*[22], *teashirt*[23], *rotund*[24,25], etc. The specific band-like expression of Bar in the tarsal segments of the evaginating leg discs further supports its function as a proximal–distal sector gene in these appendages.

The dpp^{d6} and dpp^{d12} alleles are loss-of-function alleles due to deletion in the disc region of the *dpp* gene[9]. Generally such mutant alleles are recessive lethal but due to a partial complementation, the dpp^{d6}/dpp^{d12} hetero-allelic combination permits survival of a few individuals to adult stage with severe abnormalities in appendages. Aggravation of these mutant phenotypes by the *B* mutation clearly indicates that the *B* and *dpp* genes interact in leg and antennal differentiation. Although *dpp* is expressed in a stripe that roughly parallels the anterior–posterior boundary in the leg and antennal discs[16], adult viable mutants of *dpp* show abnormalities in adult appendages restricted to distal structures. This suggests that the diffusible product of this gene has a more significant role in pattern formation in the distal segments of these appendages[10,11]. Our results showed that in spite of the spatially well defined localization of Bar homeo-box-containing proteins in leg and antennal discs, their over-expression due to the *Bar* mutation in dpp^{+} background had no effect on leg or antennal differentiation but in *dpp* loss-of-function background, the *dpp*-mutant phenotypes in these appendages were significantly aggravated. This suggests some kind of stoichiometric relationship between these two gene products such that when the Dpp protein is below a certain threshold level, as is likely to happen in the dpp^{d6}/dpp^{d12} heteroallelic combination, an overdose of Bar homeo-box proteins has an enhancing effect on the *dpp* phenotype. It appears that Bar proteins have an inhibitory effect on *dpp* expression so that in the dpp^{d6}/dpp^{d12} heteroallelic combination, the already lowered activity of Dpp gets further reduced resulting in more extreme phenotypes. The disruption in the pattern of Bar expression in leg and antennal discs in *dpp* mutant background (Figure 2 *c*) suggests a role of Dpp in regulating *B* expression: While in the eye-disc, the *B* and *dpp* express in spatially overlapping regions (see Figure 4 *b*), in the case of leg and antennal discs (Figure 4), there is only a limited overlap between the expression domains of these two genes. In spite of this limited overlap, the Bar-expressing annulus in *dpp* mutant discs was reduced and in the double mutants, it was completely disorganized. These suggest either a direct long-range field effect of Dpp on *B* expression or an indirect effect resulting from the altered expression of *wg*, *hedgehog* (*hh*) and other

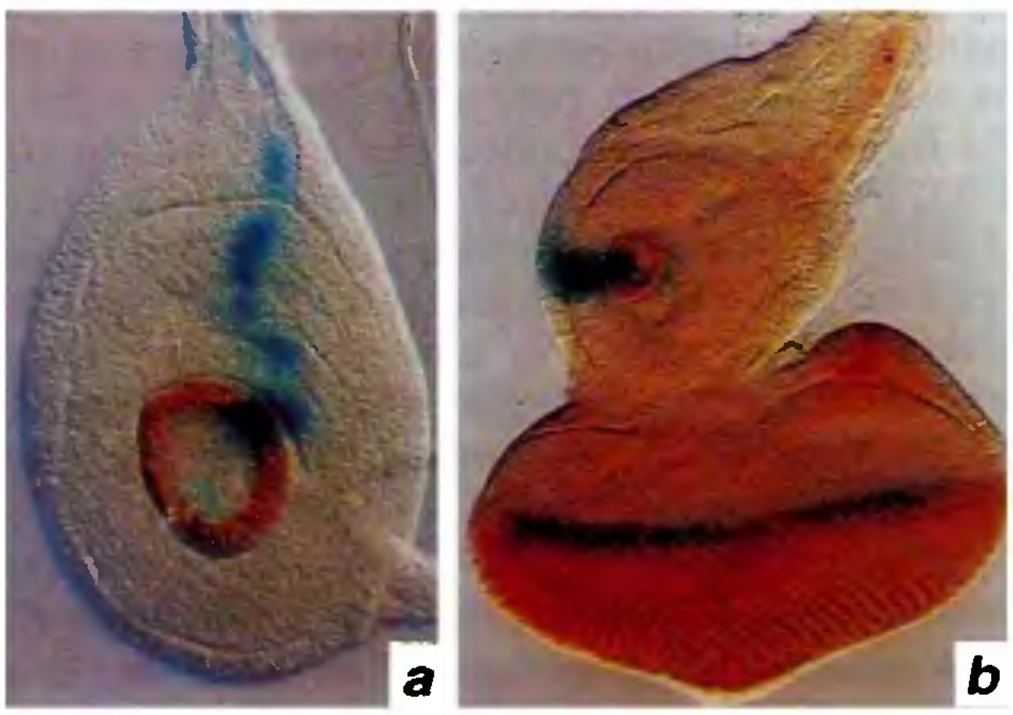

Figure 4. Mesothoracic leg (*a*) and eye-antennal (*b*) imaginal discs from the transgenic line *BS3.0* stained with X-gal to reveal the *dpp* expression pattern (bluish-green) followed by immunostaining with the S12 antibody to show the Bar expression (brown) pattern. In *b*, the lower half is the eye-disc and the upper half, the antennal disc. Note that the *dpp* expression in the leg and antennal discs is along the antero-posterior border while in the eye-disc it is restricted to the morphogenetic furrow (MF). In eye disc, Bar is expressed in the MF as well as in specific cells within each of the differentiating ommatidia arrayed posterior to the MF.

genes due to *dpp* loss-of-function mutation. This aspect is being examined further.

As has been reported earlier[26], most of the B^+; dpp^{d6}/dpp^{d12} males were completely devoid of external genitalia while a few had abnormal external genitalia. As in legs and antenna, the *Bar* mutation had an enhancing effect on male genitalia also since the external genitalia were absent in all *B*; dpp^{d6}/dpp^{d12} male flies (not shown). Interestingly, the external genitalia were not much affected in female flies of any of the genotypes. The enhancing effect of *B* mutation on male, but not female, external genitalia in *dpp* mutant background also warrants further study.

The classical view has been that the *Bar* locus has a function only in eye differentiation in view of its phenotypic effect being restricted to differentiation of ommatidia in eyes. Recently, this gene was shown to also function in the differentiation of the notal region of wing of *Drosophila*[8]. We have now shown that the *Bar* locus has roles in differentiation of legs and antennae (and possibly also the external male genitalia) as well. Thus it appears that this complex locus of homeo-box containing genes plays a much wider role in differentiation of different structures in *Drosophila*.

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