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Formation and specification of distal leg segments in *Drosophila* by dual *Bar* homeobox genes, *BarH1* and *BarH2*

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SUMMARY

Here, we show that *BarH1* and *BarH2*, a pair of *Bar* homeobox genes, play essential roles in the formation and specification of the distal leg segments of *Drosophila*. In early third instar, juxtaposition of Bar-positive and Bar-negative tissues causes central folding that may separate future tarsal segments 2 from 3, while juxtaposition of tissues differentially expressing *Bar* homeobox genes at later stages gives rise to segmental boundaries of distal tarsi including the tarsus/pretarsus boundary. Tarsus/pretarsus boundary formation requires at least two different *Bar* functions, early antagonistic interactions with a pretarsus-specific homeobox gene, *aristaless*, and the subsequent induction of *Fas II* expression in pretarsus cells abutting tarsal segment 5. *Bar* homeobox genes are also required for specification of distal tarsi. *Bar* expression requires *Distal-less* but not *dachshund*, while early circular *dachshund* expression is delimited interiorly by *BarH1* and *BarH2*.

Key words: Leg, Homeobox gene, Segmentation, *BarH1*, *BarH2*, *Fas II*, *aristaless*, *Distal-less*, *dachshund*, *Drosophila*

INTRODUCTION

The formation of compartments or domains specified by the region-specific expression of transcription factors may be essential for the body plan of insects and vertebrates (e.g. for *Drosophila*, see Azpiazu et al., 1996; Lawrence and Struhl, 1996; Sato et al., 1999). *Drosophila* leg development may provide a good system for studying region-specific expression and compartment formation, since legs are simple in structure and their formation encompasses various developmental processes.

Drosophila legs comprise the segmental units, from proximal to distal, coxa, trochanter, femur, tibia, tarsal segments 1-5 and pretarsus. Leg formation occurs through concentric folding and subsequent segmentation of monolayered epithelia of leg discs invaginated from the epidermis during embryogenesis (Cohen, 1993). According to Lecuit and Cohen (1997), *decapentaplegic* (*dpp*) and *wingless* (*wg*) expressed dorsally and ventrally, respectively, along the anteroposterior compartment boundary (AP boundary) are essential for the concentric expression of *Distal-less* (*Dll*) and *dachshund* (*dac*) in leg discs. *Dll*, encoding a homeodomain protein, is expressed in the distal region of leg discs and is required for the development of all distal structures other than the coxa (Cohen et al., 1989; Diaz-Benjumea et al., 1994). *dac*, encoding a novel nuclear protein, is expressed in the middle leg region and is essential for the formation of intermediate portions of legs (Mardon et al., 1994).

Abu-Shaar and Mann (1998) suggested that the generation of the concentric domains occurs in multiple phases. The coxopodite and telopodite regions are initially established by the expression of *Dll* and a homeobox gene, *homothorax* (*hth*). In coxopodite, the proximalmost region constituting future body wall and proximal segments, Dpp and Wg signaling are blocked by concerted function of Hth and nuclear localized Extradenticle (Exd), another homeodomain protein (Abu-Shaar and Mann, 1998; Gonzalez-Crespo and Morata, 1996; Wu and Cohen, 1999). In contrast, in the telopodite, Dpp and Wg signaling are active and required for proximodistal axis generation. Subsequently, a *dac* expression domain appears between *hth* and *Dll* domains to subdivide the telopodite region further. One more intermediate region, which expresses both *dac* and *Dll*, then arises by the early third instar.

Downstream of *Dll*, several genes participate in distal tarsal formation. A homeobox gene *aristaless* (*al*), expressed in the most distal tip of leg discs, is required for the pretarsus structures (Campbell et al., 1993; Schneitz et al., 1993; Campbell and Tomlinson, 1998). *spineless* (*ss*), which encodes a bHLH-PAS protein homologous to mammalian dioxin receptor, is expressed transiently in a future tarsus region and its absence results in deletion of distal tarsal segments 2-4 (Duncan et al., 1998). *bric á brac* (*bab*), encoding a nuclear factor having BTB domain, is expressed downstream of *ss* (Duncan et al., 1998) and is essential for the segmentation and specification of tarsal segments 2-4 (Godt et al., 1993).

Here, we show that functionally redundant homeobox genes at the *Bar* locus, *BarH1* and *BarH2* (Kojima et al., 1991; Higashijima et al., 1992a), serve as essential regulators in

numerous aspects of distal tarsus development. *BarH1* and *BarH2* expression requires *Dll*. Juxtaposition of Bar-positive and Bar-negative tissues induces initiation of central folding. Antagonistic interactions between Bar and Al or other pretarsus factors establish the pretarsus/tarsal-segment-5 boundary. *Bar* is also required for the segmentation and specification of tarsal segments 3-5.

MATERIALS AND METHODS

Fly stocks

Fly strains used are: Canton S (wild-type), *Df(1)B$^{263\text{-}20}$* (Sato et al., 1999), *Df(1)BH2* (Higashijima et al., 1992b), *In(1)B^{M2}*, *In(1)pdf, B^{S}Y* (Lindsley and Zimm, 1992), *In(1)w^{VC}* (Hotta and Benzer, 1976), *Dll^{SA1}* (Gorfinkiel et al., 1997), *dac^{3}* (Mardon et al., 1994) and *al^{1}*, *al^{2}* (Lindsley and Zimm, 1992). *al^{1}* and *al^{2}* are used in combination with *Df(2L)al* (Lindsley and Zimm, 1992). In *al^{1}/Df(2L)al* and *al^{2}/Df(2L)al* flies, aristae and claws were almost completely abolished but other pretarsus structures were not significantly affected. *en-lacZ* (ryXho25; Hama et al., 1990), *neu-lacZ* (A101; Bellen et al., 1989), *Bar-lacZ* (P058; FlyView: http://pbio07.uni-muenster.de/) and ta5-*lacZ* (BM25; T. Michiue and K. S., unpublished data). *B^{55}* was isolated by imprecise excision of P-element of P058. *ptc*-GAL4 (559.1; Brand and Perrimon, 1993), *blk*-GAL4 (40C.6; Morimura et al., 1996), *ap*-GAL4 (md554; Calleja et al., 1996), UAS-*BarH1*, UAS-*BarH2* (Sato et al., 1999) and UAS-*al*. UAS-*al* was generated by inserting *al* cDNA (Campbell et al., 1993) into pUASV (Sato et al., 1999). For FRT/FLP mosaic analyses, *hsFLP*, *FRT18A*, *FRT40A* and *FRT42D* (Xu and Rubin, 1993) were used. All other mutations and balancer chromosomes were described in Lindsley and Zimm (1992).

Gynandromorph mosaic analysis

Ten out of twenty eight mosaic legs from 83 flies of the genotype *Df(1)B$^{263\text{-}20}$*, *y/In(1)w^{VC}*, are entirely *Bar^{-}*. To isolate *Bar^{-}* discs, larvae having mouth hooks and denticle belts mosaic for *y^{-}* were dissected. Among leg discs from eighteen such larvae, six were judged as *Bar^{-}* because of the absence of anti-BarH1 antibody-staining signals.

FRT/FLP mosaic analysis

Bar^{-}, *Dll^{-}* and *dac^{-}* clones, respectively, were generated in larvae whose genotypes are *Df(1)B$^{263\text{-}20}$, y FRT18A/FRT18A*; *hsFLP*, *Sb*/+, *y w hsFLP*; *FRT42D Dll^{SA1}/FRT42D πM45F* and *y w hsFLP*; *dac^{3} FRT40A/πM36F FRT40A*. To examine the ability of *B^{S}Y* to rescue *Df(1)B$^{263\text{-}20}$* mosaic phenotypes, clones were generated in larvae of the genotype *Df(1)B$^{263\text{-}20}$, y FRT18A/FRT18A*/B^{S}*Y*; *hsFLP*, *Sb*/+. In all cases, clones were induced by a 90 minute heat shock at 37°C during late first-second instar.

Ectopic expression of *BarH1*, *BarH2* and *al*

Several independent UAS-*Bar* and UAS-*al* lines were used to misexpress *Bar* and *al*. UAS-*BarH1^{M6}* and UAS-*BarH2^{F9}* driven by *ptc*-GAL4 gave leg phenotypes similar to those of *blk*-GAL4-driven UAS-*BarH1^{M12}*, UAS-*BarH1^{M13}*, UAS-*BarH2^{F11}* or UAS-*BarH2^{M9}*. In combination with *ap*-GAL4, UAS-*BarH1^{M13}*, UAS-*BarH2^{M9}* and UAS-*BarH2^{F11}* gave leg phenotypes similar to one another. Five independent lines of UAS-*al* showed little leg defects when driven by *ptc*-GAL4 or *blk*-GAL4.

Immunohistochemistry

Antibody staining was carried out according to Sato et al. (1999). For confocal microscopy, Cy3-conjugated secondary antibody (Jackson Immune Research) or biotinilated secondary antibody (Vector) followed by avidin-FITC (Promega) were used. Antibodies used were: rabbit anti-BarH1, rabbit anti-BarH2 (Higashijima et al., 1992b), rat anti-Ap (Lundgren et al., 1995), mouse anti-Fas II (Lin et al., 1994), mouse anti-Dac (Mardon et al., 1994), rat anti-Al (Campbell et al., 1993), mouse anti-Dll (Diaz-Benjumea et al., 1994), rabbit anti-β-gal (Cappell), mouse anti-β-gal (Promega) and Rhodamine-phalloidin (Molecular Probe).

RESULTS

Concentric expression of *Bar* homeobox genes and some distal tarsus segment markers in third instar larvae

During development of *Drosophila* legs, the disc epithelium folds concentrically; genes expressed circularly in the leg disc just before the onset of folding may thus be essential for leg morphogenesis. *BarH1* and *BarH2*, may belong to such a class of genes. Staining for BarH1, BarH2 and *Bar-lacZ* (Fig. 1E,F) indicated that *BarH1* and *BarH2* are coexpressed circularly in all three types of third-instar leg discs. Since *BarH1* and *BarH2* are functionally redundant to each other (see below), they are hereafter referred to as *Bar* collectively.

Indications of circular Bar expression (Bar ring) were first evident at 76 hours AEL (after egg laying) at 25°C and became more evident at 80 hours AEL (Fig. 1A,B). The formation of the central fold, from which distalmost leg segments are generated (see below), began at 84 hours AEL as a circular indentation along the periphery of the Bar ring, starting dorsally and completing ventrally by 88 hours AEL (Fig. 1C,D,G-G″). As the indentation became deeper in mid third instar, graded Bar expression gradually became apparent along the proximodistal axis (Fig. 1H-H″). In the central fold of a 112 hours AEL leg disc, future tarsal segment 3, lacking *Bar* expression, became clearly identifiable (Fig. 1I-I″). At 5 hours APF (after puparium formation), Bar expression was closely related to lines of demarcation of tarsal segments (Fig. 1J-L,O). Bar expression was strongest in tarsal segment 5, clearly evident in tarsal segment 4 and not detected in tarsal segment 3 and the pretarsus except for future claw regions.

ta5-*lacZ* is a *lacZ* reporter driven by a tarsal-segment-5-specific *Bar* enhancer (Fig. 1J-M), while *ap* is a LIM-homeobox gene expressed in tarsal segment 4 (Fig. 1M-P; Cohen et al., 1992). Fig. 1Q shows that these markers begin to be expressed as adjacent circles within the Bar ring during the central fold formation, indicating that tarsal segments 4 and 5 are derivatives of the early Bar compartment. Because of the absence of a suitable molecular marker, we could not determine whether future tarsal segment 3 is included in the early Bar ring. However, since genetic analysis (see below) shows that *Bar* function in tarsal segments 3-5 is essential for segmentation of distal tarsus, we tentatively conclude that tarsal segments 3-5 are derivatives of the early Bar ring and distal tarsus separation starts just prior to the completion of the central fold formation at early third instar.

From late third instar stages onwards, Bar was also expressed in putative claw cells (insets of Fig. 1E,F). Staining for BarH1 and *en-lacZ* (Fig. 1R; Hama et al., 1990) showed the anterior edge of the posterior claw region coincides with that of the posterior compartment, suggesting that the center of the Bar ring is situated in the anterior compartment, between the paired claw regions. During late third instar, Bar-negative patches in future tarsal segment 5 became detectable (Fig.

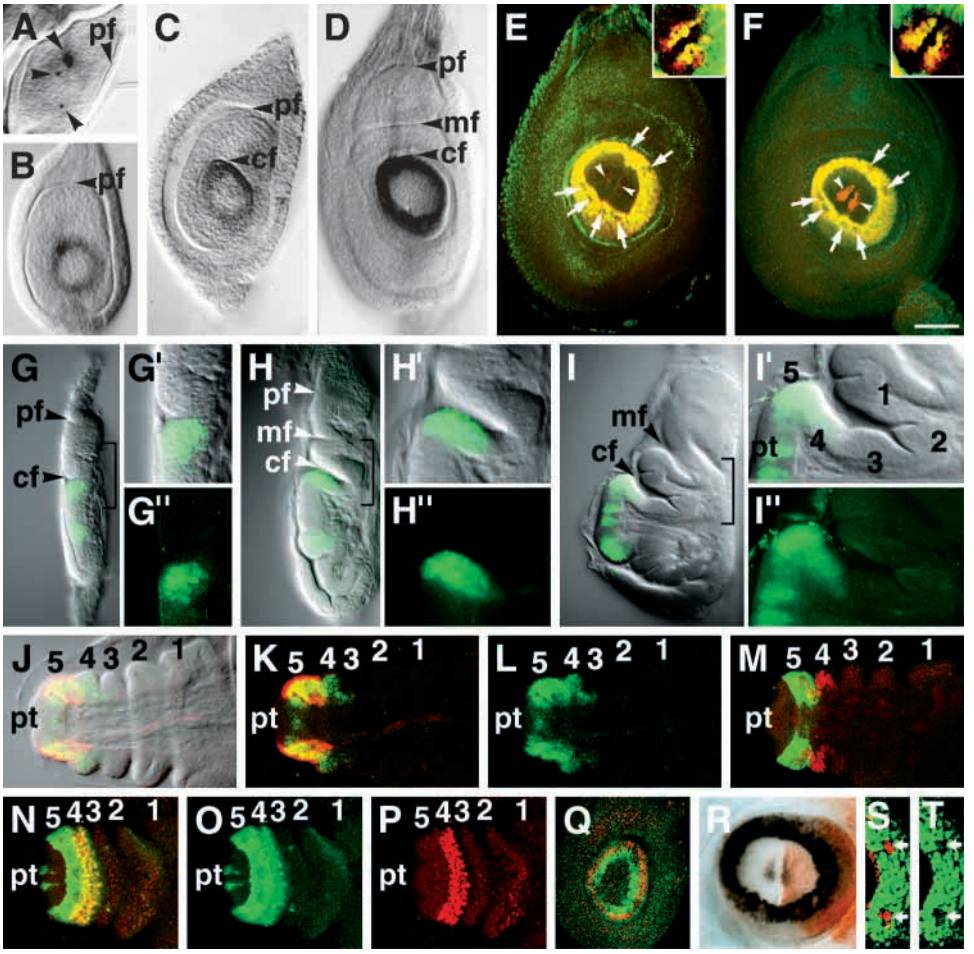

Fig. 1. Bar expression in leg discs. cf, central fold; mf, middle fold; pf, peripheral fold; pt, pretarsus; 1-5, tarsal segments 1-5. (A-D) Early (A-C) and mid (D) third instar larval leg discs stained for BarH1. (A) At 76 hours AEL. Note that strong Bar expression occurs in Keilin's organ cells (arrowheads). (B) Early Bar ring at 80 hours AEL. (C) An 84 hours AEL disc, in which cf is being formed dorsally just outside the Bar ring. mf is not yet formed. (E,F) Late third instar discs stained for *Bar-lacZ* (red) and BarH1 (E; green) or BarH2 (F; green). BarH1 and BarH2 are coexpressed in the Bar ring region. Arrows, Bar-negative patches in tarsal segment 5. Arrowheads, expression in claw regions. Although BarH1 expression in claw regions is much weaker than BarH2 expression, BarH1/BarH2 coexpression was clearly demonstrated by amplifying BarH1 signals (see the insets). (G-I) Sagittal sections of early (G), mid (H) and late (I) third instar leg discs. Confocal and Nomarsky images were merged. (G′-I′) Enlarged images of bracketed regions. (G″-I″) Enlarged confocal images of bracketed regions. Gradation of Bar expression gradually apparent until mid third instar (H-I). Note that, in I-I″, Bar-negative tarsal segment 3 is physically separated from adjacent segments. (J-P) Early pupal legs (5 hours APF) stained for BarH1, ta5-*lacZ* or Ap. Green (J-L,N,O), BarH1; red (J,K) and green (M), ta5-*lacZ*; red (M,N,P), Ap. (M) Tarsal segment 4 and proximal edge of tarsal segment 5 specifically express Ap. Note that the strongest BarH1 expression occurs in tarsal segment 5. (Q) ta5-*lacZ* (green) and Ap (red) begin to be expressed just after the onset of central folding. (R) A late third instar disc stained for *en-lacZ* (brown) and BarH1 (black). (S,T) Staining for *neu-lacZ* (red) and Bar (green) indicates Bar^{-} patches to be SOPs and/or their progenitors (arrows). For A-F,Q,R, anterior is left and dorsal is up. For other discs, distal is left and dorsal is up. Scale bar in F, 100 μm for A-Q, 70 μm for R, 50 μm for S,T.

1E,F,S,T). Staining for BarH1 and *neu-lacZ* (Bellen et al., 1989; Huang et al., 1991) showed that these correspond to sensory organ precursors and/or their derivatives (Fig. 1S,T).

Functional requirements of *Bar* for distal tarsus formation

As a first step to elucidate *Bar* functions during leg development, we carried out gynandromorphic mosaic analysis (Hotta and Benzer, 1976) using a larval-lethal deletion *Df(1)B$^{263\text{-}20}$*, which uncovers *BarH1* and *BarH2* along with *forked* (*f*; Hoover et al., 1993; Ishimaru and Saigo, 1993), *Fimbrin* (*Fim*; Ishimaru et al., unpublished data), and an unknown gene, *X2* (see Fig. 2N). In legs totally composed of cells hemizygous for *Df(1)B$^{263\text{-}20}$*, tarsal segments 2-5 were fused together into a small bulb-like, non-segmental structure having neither claws nor pulvilli, whereas other leg segments were normal (Fig. 2A,B). The first appreciable morphological event in distal leg development is central folding along the periphery of the Bar ring (see Fig. 1). As shown in Fig. 2K, no central folding occurred in *Df(1)B$^{263\text{-}20}$* leg discs; more proximal folds (mf and pf) were normally formed (for the wild-type, see Fig. 1D). Together, these results may indicate that at least one of the five genes uncovered by *Df(1)B$^{263\text{-}20}$* is essential for distal leg morphogenesis.

To determine which cells require *Df(1)B$^{263\text{-}20}$* gene activity for normal distal leg development, small *Df(1)B$^{263\text{-}20}$* clones were generated using the FRT/FLP method (Xu and Rubin, 1993). Partial fusion of tarsal segments 2-5 were frequently observed (see Fig. 3P,Q). Central fold formation was also prevented in *Df(1)B$^{263\text{-}20}$* clones generated along the periphery of the early Bar ring (data not shown), consistent with the notion that both central folding and segmentation among tarsal segments 2-5 require the *Df(1)B$^{263\text{-}20}$* gene(s). In mosaic legs normal in appearance (n=132), all mutant clones, except for a single case, in which a mutant bristle was situated at the proximal tip of tarsal segment 3, were observed outside tarsal segments 3-5 (Table 1), indicating that *Df(1)B$^{263\text{-}20}$* gene activity in future tarsal segments 3-5, which are the presumed derivatives of the early Bar ring, is essential for normal leg development.

Among five *Df(1)B$^{263\text{-}20}$* genes, *f*, *Fim*, *BarH2* and *X2* appeared dispensable for distal leg development other than

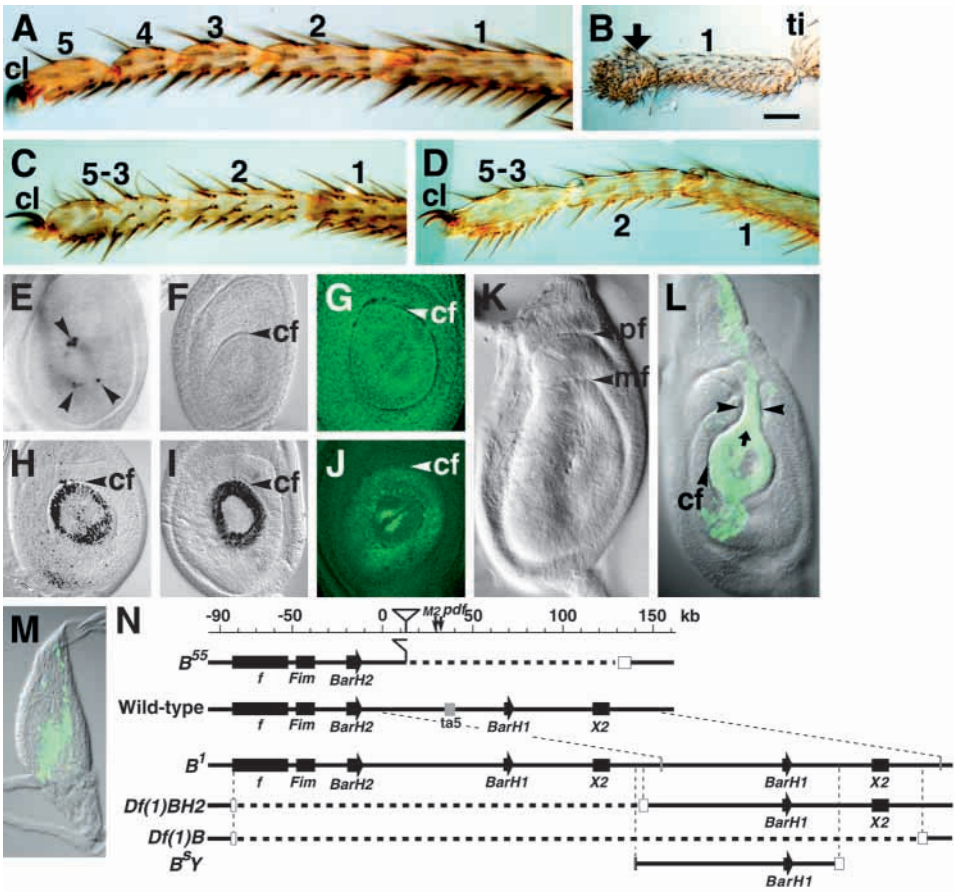

Fig. 2. Leg phenotypes of *Bar* locus mutants and effects of *BarH1* misexpression on leg morphology. 1-5, tarsal segments 1-5; cl, claws; ti, tibia; cf, central fold; mf, middle fold; pf, peripheral fold. (A) A distal part of the wild-type leg; (B) a gynandromorph mosaic leg consisting of Bar^- ($Df(1)B^{263\text{-}20}$) cells. Arrow, fusion of segments distal to tarsal segment 1. Neither claws nor pulvilli can be seen. (C,D) $B^{M2}/Df(1)B^{263\text{-}20}$ (C) and B^{55} (D) legs. Tarsal segments distal to tarsal segment 2 are fused. (E-J) Leg discs of B^{M2} (E,H) and B^{55} (F,G,I,J) stained for BarH1 (E-G) or BarH2 (H-J). In early third-instar-larval B^{M2} discs (E,H), BarH2 is almost normally expressed, but virtually no BarH1 ring is observed. Arrowheads, Keilin's organ cells. In B^{55} discs, BarH1 is hardly expressed at both early (F) and late (G) third instar larval stages, while early BarH2 expression is almost normal (I); late circular BarH2 expression is significantly reduced (J). Note that the central folding occurred normally. (K) A mid third-instar disc of a gynandromorph mosaic larva, lacking BarH1 staining. No central fold formation occurs (compare (K) with Fig. 1D). (L,M) *blk*-GAL4/UAS-$BarH1^{M13}$ discs stained for BarH1 (green). Confocal and Nomarsky images were merged. At mid third instar (L), ectopic fold formation occurs along the boundary between Bar-positive and Bar-negative tissues (arrowheads), while the formation of the authentic central fold is interrupted by *Bar*-misexpression (arrow). At late second instar (M), no folding occurs irrespective of *BarH1* misexpression. (N) Physical map of the *Bar* locus and *Bar* mutant structures. The P insertion site in P058 is shown by a triangle. Distal inversion breakpoints in B^{M2} and *pdf* are indicated by vertical arrows. Filled boxes and thick arrows, transcriptional units. A stippled box, tarsal-segment-5-specific enhancer. Open boxes, breakpoints in B^1, $Df(1)B^{263\text{-}20}$, $Df(1)BH2$, B^SY and B^{55}. B^1 is a duplication of the *Bar* locus (Lindsley and Zimm, 1992). $Df(1)B^{263\text{-}20}$ is a deletion of B^1 and lacks *f*, *Fim*, *BarH2*, *BarH1* and *X2*. $Df(1)BH2$ is a deletion mutant lacking *f*, *Fim* and *BarH2* (Higashijima et al., 1992b). B^SY originates from B^1 chromosome (Brosseau et al., 1958) and possess *BarH1* but not other $Df(1)B^{263\text{-}20}$ genes. (A-D) Distal is left; (E-M) anterior is left and dorsal is up. Scale bar in B: 50 μm for A-D, 80 μm for E-M.

bristle morphogenesis at least in the presence of *BarH1*. Indeed, no defect in gross morphology of distal legs was observed when $Df(1)B^{263\text{-}20}$ homozygous clones were generated in flies carrying B^SY, a Y chromosome with a *Bar* locus fragment including *BarH1* but not other $Df(1)B^{263\text{-}20}$ genes (Fig. 2N, unpublished data). Consistently, no defect in distal leg gross morphology was observed in $Df(1)BH2$, uncovering *f*, *Fim* and *BarH2* (Fig. 2N, unpublished data; Higashijima et al., 1992b).

BarH1 may be functionally redundant to *BarH2* as has been shown in other developmental contexts (Hayashi et al., 1998; Sato et al., 1999). B^{M2} and *pdf* are inversion mutants with a distal breakpoint between *BarH1*- and *BarH2*-coding sequences (Fig. 2N; Tsubota et al., 1989). In these mutants, early BarH1 expression was almost completely missing but early BarH2 expression along with central folding occurred normally (Fig. 2E,H). We conclude that the genes essential for distal leg morphogenesis are *BarH1* and *BarH2*, which are functionally redundant to each other.

In *pdf* and $B^{M2}/Df(1)B^{263\text{-}20}$ flies, tarsal segments 3-5 were partially fused with each other (Fig. 2C). B^{55} is a newly isolated *Bar* deletion mutant uncovering *BarH1*, *X2* and a late *Bar* enhancer (ta5) but not *BarH2* (Fig. 2N). In B^{55} mutants, BarH1 expression is missing throughout development (Fig. 2F,G), while BarH2 expression was normal in early third instar larval stages but extensively reduced afterwards (Fig. 2I,J). As with *pdf* flies, tarsal segment 3 and more distal segments were fused together

Table 1. FRT/FLP mosaic analysis

Segment	No.*	%
coxa	40	30
trochanter	58	44
femur	97	73
tibia	79	60
tarsal segment 1	40	30
tarsal segment 2	29	22
tarsal segment 3	1‡	1
tarsal segment 4	0	0
tarsal segment 5	0	0

*The number of Bar^- clone-possessing segments. A total of 132 legs normal in gross morphology were examined.
‡A y^- bristle was observed at the proximal tip of tarsal segment 3.

(Fig. 2D), while tarsal segment 2 and central fold were normally formed (Fig. 2D,F,I). The *pdf* and B^{55} phenotype was eliminated by $B^{S}Y$ (Lindsley and Zimm, 1992; unpublished data). Based on these results, we conclude that late *Bar* function is involved in segmentation among tarsal segments 3-5.

Induction of folding by juxtaposition of Bar-positive and Bar-negative tissues

Juxtaposition of Bar-positive and Bar-negative tissues may result in folding. To test this idea, *BarH1* or *BarH2* was misexpressed along the AP border using the UAS/GAL4 system (Brand and Perrimon, 1993). *blk*-GAL4 or *ptc*-GAL4 was used as a GAL4 driver. As anticipated, no appreciable difference in morphological changes were detected between *BarH1* and *BarH2* misexpression. Fig. 2L shows that ectopic folding was formed along outer circumference of *Bar*-misexpressing region proximal to the Bar ring, while the formation of the authentic central fold was prevented in the regions where endogenous Bar-expressing cells and cells solely expressing exogenous Bar were juxtaposed to each other. It would thus follow that central folding along the early Bar ring is an outcome of the juxtaposition of Bar-positive and Bar-negative tissues. It should, however, be emphasized that no ectopic folding ever occurred either prior to the onset of central folding (Fig. 2M), or within the Bar ring and future pretarsus (Fig. 2L), suggesting the involvement of temporal and regional factors other than Bar.

Requirements of *Bar* for specification of tarsal segments 3-5

Using mosaic analysis and misexpression, we examined whether *Bar* activity is required for the expression of markers for tarsal segments 4 (*ap*) and 5 (ta5-*lacZ*). Neither Ap nor ta5-*lacZ* expression was detected in Bar^{-} clones (Fig. 3A-F). *Bar* misexpression along the AP border caused ectopic expression of either ta5-*lacZ* or Ap or both in *Bar*-misexpressing regions proximal to the Bar ring (Fig. 3G-I). Furthermore, Bar^{-} clones within tarsal segments 4 and 5 were occasionally associated with campaniform sensilla (arrowhead in Fig. 3P), normally situated only at the dorsodistal tip of tarsal segment 3 (Fig. 3O). Based on these observations, we conclude that Bar is essential for *ap* and ta5-*lacZ* (*Bar*) expression in distal tarsi and prevents cells expressing *Bar* at later stages from adopting the tarsal segment 3 fate.

ap-GAL4 is a GAL4 enhancer trap of the *ap* locus (Calleja et al., 1996). In *ap*-GAL4/UAS-*Bar* leg discs, not only did ta5-*lacZ* misexpression occur weakly in presumptive tarsal segment 4 (Fig. 3M,N), but also tarsal segment 4 occasionally

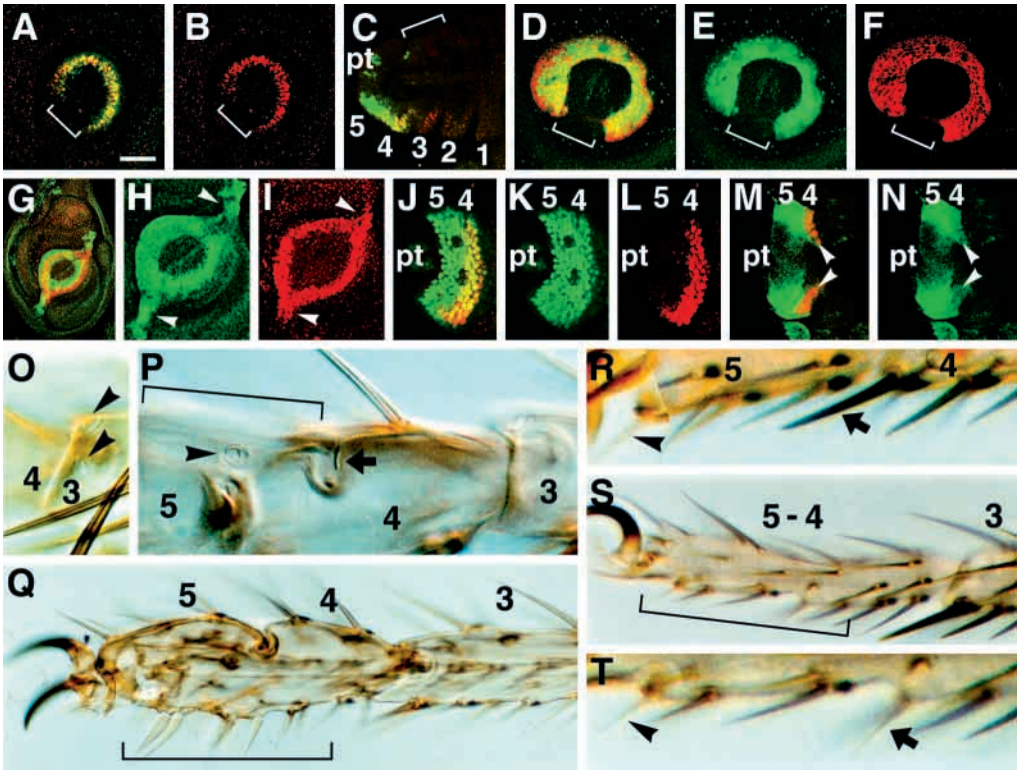

Fig. 3. Defects in tarsal segments 3-5 due to the absence or overexpression of *Bar*. 1-5, tarsal segments 1-5; pt, pretarsus. (A-F) Late third (A,B,D-F) or early pupal (C) leg discs having Bar^{-} clones stained for BarH1 (A,C,D,E, green) and Ap (A-C, red) or ta5-*lacZ* (D,F, red). Focal planes of A,B and D-F are at tarsal segment 4 and tarsal segment 5, respectively. Ap and ta5-*lacZ* signals are abolished in Bar^{-} clones (brackets). (G-I) A late-third-instar *ptc*-GAL4/UAS-$BarH1^{M6}$ leg disc stained for ta5-*lacZ* (G,H, green) and Ap (G,I, red). Note that the central knob is slightly squeezed. Both ta5-*lacZ* and Ap misexpression are induced (arrowheads). (J-N) *ap*-GAL4/UAS-$BarH1^{M13}$ leg discs. (J-L) An early pupal leg stained for BarH1 (K, green) and Ap (L, red). (J) Merged figure. Note that Bar expression levels in tarsal segment 4 are virtually identical to those in tarsal segment 5 (compare K with Fig. 1O). No appreciable Ap repression can be seen. (M,N) A sagittal section of a late third instar disc stained for ta5-*lacZ* (M,N, green) and Ap (M, red). Weak but significant ta5-*lacZ* expression is observed in tarsal segment 4 (arrowheads), suggesting partial fate change of tarsal segment 4. (O) A dorsal joint between wild-type tarsal segments 3 and 4. It is associated with a pair of campaniform sensilla (arrowheads). (P,Q) Legs possessing Bar^{-} clones (bracketed regions). Ectopic joint (arrow) and campaniform sensillum (arrowhead) are formed in P, while partial fusion of tarsal segments 4 and 5 occurs in Q. (R) Ventral region of tarsal segments 4 and 5 in wild-type leg. The distalmost bristle of tarsal segment 5 (arrowhead) is colorless and thin, while that in tarsal segment 4 (arrow) is black and thick. (S,T) An *ap*-GAL4/UAS-$BarH1^{M13}$ leg possessing tarsal segment 4/5 fusion. The bracketed region is enlarged in T. The black thick bristle at the distal edge of tarsal segment 4 (arrow) appeared transformed to that of tarsal segment 5 type, thin and less-pigmented. Arrowhead, authentic tarsal-segment-5 bristle. (A,B,D-I) Anterior is left and dorsal is up; (C,J-T) distal is left. Scale bar in A: 60 μm for A-F,H-N; 120 μm for G; 20 μm for O,P,R,T; 40 μm for Q,S.

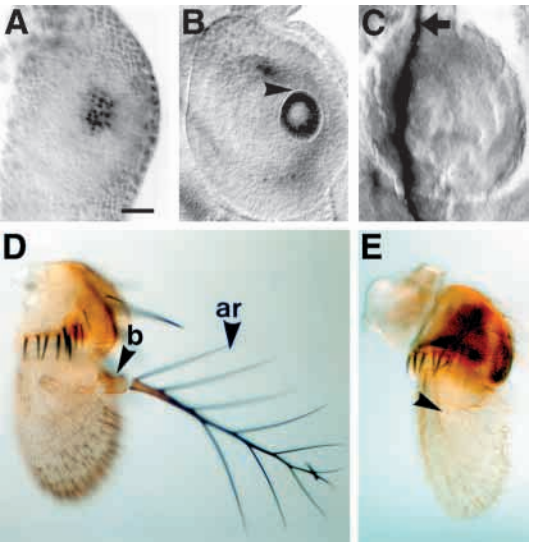

Fig. 4. Requirements of *Bar* for antennal development. (A,B) Wild-type early (A) or late (B) third instar antennal discs stained for BarH1. Central fold is formed just outside the Bar ring (B, arrowhead). (C) A late third instar antennal disc of a gynandromorph mosaic larva lacking BarH1 signals. No central fold is formed. Arrow, Bolwig's nerve stained with a neuron-specific antibody. (D) A wild-type antenna. ar, arista; b, basal cylinder. (E) A gynandromorph mosaic antenna consisting of *Bar*$^{-}$ cells. The arrowhead shows the absence of arista and basal cylinder. (A-C) Posterior is up and ventral is right. Scale bar in A: 50 μm for A-C; 30 μm for D,E.

showed partial transformation into tarsal segment 5. The ventralmost bristle situated at the distal tip of tarsal segment 4, normally thick and black (arrow in Fig. 3R), was frequently transformed into a thin and less-pigmented bristle (Fig. 3T) characteristic of tarsal segment 5 (arrowheads in Fig. 3R,T). Specific expression of *ap* or *ap*-GAL4 in future tarsal segment 4 reflects that subdivision of the early Bar ring has already occurred. Thus, the above findings may indicate that *Bar* misexpression in future tarsal segment 4 at later stages is still capable of causing future tarsal segment 4 cells to adopt tarsal segment 5 fate at least partly, and hence, suggests that late strong Bar expression in wild-type tarsal segment 5 primordia is important for them to acquire and maintain the tarsal segment 5 identity. It should, however, be noted that *ap* expression is not eliminated upon *Bar* misexpression (Fig.

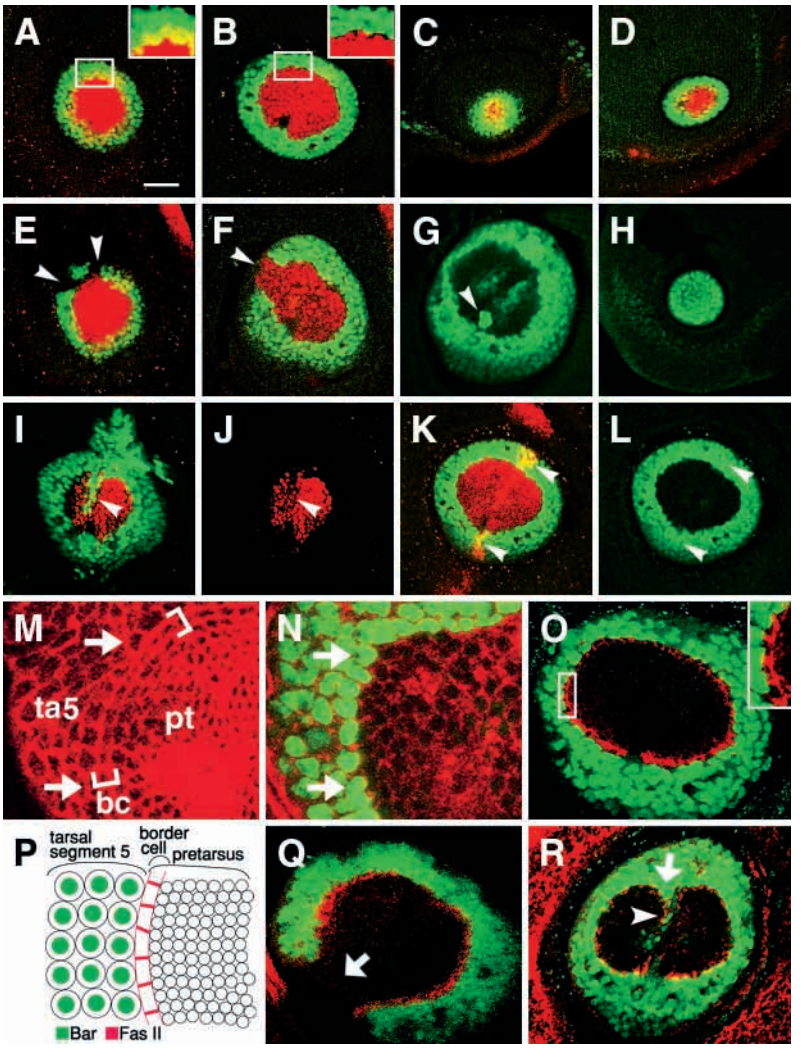

Fig. 5. Antagonistic interactions between *Bar* and *al* and tarsal segment 5/pretarsus boundary formation. Leg (A,B,E-G,I-O,Q,R) or antennal (C,D,H) discs are stained for BarH1 (green) and Al (A-F,I-K, red), Fas II (O,Q,R, red) or with Rhodamine-phalloidin (M,N, red). (A-D) Wild-type discs. In leg discs, Bar and Al domains are overlapped each other in early third instar (A) but not in mid third instar (B). Insets, enlargements of boxed regions. In antenna discs, Bar and Al are initially expressed in the center (C) in that Al is expressed only within the Bar domain. Later, Bar expression but not Al expression is excluded from the center, so that Bar and Al domains are only slightly overlapped each other (D). (E,F) Leg discs having *Bar*$^{-}$ clones. The Al domain apparently invades into the *Bar*$^{-}$ clone (arrowhead) when observed in late third instar (F). No invasion occurs in early third instar (E). (G,H) al^{2}/*Df(2L)al* discs. Patches of ectopic Bar expression are seen (arrowhead) in the leg disc at late third instar (G) and Bar expression in the center persists even at late third instar in the antennal disc (H), suggesting the partial repression of *Bar* expression by Al. (I,J) A *blk*-GAL4/UAS-$BarH1^{M13}$ disc at mid third instar. *Bar* misexpression causes *al* repression (see arrowheads). (K,L) A *ptc*-GAL4/UAS-al^{3} disc at late third instar. Bar expression is not affected by *al* misexpression (arrowheads). (M-O) Wild-type late third instar discs. (M,N) Apical (M) and slightly basal (N) sections. Large arrows, position markers. Pretarsus cells (pt) other than rectangular border cells (bc) are tightly packed in the apical region, while the tarsal segment 5 (ta5) cells are loosely packed. (O) Fas II expression in border cells. The boxed region is enlarged in the inset. Fas II is concentrated along border-cell boundaries. (P) An illustration of a part of the tarsal segment 5/pretarsus boundary. (Q) Fas II expression is interrupted by a *Bar*$^{-}$ clone (arrow). (R) A *ptc*-GAL4/UAS-$BarH1^{M6}$ disc. Ectopic Fas II expression is induced along the ectopic Bar domain (arrowhead), while normal Fas II expression is partially abolished by Bar misexpression (arrow). Anterior is left and dorsal is up. Scale bar in A: 60 μm for A-L; 18 μm for M,N; 35 μm for O,Q,R.

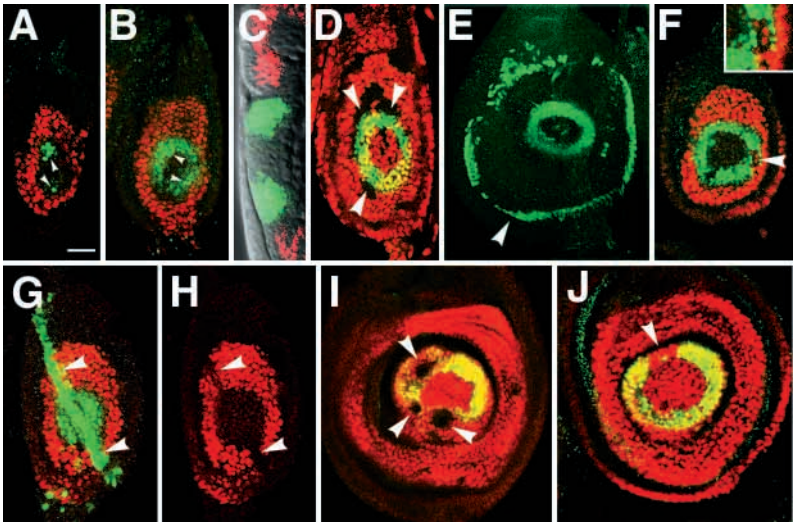

Fig. 6. *dac*/*Bar* and *Dll*/*Bar* interactions. Late second (A), early third (B-D,F-H) or late third (E,I,J) instar leg discs are stained for BarH1(green) and Dac (A-C,F-H, red), Myc (D, red) or Dll (I,J, red). (A) Dac is expressed before the early Bar ring becomes discernible. Arrowheads, Keilin's organ cells. (B) Early Bar expression occurs just inside the Dac domain. Arrowheads, Keilin's organ cells. (C) Just before the onset of the central folding, region not expressing Dac appears. Confocal and Nomarsky images were merged. (D) In *dac*$^{-}$ clones (arrowheads), Bar expression is normal. (E) In the *dac*^{3} disc, Bar is derepressed in trochanter (arrowhead). (F) Dac misexpression is observed in a *Bar*$^{-}$ clone (arrowhead and inset). (G,H) Dac expression is repressed by *Bar* misexpression (arrowheads) in a *ptc*-GAL4/UAS-*BarH1*^{M6} disc. (I) In *Dll*$^{-}$ clones (arrowheads), Bar expression is abolished. (J) In *Bar*$^{-}$ clones (arrowhead), Dll is normally expressed. Anterior is left and dorsal is up except for C, distal is left and dorsal is up. Scale bar in A: 50 μm except for C, 100 μm.

3I,L,M), suggesting that a factor other than Bar is involved in repression of *ap* in tarsal segment 5. Together, these findings indicate that graded Bar expression at later stages may be essential for specification of tarsal segments 3-5.

Ectopic incomplete segmental joints were frequently formed in the vicinity of the boundary between *Bar*$^{-}$ clones and *Bar*-expressing tarsal segments 4-5 tissues (Fig. 3P). Tarsal segments 4 and 5 were partially fused with each other in *ap*-GAL4/UAS-*Bar* legs (Fig. 3S). Thus, the juxtaposition of tissues expressing different levels of Bar may be essential for proper segmentation in distal tarsi.

Requirements of *Bar* for distal antennal structure formation

In *Drosophila*, antennae possess segmental structures homologous to those in legs and similarly differentiate through circular folding. Arista and basal cylinder, probably corresponding to pretarsus and distal tarsus (Postlethwait and Schneiderman, 1971), are derivatives of the central knob of antennal discs. After the onset of third instar, Bar expression occurred initially in a central region of the antennal disc and gradually became a ring similar to that observed in the leg disc (Fig. 4A,B). As with legs, central folding occurred just outside the Bar ring (Fig. 4B). In antennal discs lacking *Bar* activity, no central fold was formed (Fig. 4C); *Bar*$^{-}$ antennae frequently lost arista and basal cylinder (Fig. 4D,E). Thus, *Bar* is concluded to be essential not only for leg but also for antennal development.

Establishment of the tarsus/pretarsus boundary

al is a homeobox gene expressed at the center of leg and antennal discs from early third instar onwards (Campbell et al., 1993; Schneitz et al., 1993). Initially, the Al expression domain and early Bar ring overlapped slightly in leg discs (Fig. 5A). Al/Bar overlapping could be more clearly seen in early antennal discs (Fig. 5C). Up to 90 hours AEL, the central part of the leg disc was strictly divided into two regions, Bar-positive/Al-negative and Bar-negative/Al-positive circular domains (Fig. 5B). In antennal discs, such discrimination in Bar/Al expression may be incomplete (Fig. 5D).

Regionally exclusive expression of Bar and Al may be due to mutually antagonistic interactions between Bar and Al. When Al expression was examined in mid to late third instar larval leg discs having *Bar*$^{-}$ clones, Al expression invaded a *Bar*$^{-}$ presumptive tarsus region (Fig. 5F), while Al expression was considerably attenuated by *Bar* misexpression along the anteroposterior compartment border in mid third instar discs (Fig. 5I,J). Ectopic patches of Bar expression were frequently observed in the presumptive pretarsus of hypomorphic *al* leg discs in late third instar (Fig. 5G). *Bar* derepression due to reduction in Al activity is more clearly observed in antennal discs; on a hypomorphic *al* mutant background, Bar was

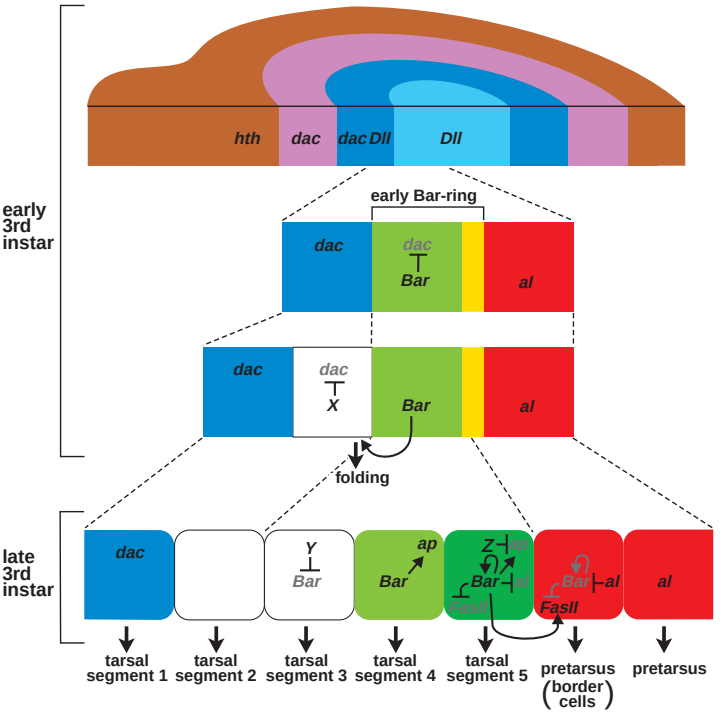

Fig. 7. Distal leg development is schematically summarized. Black, expressed genes. Gray, repressed genes. For detail, see text.

expressed in the centralmost region even at late third instar larval stages (Fig. 5H). That no appreciable repression of Bar was detected when *al* was misexpressed by *ptc*-GAL4/UAS-*al* (Fig. 5K,L) may indicate the involvement of factors other than Al in *Bar* repression in presumptive pretarsus. In addition, the lack of Al invasion into *Bar*⁻ clones in leg discs at early third instar larval stages (Fig. 5E) may indicate that *Bar* is dispensable for repressing *al* when their expression is initiated.

At late third instar, cells in the distalmost region of leg discs are densely packed at the apical surface and distinguishable from surrounding loosely packed cells (Condic et al., 1991; Fig. 5M). Double-staining with rhodamine-phalloidin and anti-BarH1 antibody revealed that the former corresponds to Bar-negative pretarsus cells, and the latter, Bar-positive tarsus cells (Fig. 5M,N). Proximalmost pretarsus cells (border cells) were frequently rectangular in apical shape (Fig. 5M). Staining for Fasciclin II (Fas II; Grenningloh et al., 1991) showed that border cells prominently express Fas II at late third instar (Fig. 5O). Fas II expression was interrupted by *Bar*⁻ clones (Fig. 5Q). Fas II misexpression was induced along *Bar*-misexpressing presumptive pretarsus, while endogenous Fas II expression was repressed (Fig. 5R). Thus, Bar would upregulate and downregulate Fas II expression in Bar-negative border cells and Bar-positive non-border cells, respectively. We, thus, conclude that *Bar* is essential for the establishment of the boundary between tarsal segment 5 and pretarsus.

Interactions between *Bar* and *dac* or *Dll*

Circular Dac expression appeared in second-instar leg discs before Bar ring appearance (Fig. 6A). This early Dac-ring was associated interiorly with Bar-positive Keilin's organ cells, which are situated along the interior circumference of or within the early Bar ring (see Figs 1A, 6A,B). Although they were separated from each other by a Bar-negative, Dac-negative region just before the onset of central fold formation (Fig. 6C), Dac and Bar rings were immediate neighbours at earlier stages (Fig. 6B). Dac expression was derepressed in *Bar*⁻ clones observed in early third instar (Fig. 6F), while repressed by *Bar* misexpression (Fig. 6G,H), indicating that *Bar* is essential for distal restriction of Dac expression. Since early Bar expression normally occurred in *dac*⁻ clones (Fig. 6D), *dac* appears dispensable for proximal restriction of the early Bar ring. Interestingly, Bar misexpression occurred in future trochanter in *dac*⁻ mutants (Fig. 6E), indicating that Dac represses *Bar* in future trochanter.

Dll is expressed from the beginning of leg development (Abu-Shaar and Mann, 1998). The Dll domain includes all *Bar*-expressing cells (see Fig. 6I,J). *Bar* expression was abolished in *Dll*⁻ clones (Fig. 6I), while Dll continued to be expressed in *Bar*⁻ clones (Fig. 6J). *Bar* expression thus appears to require *Dll* activity but *Dll* does not require *Bar*.

DISCUSSION

Mechanism of distal leg development

Distal leg segmentation is a multiple-step process involving various aspects of development. Our results are summarized in Fig. 7. Most events of distal leg segmentation occur during third instar larval stages.

By early third instar, the leg disc has been divided into four domains through *hth*, *dac* and *Dll* expression (Abu-Shaar and Mann, 1998). At early third instar, circular expression of *Bar* and *al* begins within the *Dll* domain; *Dll* is required for the expression of *Bar* (Fig. 6I and unpublished data) and *al* (Campbell and Tomlinson, 1998). Future tarsal segment 2 may be generated in the distalmost region of the Dac ring possibly through repression of *dac* expression by an unknown factor, X (Fig. 7), since Dac is expressed in the region immediately proximal to the early Bar ring (future tarsal segments 3-5) at early stages (Fig. 6B) but not in tarsal segment 2 at later stages (Abu-Shaar and Mann, 1998; Lecuit and Cohen, 1997).

Expression of molecular markers for tarsal segments 5 (ta5-*lacZ*) and 4 (*ap*) becomes apparent within the Bar ring just after the onset of central folding but before distal tarsus segmentation (Fig. 1Q). Cells in the proximalmost region of the early Bar ring may also be committed to become tarsal segment 3 at this stage. Bar expression within the early Bar ring is nearly homogeneous (Fig. 1B) and thus the initial subdivision of this ring into future tarsal segments 3-5 may require factor(s) other than Bar. *ss* is expressed transiently in the future tarsus region in late second to early third instar and *ss* mutant legs lack tarsal segments 2-4 but not 5 (Duncan et al., 1998). *ss* may thus be responsible for differential gene expression between future tarsal segments 4 and 5. Repression of Bar expression in future tarsal segment 3 may be due to a putative Bar repressor, Y (Fig. 7).

At later stages, Bar expression is strong in tarsal segment 5 (Fig. 7, deep green), moderate in tarsal segment 4 (light green) and absent from tarsal segment 3. Genetic and morphological analyses (Table 1; Fig. 3) strongly suggest that Bar upregulates *ap* and/or its own expression in tarsal segments 4 and 5. Since (1) only partial transformation of tarsal segment 4 into 5 occurs upon *Bar* overexpression in tarsal segment 4 (Fig. 3T) and (2) *Bar* misexpression fails to repress Ap expression (Fig. 3G-I), an unknown factor (Z) is likely involved in *ap* repression in future tarsal segment 5 (Fig. 7).

Duncan et al. (1998) suggest that tarsal development takes place in two steps: establishment of a uniform tarsal region followed by subdivision of this ring into segments. Our results indicate that there are several more intermediate steps in this process. Abu-Shaar and Mann (1998) propose three phases of leg-disc subdomain formation during early development probably prior to the onset of Bar expression. Thus, leg segmentation requires repeated subdivision of leg epithelium along the proximodistal axis with the result that smaller region-specific transcription factor domains are generated from larger ones in all instances of subdivision.

Genetic interactions of *Bar* with *al*

Bar and Al expression begins essentially at the same time at early third instar. Initially, Al expression partially overlaps Bar expression (Figs 5A,C, 7, yellow), and no invasion of Al into Bar⁻ clones was found when mosaic clones were observed at this stage (Fig. 5E). However, at slightly later stages, Al and Bar expression became mutually exclusive (Fig. 5B,D) and Al invaded into Bar⁻ clones (Fig. 5F). It may thus follow that *al* expression is initiated *Bar* independently and, after a while, Bar protein accumulated to some extent begins to repress *al* expression. *al* may also regulate *Bar* expression in a similar fashion. Indeed, Bar misexpression occasionally occurred in leg and antennal discs of *al* hypomorphic mutants (Fig. 5G,H),

although *Bar* expression was not repressed by *al* misexpression (Fig. 5K,L). The failure of *Bar* repression by *al* may suggest the involvement of other pretarsus gene(s) that function cooperatively with *al*. We actually identified two new genes that function in the pretarsus and show mutant phenotypes similar to *al* (T. Tsuji, T. K. and K. S., unpublished data).

Bar misexpression experiments (Fig. 3G-I) showed pretarsus to be the region where ta5-*lacZ* expression is very difficult. Al and/or other pretarsus factors may thus possibly compete with Bar for the late *Bar* enhancer, ta5, to repress pretarsus *Bar* expression (see Fig. 7).

Folding, segmentation and tarsus/pretarsus boundary formation by Bar

Fig. 5O shows that Fas II, a protein mediating homophilic adhesion (Grenningloh et al., 1990), is concentrated in border cells demarcating the proximal pretarsus border. Our results (Fig. 5Q,R) also shows that Bar is capable of inducing Fas II expression in cells distally adjacent to Bar-expressing cells. Thus, Bar may establish the boundary between the pretarsus (Bar-negative) and tarsal segment 5 (Bar-positive) by regulating the expression of cell adhesion molecules such as Fas II. Interestingly, *BarX2*, a mouse gene encoding a Bar-related homeodomain protein, has been reported to regulate the expression of Fas II-like cell adhesion molecules (Jones et al., 1997).

At early third instar, proximal neighbors of the Bar ring initiate folding in a *Bar*-dependent manner (Fig. 1G-G″). Similarly, Bar concentration differences in future tarsal segments might be essential for the normal development of tarsal segments 3-5 (see Fig. 3P,S). Since folding and/or segmentation are likely to be caused by change in local cell adhesiveness, *Bar* may also regulate some cell adhesion molecule(s) responsible for central folding and/or distal-tarsus segmentation.

Similarity in mechanism between *Drosophila* and vertebrate limb development

Mechanisms similar to antagonistic interactions between *Bar* and *al* may also be involved in vertebrate limb development. *Hoxa11* and *Hoxa13* are homeobox genes expressing in a region-specific manner in vertebrate limb buds. At early developmental stages, both *genes* are expressed in the distalmost region of the limb, although *Hoxa11* expression expands more proximally than that of *Hoxa13* (Yokouchi et al., 1991). At later stages, *Hoxa11* and *Hoxa13* expression domains are separated from each other through *Hoxa11* repression in the *Hoxa13* expression domain. As in the case of Bar expression in *Drosophila* pupal legs, the boundary between *Hoxa11* and *Hoxa13* expression domains appears intimately related to cartilage segmentation (Yokouchi et al., 1991).

As with *Bar* and *Dll* in *Drosophila* (this work; Campbell and Tomlinson, 1998; Wu and Cohen, 1999), *Hoxa13* has been reported to regulate local cell adhesiveness (Yokouchi et al., 1995). Thus, the control of genes encoding cell adhesion proteins by region-specific transcription factors may be one of fundamental mechanisms involved in both insect and vertebrate development.

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