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Analysis in *Drosophila melanogaster* of the Interaction Between Sex Combs Reduced and Extradenticle Activity in the Determination of Tarsus and Arista Identity

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ABSTRACT

Sex Combs Reduced (SCR) activity is proposed to be required cell nonautonomously for determination of tarsus identity, and Extradenticle (EXD) activity is required cell autonomously for determination of arista identity. Using the ability of Proboscipedia to inhibit the SCR activity required for determination of tarsus identity, we found that loss-of-EXD activity is epistatic to loss-of-SCR activity in tarsus *vs.* arista determination. This suggests that in the sequence leading to arista determination SCR activity is OFF while EXD activity is ON, and in the sequence leading to tarsus determination SCR activity is ON, which turns EXD activity OFF. Immunolocalization of EXD in early third-instar larval imaginal discs reveals that EXD is localized in the nuclei of antennal imaginal disc cells and localized in the cytoplasm of distal imaginal leg disc cells. We propose that EXD localized to the nucleus suppresses tarsus determination and activates arista determination. We further propose that in the mesodermal adepithelial cells of the leg imaginal discs, SCR is required for the synthesis of a tarsus-inducer that when secreted acts on the ectoderm cells inhibiting nuclear accumulation of EXD, such that tarsus determination is no longer suppressed and arista determination is no longer activated.

THE conserved *Hox* genes function in laying out the body plan along the anterior/posterior axis of many, and maybe most, animal phyla (Slack *et al.* 1993; Carroll 1995). All *Hox* genes encode proteins that contain the DNA-binding protein domain, the homeodomain (McGinnis and Krumlauf 1992). The activity of the *Hox* genes was initially identified in Drosophila by the phenotypes of loss-of-function or gain-of-function alleles. A mutation in a Drosophila *Hox* gene results in the transformation of one body part into another (Lewis 1978; Kaufman *et al.* 1990).

Two HOX activities, Proboscipedia (PB) and Sex Combs Reduced (SCR), are required for the determination of the maxillary palps, proboscis, prothorax, and tarsi (Kaufman *et al.* 1990; Percival-Smith *et al.* 1997). PB activity is required for determination of maxillary palp identity. PB and SCR activities are proposed to form a complex that is required for determination of proboscis identity (Percival-Smith *et al.* 1997). Proposal of complex formation between PB and SCR is based on the interpretation of genetic experiments and has not been observed directly in biochemical assays. SCR activity is required for determination of prothorax identity and also for the determination of all tarsi. SCR activity is proposed to be required cell nonautonomously in tarsus determination (Percival-Smith *et al.* 1997). Specifically proposed is that SCR activity expressed in the mesodermal adepithelial cells of all leg imaginal discs is required for the synthesis of a yet unidentified signal factor, the tarsus-inducer, which after secretion from the adepithelial cells acts on the overlaying ectodermal cells to determine tarsus identity. This cell-nonautonomous model explains the results of two distinct sets of experiments (Percival-Smith *et al.* 1997).

In the first set of experiments, SCR activity required for tarsus determination is manipulated directly (Percival-Smith *et al.* 1997). In a mosaic analysis, clones of *Scr* mutant tarsal ectoderm cells do not adopt aristal identity, but remain tarsal. The cell-nonautonomous model explains this result nicely because it proposes that SCR activity is not required in the ectoderm. Furthermore, clones of aristal ectodermal cells that ectopically express SCR protein fail to adopt tarsal identity. An additional observation made in this ectopic expression experiment was that transformations of the arista to tarsus were not associated with the apparent expression of SCR activity in the ectodermal cells. The model proposes that in tarsus determination the mesoderm signals to the ectoderm with a SCR-dependent, tarsus-inducing factor. The model also proposes a role for the SCR protein expressed in the mesodermal adepithelial cells of all three pairs of leg imaginal discs (Glickman and Brower 1988).

In the second set of experiments, SCR activity required for tarsus determination is manipulated indirectly with

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PB activity (Percival-Smith *et al.* 1997). For proboscis determination, PB and SCR are proposed to interact as a protein complex. Hence, SCR has two roles: SCR activity alone determines tarsus identity and not proboscis identity; whereas, SCR protein complexed with PB protein determines proboscis identity and not tarsus identity. Ectopic expression of PB or PB^{Q50K} results in tarsus-to-arista transformations on all three pairs of legs. The explanation for these transformations using the cell-nonautonomous model is that expression of PB or PB^{Q50K} in the mesoderm complexes with SCR and inhibits the SCR activity required for tarsus determination. The proboscis of a completely *pb* null mutant organism is transformed to tarsi. For this transformation, the model suggests that SCR protein alone determines tarsus identity in a cell-nonautonomous fashion. Evidence for this cell-nonautonomous mechanism comes from a mosaic analysis with a null *pb* allele in which the predominant transformation observed is proboscis-to-arista. The cell-nonautonomous model is used to explain this result by suggesting that the *pb* mutant ectodermal clone adopts arista identity because in the *pb*$^+$ mesoderm cells of the proboscis, PB protein interacts with SCR protein and no tarsus-inducer is synthesized. This same mechanism is proposed to occur also in a mosaic analysis with a double *pb Scr* null mutant chromosome. The *pb Scr* clone adopts antennal identity because in the *pb*$^+$ *Scr*$^+$ cells surrounding the clone, PB interacts with SCR to determine proboscis identity and no tarsus-inducer is synthesized. This can be shown, as *pb Scr* clones of proboscis cells induced in a *pb* mutant genetic background adopt tarsal identity. The model explains this tarsal transformation as resulting from the *pb Scr* clone of cells being surrounded by *pb Scr*$^+$ cells that synthesize the tarsus-inducer (Percival-Smith *et al.* 1997).

Extradenticle (EXD) activity is required across the anterior/posterior axis for segmental patterning of Drosophila (Peifer and Wieschaus 1990). The HOX proteins, Ultrabithorax (UBX), Deformed (DFD), and Labial (LAB), form complexes with EXD that are required for the expression of downstream genes (Chan *et al.* 1994, 1996; Pinsonneault *et al.* 1997). These cases of HOX-EXD interactions are examples of a codeterminant mechanism for assigning segmental identity (Peifer and Wieschaus 1990). In the codeterminant model, the HOX protein and EXD protein are expressed and co-localize to the nuclei of certain cells to assign their segmental identity. EXD activity is also regulated by a second mechanism that is independent of complex formation with HOX proteins. This mechanism is intracellular localization; EXD protein can be found in the cytoplasm or nucleus (Mann and Abu-Shaar 1996; Aspland and White 1997). Recently it has been demonstrated that nuclear localization of EXD correlates with the expression pattern of the gene *homothorax* (*hth*) (Rieckhof *et al.* 1997; Pai *et al.* 1998). Homothorax (HTH) activity is required for nuclear localization of EXD. Where EXD activity is cytoplasmically localized, EXD is proposed to be inactive (Rieckhof *et al.* 1997). EXD activity is required for the determination of arista and antenna identity; loss-of-EXD activity results in an antenna-to-second-leg transformation (González-Crespo and Morata 1995; Rauskolb *et al.* 1995).

SCR activity is required for tarsus determination, and EXD activity is required for arista determination (González-Crespo and Morata 1995; Rauskolb *et al.* 1995; Percival-Smith *et al.* 1997). The opposite phenotypes of loss-of-SCR and EXD activity present the perfect situation for the analysis of genetic epistasis. However, the cell-nonautonomous requirement of SCR activity *vs.* the cell-autonomous requirement of EXD activity makes this analysis difficult (González-Crespo and Morata 1995; Percival-Smith *et al.* 1997). Fortunately, the ability to manipulate SCR activity required for determination of tarsus identity with PB allows an analysis of epistasis. In mosaic analyses with *pb* or *pb Scr*, the SCR activity required for determination of tarsus identity is proposed to be OFF such that aristal identity is adopted by proboscis cells (Percival-Smith *et al.* 1997). Hence, the test of epistasis is whether clones of *pb* or *pb Scr* proboscis cells will remain aristal or will adopt tarsal identity when also mutant for *exd.* Here we present evidence that the determination of tarsus identity in Drosophila requires SCR activity in the mesodermal adepithelial cells, which leads to the cytoplasmic localization of EXD protein in the adjacent ectodermal epithelial cells.

MATERIALS AND METHODS

Stock construction: For a description of the genetic markers and balancer chromosomes used in this article, see Lindsley and Zimm (1992). Flies were maintained on standard Drosophila media supplemented with Baker's yeast. The null *pb*^{27} allele is a small deletion that removes the second exon of *pb*, which encodes a significant portion of the PB protein. The *pb*^{27} allele is also a protein minus allele (Pultz *et al.* 1988; Cribbs *et al.* 1992). The *Scr*^{2} allele is a genetically defined null allele (Pattatucci *et al.* 1991). The *exd*^{1} allele is a nonsense null allele (Rauskolb *et al.* 1995).

Flip-mediated mitotic recombination: Stocks DJ103 and DH52 were crossed (Hyduk and Percival-Smith 1996; Table 1) and lobed males selected. These lobed males were crossed with stock DJ400, and male progeny of the genotype *y w; P{hspFLP}/L; P{ ry*$^+$*, neo*^{r}*, FRT}82B M(3)95A*^{2} *P{y*$^+$*, ry*$^+$*}96E P{w*$^+$*, exd*$^+$*}/Ki ftz*^{11} or *TM6B, P{*walL*y}* were selected. For the three experiments, these males were crossed with stock APS401 for generating *exd*^{1} clones, with stock APS402 for generating *pb*^{27} *exd*^{1} clones, and with stock APS 403 for generating *pb*^{27} *Scr*^{2} *exd*^{1} clones (Table 1). The larval progeny of these three sets of crosses were grown at 25°, and larvae between 36 and 60 hr after egg laying of age were heat-shocked for 20 min at 36.5°. Because of the mutational load, the *P{w*$^+$*, exd*$^+$*}* element did not rescue the exd phenotype to eclosed adults; hence, male pharate adults were screened for the y$^+$, Tb$^+$, f, and L$^+$ phenotype that corresponds with the genotype *y w exd*^{1} *f*^{36a}*; P{hspFLP}/+; P{ry*$^+$*, neo*^{r}*, FRT}82B X/P{ry*$^+$*, neo*^{r}*, FRT}82B M(3)95A*^{2} *P{y*$^+$*, ry*$^+$*}96E P{w*$^+$*, exd*$^+$*}*, where *X* can be *P{w*$^+$*, ry*$^+$*}90E, pb*^{27} or *pb*^{27} *Scr*^{2} *p*^{p} *cu P{w*$^+$*, ry*$^+$*}90E.* In the same

TABLE 1

Table of stocks

Name	Genotype	Origin
DJ103	*y w; P{ry⁺, neoʳ, FRT}82B M(3) 95A² P{y⁺, ry⁺}96E P{w⁺, exd⁺}/TM6B, P{w*allL*y}*	This study
DH52	*y w; L/CyO; Ki ftz¹¹/TM6B, P{w*allL*y}*	Hyduk and Percival-Smith (1996)
DJ400	*y w; P{hspFLP}; Ki ftz¹¹/TM6B, P{w*allL*y}*	Percival-Smith *et al.* (1997)
APS401	*y w exd¹ f³⁶ᵃ/FM7c;P{ry⁺, neoʳ, FRT}82B P{w⁺, ry⁺}90E*	This study
APS402	*y w exd¹ f³⁶ᵃ/FM7c; P{ry⁺, neoʳ, FRT}82B pb²⁷/TM6B, P{w*allL*y}*	This study
APS403	*y w exd¹ f³⁶ᵃ/FM7c; P{ry⁺, neoʳ, FRT}82B pb²⁷ Scr² pᵖ cu P{w⁺, ry⁺}90E/TM6B, P{w*allL*y}*	This study
APS17	*y w; L/CyO; Ki pb⁴ pᵖ/TM6B, P{w*allL*y}*	This study
APS18	*y w; L/CyO; pb²⁷/TM6B, P{w*allL*y}*	This study
APS303	*y w; P{hspFLP}; pb²⁰/TM6B, P{w*allL*y}*	Percival-Smith *et al.* (1997)

experiment, we were able to score *exd⁺* clones by screening *y w/y w exd¹ f³⁶ᵃ* females, *FM7c* males, or *y w* males. *y w* males arise because of a high frequency of nondisjunction of the first chromosome. The heads were dissected and mounted in 50% Hoyer's:50% lactic acid (Wieschaus and Nüsslein-Volhard 1986).

Immunolocalization: EXD SCR immunolocalization was performed by standard procedures (Aspland and White 1997). A mouse monoclonal anti-EXD antibody and a rabbit polyclonal anti-SCR antibody were used to visualize EXD and SCR localization in late third-instar larval imaginal discs (Lemotte *et al.* 1989; Aspland and White 1997). A rabbit polyclonal anti-EXD antibody was used to visualize EXD in early third-instar larval imaginal discs (Mann and Abu-Shaar 1996). Texas red-conjugated donkey anti-mouse or anti-rabbit secondary antibodies were used to visualize EXD, and FITC-conjugated donkey anti-rabbit secondary antibody was used to visualize SCR (Jackson ImmunoReseach Laboratories, Inc., West Grove, PA). Images were collected on a laser scanning microscope (Carl Zeiss, Inc., Thornwood, NY).

Collection of *pb⁴/pb²⁰* and *pb²⁷/pb²⁰* early third-instar larval labial imaginal discs was performed by dissecting larvae with yellow head skeletons from crosses APS 17 × APS303 and APS 18 × APS303, respectively.

RESULTS

The methodology for generating the *pb exd* and *pb Scr exd* clones: Addressing the epistatic interaction between *Scr* and *exd* required a mosaic analysis using Flip-mediated mitotic recombination (Golic 1991; Xu and Rubin 1993). The locations of the *pb Scr* loci on the third chromosome and the *exd* locus on the first chromosome are a major technical problem. This problem was overcome by moving the *exd⁺* locus from the first chromosome to the right arm of the third chromosome. To move the *exd⁺* locus, Cordelia Rauskolb kindly supplied a *P{w⁺, exd⁺}* insertion on the third chromosome (Rauskolb *et al.* 1993), which we mapped genetically to the distal tip of *3*R (3-107.8). The position of *P{w⁺, exd⁺}* allowed the construction of the marked *P{ry⁺, neoʳ, FRT}82B M(3)95A² P{y⁺, ry⁺}96E P{w⁺, exd⁺}* chromosome. This chromosome was used to set up the genotype shown in Figure 1 (see materials and methods). When a mitotic recombination event occurs between the FRT sites, the yellow clones of cells are *exd¹*, because *P{w⁺, exd⁺}* is lost, and are homozygous for *pb²⁷* or *pb²⁷ Scr²*.

We tested whether this system for generating *exd¹* clones would reproduce previously observed phenotypes. The adult phenotypes of loss-of-EXD activity have been documented previously (González-Crespo and Morata 1995; Rauskolb *et al.* 1995). All the head phenotypes were reproduced. This includes the arista-to-tarsus transformation (Figure 2a), the antenna-to-second-leg transformation (Figure 2b), and ectopic eye formation, which is sometimes associated with a leg (Figure 2c). However, clones induced at the first/second-instar larval stage with the *P{ry⁺, neoʳ, FRT}82B M(3)95A² P{y⁺, ry⁺}96E P{w⁺, exd⁺}* chromosome did not include the transformation of proboscis tissue into knobs of tissue with bracted bristles, as was reported in a gnandromorph analysis (Rauskolb *et al.* 1995). This transformation was observed when clones were induced during embryogenesis with the *P{ry⁺, neoʳ, FRT}82B Sb⁶³ᵃ P{w⁺, πM}87E P{y⁺, ry⁺}96E P{w⁺, exd⁺}* chromosome (Hayden 1996). Clones generated by the loss of the ring *X* chromosome are generated early during embryogenesis, suggesting that, for this transformation of the proboscis, *exd¹* clones must be generated during embryogenesis (Rauskolb *et al.* 1995).

Loss-of-EXD activity is epistatic to loss-of-SCR activity: Epistasis was tested by determining whether *pb exd* and *pb Scr exd* proboscis cells adopted aristal or tarsal identity. Clones of *exd¹* proboscis cells induced during the first/second larval stage were wild type (Figure 3b). Both *pb²⁷* and *pb²⁷ Scr²* clones of ectodermal proboscis cells adopted aristal identity (Figure 3, c and e; Table 2). Both *pb²⁷ exd¹* and *pb²⁷ Scr² exd¹* clones of ectodermal proboscis cells adopted tarsal identity (Figure 3, d and f; Table 2). Hence, loss-of-EXD activity is epistatic to loss-of-PB and loss-of-PB SCR activity. We have observed the same result with the *P{ry⁺, neoʳ, FRT}82B Sb⁶³ᵃ P{w⁺,*

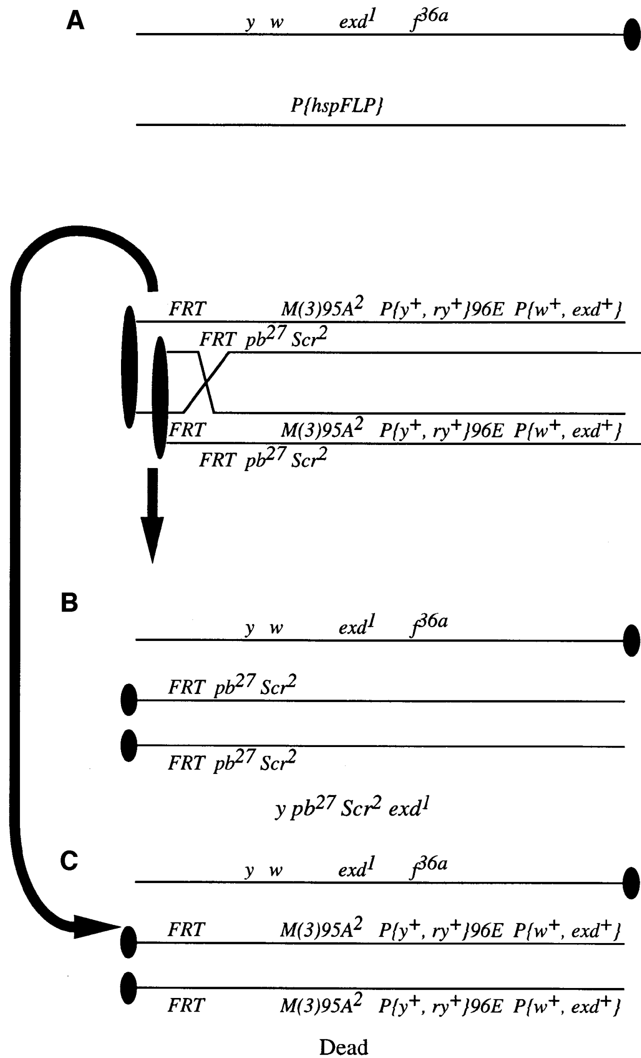

Figure 1.—Generation of clones for the analysis of epistasis. In the example shown, *pb^{27} Scr^{2} exd^{1}* clones are being generated. The genotype of the first, second, and right arm of the third chromosomes are indicated in A. The right arm of the third chromosome is shown at the G2 phase of the cell cycle when site-specific recombination generates detectable mitotic recombination events. The results of these events after cytokinesis are shown at the bottom in B and C with only the genetic constitution of the first and third chromosomes indicated.

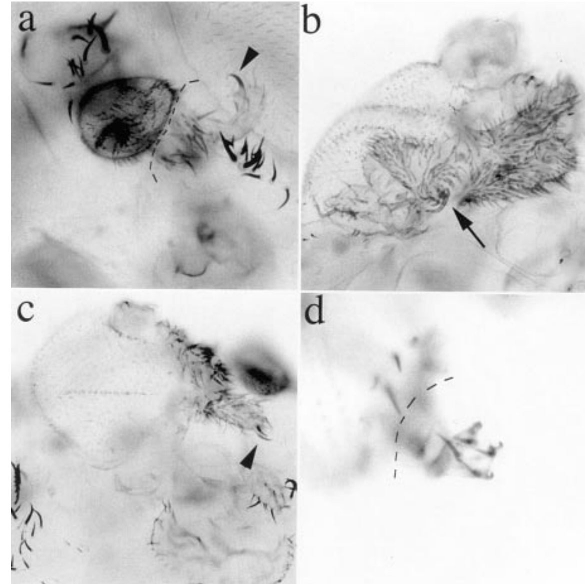

Figure 2.—Mosaic analysis with FLP-mediated mitotic recombination reproduces previously observed phenotypes. (a–c) Clones generated with the *P{ry^{+}, neo^{r}, FRT} M(3)95A^{2} P{y^{+}, ry^{+}} 96E P{w^{+}, exd^{+}}* chromosome and induced at 36–60 hr after egg laying. a shows an arista-to-tarsus transformation, b shows an antenna-to-second-leg transformation, and c shows a rostrum-to-ectopic eye, with associated leg, transformation. (d) A clone induced during embryogenesis and generated with the *P{ry^{+}, neo^{r}, FRT} Sb^{63b} P{w^{+}, πM}87E P{y^{+}, ry^{+}} 96E P{w^{+}, exd^{+}}* chromosome. The clone of *exd^{1}* proboscis cells has bristles with bracts. The clones in a and d are enclosed with dashed lines, tarsal claws are shown by arrowheads, and the apical bristle by an arrow.

πM}87E P{y^{+}, ry^{+}}96E P{w^{+}, exd^{+}} chromosome inducing clones during embryogenesis (Hayden 1996).

In a *pb^{27} Scr^{2}* mosaic analysis, the proboscis is transformed into a complete antenna that includes the third antennal segment and arista (Percival-Smith *et al.* 1997). However, clones of *pb^{27} Scr^{2} exd^{1}* cells did not adopt third-antennal-segment identity (Table 2). This indicates that *pb^{27} Scr^{2} exd^{1}* clones of ectodermal proboscis cells were transformed to second-leg identity; however, we were unable to assign the exact identity of *pb^{27} Scr^{2} exd^{1}* clones in the proboscis because an unambiguous apical bristle, which is a specific marker of second-leg identity, was not observed.

SCR activity required for sex comb determination does not require EXD activity: Clones of *pb^{27}* cells in the proboscis form sex combs. We suggest that this is because SCR activity is released from an interaction with PB (Percival-Smith *et al.* 1997). SCR activity is required cell autonomously for sex comb determination (Struhl 1982). Clones of *pb^{27} exd^{1}* cells in the proboscis were transformed to tarsal identity (Figure 3d), and in addition these clones formed sex combs (Figure 4; Table 2). This indicated that SCR activity does not require EXD activity in determination of sex combs, and *pb^{27} exd^{1}* clones in the proboscis were transformed into tarsi with first-leg identity. The transformation of the proboscis to first-leg identity requires SCR activity, as no sex comb formation was observed in *pb^{27} Scr^{2} exd^{1}* clones (Table 2).

Intracellular localization of EXD protein: Our genetic analysis suggests that EXD activity is turned OFF in the ectoderm as a result of SCR expression in the mesoderm and that SCR activity does not require EXD activity for sex comb determination. We investigated whether the ON/OFF state of EXD activity correlated with a change in accumulation or localization of EXD protein. We performed double immunolocalization of SCR and EXD in late third-instar larval leg and antennal imaginal

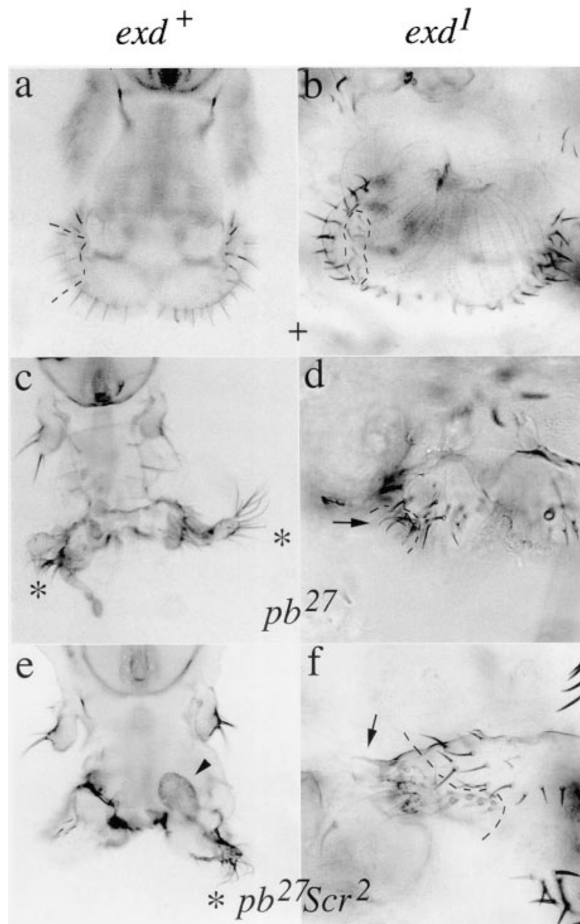

Figure 3.—Analysis of epistasis of pb^{27} and pb^{27} Scr^{2} with exd^{1}. (a–f) Clones in the proboscis representing a distinct genotype. a and b are pb^{+} Scr^{+}, c and d are pb^{27} Scr^{+}, and e and f are pb^{27} Scr^{2}. a, c, and e are exd^{+}, and b, d, and f are exd^{1}. Clones of yellow ectodermal tissue are enclosed within dashed lines in a, b, d, and f. The asterisks show aristal structures, arrows show tarsal claws and the arrowhead shows third antennal segment identity.

discs (Figure 5, a–h). In all leg imaginal discs, EXD protein was cytoplasmically localized in the central (distal) region of the discs (González-Crespo and Morata 1996; Aspland and White 1997). SCR was expressed and localized in the nuclei of the ectodermal cells of the T1 leg imaginal disc (Glickman and Brower 1988). In the region that includes the primordium of the sex combs, SCR protein is highly expressed, but EXD protein is cytoplasmically localized (Figure 5, a–c; Glickman and Brower 1988; Pattatucci and Kaufman 1991). Since EXD activity is proposed to be inactive when cytoplasmically localized (Rieckhof *et al.* 1997), EXD may be inactive in the sex comb primordia of the ectoderm, which may explain why SCR activity required for sex comb determination does not require EXD activity. SCR activity is required at late third-instar larval stage for sex comb determination (Pattatucci *et al.* 1991).

In all late third-instar larval leg imaginal discs, SCR was expressed in the adepithelial cells and EXD was cytoplasmically localized in the distal region of the discs (Figure 5, d–f; Glickman and Brower 1988; Aspland and White 1997). This suggests that EXD activity is OFF in the determination of the tarsi, and, as SCR is not expressed in the adepithelial cells of antennal discs of late third-instar larvae (Glickman and Brower 1988; Figure 5, g and h), it may have been expected that EXD protein would be nuclearly localized. SCR was expressed in the disc stack of the antennal disc, but not the adepithelial cells (Figure 5, g and h); however, EXD was not nuclearly localized throughout the antennal disc, but rather was cytoplasmically localized in the distal region that is the primordium of the arista (Figure 5, g and h; González-Crespo and Morata 1995; Rieckhof *et al.* 1997). This suggests that EXD activity is OFF in the arista primordia of late third-instar larval discs; yet EXD activity is required for aristal determination (González-Crespo and Morata 1995; Rauskolb *et al.* 1995; Figure 2a). This paradox is resolved by the observation that SCR activity is required at the late second/early third-instar larval stage for tarsus determination (Percival-Smith *et al.* 1997). When we assayed EXD expression and localization in early third-instar larval imaginal discs, EXD protein was nuclearly localized both proximally and distally in the ectoderm of antennal discs (Figure 5, i and j), but was cytoplasmically localized distally in leg imaginal discs (Figure 5, k and l).

To confirm that the localization of EXD correlates with determination of tarsus *vs.* arista identity, EXD localization was assayed in genetically manipulated situations. Ectopic expression of PB^{Q50Ka} at early third-instar larval stage was induced by administering a 20-min, 36.5° heat-shock to $P\{hsp/pb^{Q50Ka}, ry^{+}\}A$ larvae; the larvae were allowed to recover for 3 hr. EXD was nuclearly localized in the leg imaginal discs of these larvae (Figure 5m), which correlates with the tarsus-to-arista transformation observed with ectopic expression of PB^{Q50Ka} (Percival-Smith *et al.* 1997). We also assayed EXD localization in labial imaginal discs of early third-instar larvae derived from pb^{4}/pb^{20} and pb^{27}/pb^{20} mutants. We found that EXD was nuclearly localized in pb^{4}/pb^{20} labial discs (Figure 5n) and cytoplasmically localized in the distal cells of pb^{27}/pb^{20} labial discs (Figure 5o), correlating with the aristal and tarsal identity adopted by these transformed labial imaginal discs, respectively. The cytoplasmic localization of EXD in pb^{27}/pb^{20} labial imaginal discs was restricted to the most distal cells and was not as extensive as observed in normal leg imaginal discs (compare Figure 5, o with k). This difference in localization of EXD may explain the difference between the structure of a normal T1 leg and the transformed proboscis of *pb* null mutants. The leg transformation of *pb* null mutants is a near perfect tarsus but the tibia and femur are short and fused (Kaufman 1978). The clear cytoplasmic location of EXD in the center of the pb^{27}/pb^{20} labial disc explains the strong tarsus transformation of the proboscis, and the mixed and nuclear localization of EXD in

TABLE 2

The phenotypes of the clones generated

Genotype of clones in the antenna	Total number of clones	Wild type	Mixed aristal tarsal[a]	Tarsal	Apical bristle	Bracted bristles
pb^{+} Scr^{+} exd^{+}	45	45	0	0	0	0
pb^{+} Scr^{+} exd^{1}	29	0	0	9	4	29
pb^{27} Scr^{+} exd^{+}	47	47	0	0	0	0
pb^{27} Scr^{+} exd^{1}	44	0	0	21	4	41
pb^{27} Scr^{2} exd^{+}	38	38	0	0	0	0
pb^{27} Scr^{2} exd^{1}	39	0	0	21	8	39

Genotype of clones in the proboscis	Total number of clones	Aristal	Mixed aristal-tarsal	Tarsal	Wild type	Sex combs	Third antennal segment
pb^{+} Scr^{+} exd^{+}	18	0	0	0	18	0	0
pb^{+} Scr^{+} exd^{1}	7	0	0	0	7	0	0
pb^{27} Scr^{+} exd^{+}	42	10	3	7	0	6	0
pb^{27} Scr^{+} exd^{1}	20	0	0	6	0	5	0
pb^{27} Scr^{2} exd^{+}	51	12	0	0	0	0	31
pb^{27} Scr^{2} exd^{1}	25	4	3	5	0	0	0

[a] Aristal phenotype refers to the presence of aristal-like structures in the y clone of cells; tarsal phenotype refers to the presence of either a claw or pulvillus in the y clone of cells; the presence of bracted bristles was not used as a marker for tarsal transformations.

the region around the center of the pb^{27}/pb^{20} labial disc may explain the poor formation of the tibia and femur (Figure 5o).

DISCUSSION

A model for tarsus *vs.* arista determination: We have shown here that loss-of-EXD activity, which is required for arista determination, is epistatic to loss-of-SCR activity, which is required for tarsus determination. We have also shown that in early third-instar larval imaginal discs EXD is nuclearly localized in the antennal disc and cytoplasmically localized in the distal regions of the leg imaginal discs. SCR activity is proposed to be required cell nonautonomously for tarsus determination (Percival-Smith *et al.* 1997). From these data, we propose a model for the determination of arista and tarsus identity (Figure 6). In the ectoderm layer of aristal precursor cells, nuclear-localized EXD suppresses tarsus determination and activates arista determination. In the mesodermal adepithelial cells of the leg imaginal discs, SCR activity is required for the synthesis of a secreted, tarsus-inducing factor that acts on the distal ectoderm cells and inhibits the nuclear accumulation of EXD, preventing suppression of tarsus determination and activation of arista determination. This is a determinant model for a HOX -EXD activity interaction: SCR is required in the mesoderm for the inhibition of nuclear localization of EXD protein in the ectoderm. This model broadens the range of mechanisms employed in HOX-EXD interactions (Peifer and Wieschaus 1990; Chan *et al.* 1994).

Recently it has been shown that ectopic expression of UBX or HOXD10 can lead in a cell-autonomous fashion to the cytoplasmic localization of EXD in the antennal imaginal disc (Azpiazu and Morata 1998). Ectopic expression of UBX or HOXD10 results in an arista-to-tarsus transformation; however, the localization of EXD in the aristal primordia was not assayed in the late third-instar larval imaginal discs because by this time it would have been cytoplasmic in location (Figure 5, g

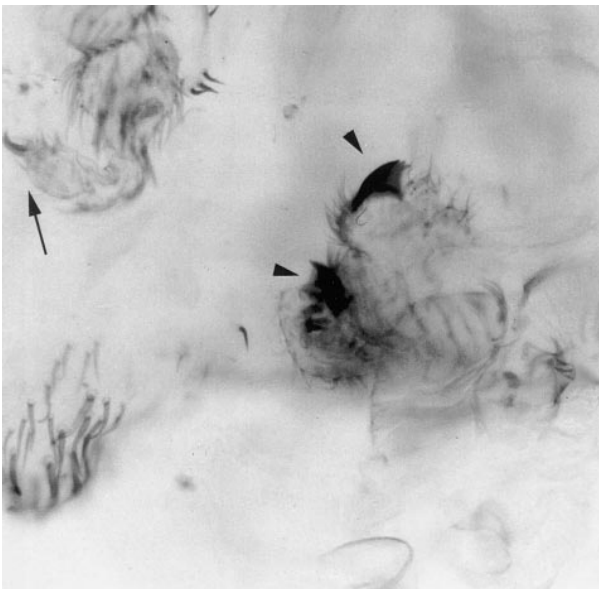

Figure 4.—EXD activity is not required for sex comb formation. Shown are pb^{27} Scr^{+} exd^{1} clones. The arrowheads indicate the sex combs in the proboscis, and the arrow indicates the arista-to-tarsus transformation in the antenna.

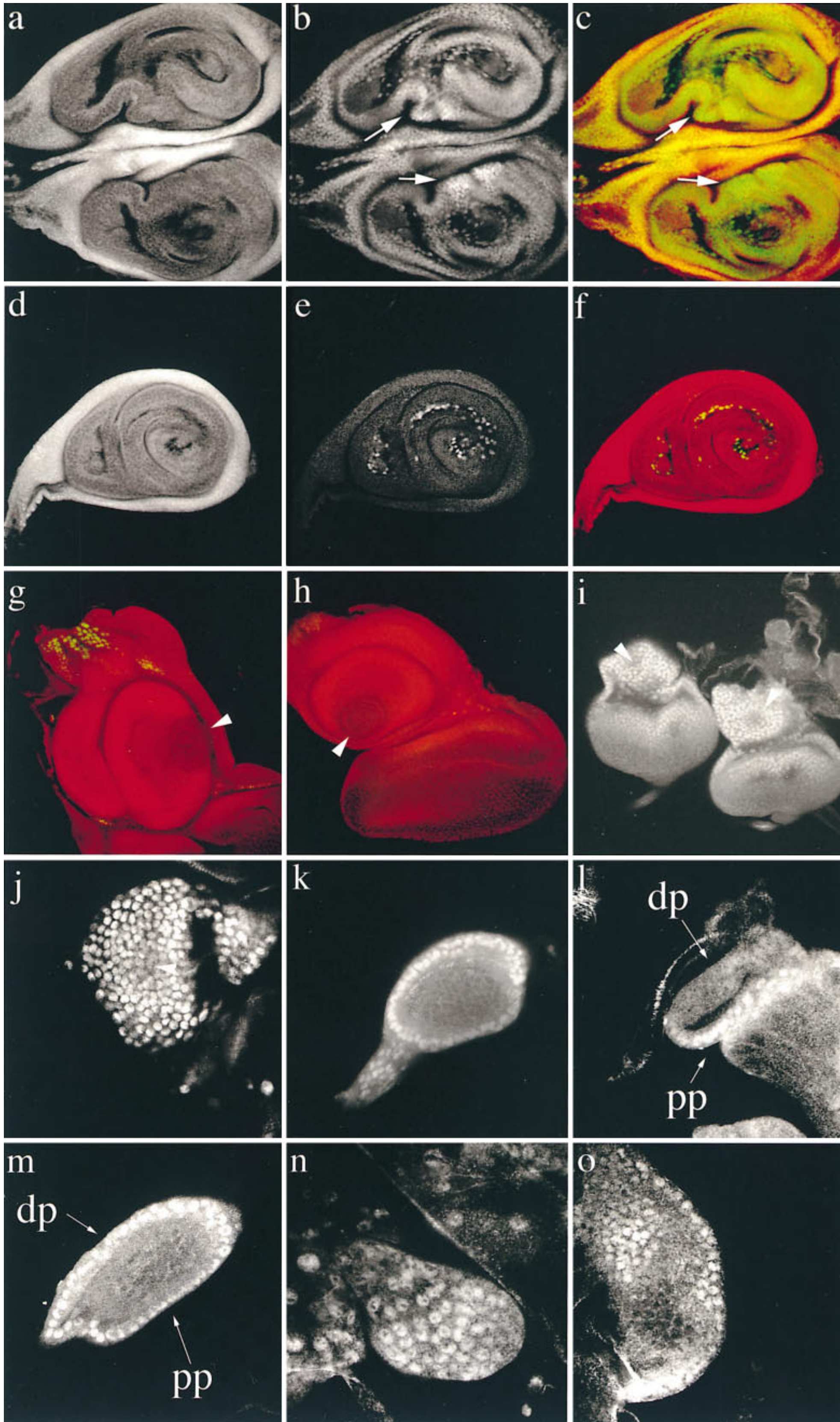

Figure 5.—EXD and SCR immunolocalization. (a–c) Late third-instar larval first leg imaginal discs; (d–f) Late third-instar larval second- or third-leg imaginal discs; (g and h) Late third-instar larval eye antennal imaginal discs; (i and j) Early third-instar larval eye antennal discs; (k–m) Early third-instar larval leg discs; (n and o) Early third-instar larval labial discs. a–h were treated with a polyclonal rabbit anti-SCR antibody and a mouse monoclonal anti-EXD antibody. i–o were treated with the rabbit polyclonal anti-EXD antibody only. The EXD signal is displayed in a, d, and i–o, the SCR signal is displayed in b and e, and both the SCR signal (green) and the EXD signal (red) are displayed in c, f, g, and h. (a–l) Discs dissected from wild-type larvae. (m) A leg disc from a heat-shocked *P{ry⁺, hsp/pb^Q50Ka}A* larva. (n) A labial disc from a pb^4/pb^{20} larva. (o) A labial disc from a pb^{27}/pb^{20} larva. Arrows show sex comb primordia (b and c) and arrowheads (g and h) show center of the antennal discs. l and m show a cross-section through a leg disc, which is required for m because ectopic expression of PB^{Q50K} balloons the leg discs. dp, disc proper; pp, peripodial membrane.

and h). This assay should have been performed at early third-instar larval stage where EXD is nuclear in the aristal primordia of wild-type discs. The region assayed for the localization of EXD in the study by Azpiazu and Morata (1998) corresponds to the first-to-third-antennal-segment primordia and is the region not transformed by ectopic expression of UBX or HOXD10.

Our model proposes that the tarsus-inducer affects

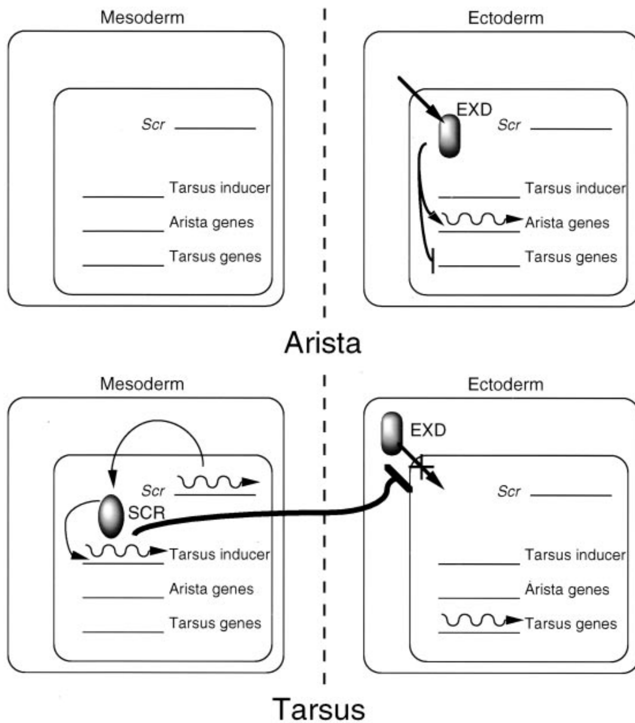

Figure 6.—Model for determination of the arista and the tarsus. The model depicts the mesodermal adepithelial cells and the ectoderm cells that will form two distal structures, the arista or the tarsus. SCR is not expressed in the nucleus of the mesodermal cells of the antennal disc, such that the hypothetical SCR-dependent, tarsus-inducing signal molecule is not expressed. Nuclear EXD activity in the overlying ectoderm is active and determines arista development. In the leg imaginal discs, expression of SCR in the mesodermal cells results in the synthesis of the secreted tarsus-inducer. This molecule acts on the overlying ectoderm cells preventing nuclear localization of EXD, thereby inhibiting its activity in determination of arista development, such that tarsal development is determined. The arrows indicate a positive interaction, and the flathead indicates a negative interaction.

the nuclear:cytoplasmic ratio of EXD localization. An effect of signaling molecules on EXD localization has been observed with Decapentaplegic (DPP) and Wingless (WG) (Mann and Abu-Shaar 1996). However, the difference in this report is that the tarsus-inducer inhibits nuclear localization of EXD; whereas, DPP and WG potentiate nuclear localization of EXD.

This model assigns very neatly a role for two proteins, SCR and EXD, in tarsus *vs.* arista determination; however, there are a number of unknown factors that must function along the pathway between the action of SCR in the adepithelial cells and the intracellular localization of EXD in the ectodermal cells. What encodes the hypothetical secreted tarsus-inducer, as well as its receptor and signal transduction pathway? We have not assayed how the recently identified activity, Homothorax (HTH) is regulated in tarsus *vs.* arista determination. HTH activity is required for EXD nuclear localization and arista determination (Casares and Mann 1998; Pai *et al.* 1998). The expression of *hth* correlates with nuclear localization of EXD protein (Rieckhof *et al.* 1997; Pai *et al.* 1998). It is presently possible that the tarsus-inducer may repress the expression of HTH protein, or may posttranslationally inhibit HTH activity. However, recent experiments characterizing how Antennapedia is required for second-leg determination favor a model where HTH expression is affected (Casares and Mann 1998). Although our study suggests a determinant model for a HOX-EXD interaction, there is a possible SCR-EXD interaction that we are unable to address in our assays. We are unable to determine whether a SCR-EXD interaction in the adepithelial cells is required for the synthesis of the tarsus-inducer. This is raised as a possibility because deletion of the region containing the YPWM motif of HOXA5 results in the inability to induce the arista-to-tarsus transformation when ectopically expressed in Drosophila (Zhao *et al.* 1993, 1996). This observation suggests that the SCR mouse homologue HOXA5 requires an interaction with EXD to determine tarsus identity.

Significance of these observations for adult pattern formation: This study makes a number of observations on the determination of adult segmental identities. In many models of the roles of HOX activities, antennal identity is proposed to be the default state (Stuart *et al.* 1991; Percival-Smith *et al.* 1997). Here we show that this default state, actively maintained by EXD activity, is affected by SCR activity suppressing EXD activity resulting in determination of tarsus identity. Interestingly, loss-of-EXD activity results in an antenna-to-second-leg transformation (González-Crespo and Morata 1995; Rauskolb *et al.* 1995), which is the proposed ground state of the Lewis model (Lewis 1978; Struhl 1982). These observations suggest that a true ground/default state does not exist, but that all identities are actively established through a series of regulatory interactions.

We have proposed previously that SCR activity was involved in the regulation of three pathways: cell nonautonomous tarsus determination, proximal leg determination, and modification of the basic leg plan conferring first-leg identity (Percival-Smith *et al.* 1997). Here we show that for determination of first-leg identity SCR does not require EXD activity. This observation explains why EXD activity can be cytoplasmically localized in the region of the sex comb primordia at the time that SCR is required for sex comb determination (Pattatucci *et al.* 1991). This suggests that HOX activities do not always require EXD activity to assign segmental identity.

Arista identity is very sensitive to ectopic expression of HOX activity. Ectopic expression of SCR, Antennapedia (ANTP), UBX, Abdominal-A (ABD-A), and Abdominal-B (ABD-B) all result in an arista-to-tarsus transformation (Schneuwly *et al.* 1987; Gibson *et al.* 1990; Mann and Hogness 1990; Casares *et al.* 1996). Only SCR, however, has been shown to be required for normal tarsus determination (Percival-Smith *et al.* 1997). Hence, it

is not readily apparent why ANTP, UBX, ABD-A, and ABD-B result in an arista-to-tarsus transformation. Ectopic expression of UBX or ANTP at late second/early third-instar larval stage, the same interval in which SCR activity is required for tarsus determination, results in this transformation. (Gibson and Gehring 1988; Mann and Hogness 1990; Percival-Smith *et al.* 1997). The driver E132 used to ectopically express UBX, ABD-A and ABD-B from *GAL4 UAS* fusion genes is ectoderm-specific (Halder *et al.* 1995; Casares *et al.* 1996). These observations raise the possibility that UBX (and potentially other HOX proteins except SCR) binds to EXD in the nucleus of ectoderm cells and inactivates EXD activity required for arista determination, such that tarsus identity is determined. This type of mechanism may also occur when HOXA5 is being ectopically expressed in Drosophila, suggesting that the mechanism of tarsus induction by SCR and HOXA5 may be distinct (Zhao *et al.* 1996).

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