quencies and estimated values for the shape parameter for among-site rate variation (α = 0.119) and the transition/transversion ratio (1.89). Bootstrap resampling support was based on 100 iterations.
22. J. D. Thompson, T. J. Gibson, F. Plewniak, F. Jeanmougin, D. G. Higgins, *Nucleic Acids Res.* **25**, 4876 (1997).
23. D. L. Swofford, *PAUP*4.0b2: Phylogenetic Analysis Using Parsimony* (Sinauer, Sunderland, MA, 1998).
24. Population genetic analyses were done with Arlequin 2.000 software (*25*): Hierarchical AMOVA for pairs of individual locales were used when both had $n > 4$; larger regional comparisons used all individuals from all sites. Kimura-2 parameter distances were used for AMOVA (with 16,000 permutations for significance tests), population pairwise F_{ST}'s (10,000 permutations), and average pairwise differences; exact tests of population differentiation based on haplotype frequencies used 100,000 steps in the Markov chain and 4000 dememorization steps (*32*). Mantel tests used 10,000 permutations for significance testing (*26*). Nucleotide sequence rate constancy was tested for the three elephant taxa with the method of Tajima in MEGA 2 (*28*, *29*). The forest-savannah elephant divergence date was calculated with the use of the ratio of between-group averages determined in MEGA 2 (*27*, *29*), using 70 individuals that had no more than two heterozygous sites in their concatenated sequence, with standard error estimated by the bootstrap method (500 replications).
25. S. Schneider, D. Roessli, L. Excoffier, *Arlequin: A Software for Population Genetics Data Analysis* (University of Geneva, Geneva, Switzerland, 2000).
26. N. Mantel, *Cancer Res.* **27**, 209 (1967).
27. M. Nei, W. H. Li, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5269 (1979).
28. F. Tajima *et al.*, *Genetics* **135**, 599 (1993).
29. S. Kumar, K. Tamura, I. Jakobsen, M. Nei, *MEGA 2: Molecular Evolutionary Genetics Analysis Program* (Pennsylvania State Univ., University Park, PA, 2000).
30. J. C. Avise, *Molecular Markers, Natural History and Evolution* (Chapman & Hall, New York, 1994).
31. W. E. Johnson, S. J. O'Brien, *J. Mol. Evol.* **44**, S98 (1997).
32. M. Raymond, F. Rousset, *Evolution* **49**, 1280 (1995).
33. D. Backhaus, *Säugetierk. Mitt.* **6**, 166 (1958).
34. E. Mayr, *Principles of Systematic Zoology* (McGraw-Hill, New York, 1969).
35. S. J. O'Brien, E. Mayr, *Science* **251**, 1187 (1991).
36. N. H. Barton, G. M. Hewitt, *Nature* **341**, 497 (1989).
37. J. F. Blumenbach, *Handbuch der Naturgeschichte* (Dieterich, Göttingen, Germany, 1797).
38. R. F. W. Barnes, *Mammal Rev.* **29**, 175 (1999).
39. S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, *J. Mol. Biol.* **215**, 403 (1990).
40. We thank E. R. Wilson, W. J. Murphy, M. P. Gough, E. Eizirik, M. J. Malasky, R. L. Hill, A. Robert, S. Cevario, A. Snyder, M. Roelke-Parker, G. Nelson, J. Sakwa, G. K. Pei, J. Brown, T. Schroyer, B. M. Gough, and K. M. Helgen. We thank A. Turkalo, J. M. Fay, R. Weladji, W. Karesh, M. Lindeque, W. Versvelt, K. Hillman Smith, F. Smith, M. Tchamba, S. Gartlan, P. Aarhaug, A. M. Austmyr, Bakari, Jibrila, J. Pelleteret, L. White, M. Habibou, M. W. Beskreo, D. Pierre, C. Tutin, M. Fernandez, R. Barnes, B. Powell, G. Doungoubé, M. Storey, M. Phillips, B. Mwasaga, and A. Mackanga-Missandzou for assistance with African elephant sample collection, and B. York and A. Baker at the Burnet Park Zoo and M. Bush at the National Zoological Park for Asian elephant samples. We thank the governments of Botswana, Cameroon, the Central African Republic, Congo (Brazzaville), Congo (Kinshasa), Gabon, Kenya, Namibia, South Africa, Tanzania, and Zimbabwe for permission to collect samples. Tissues were obtained in full compliance with specific Federal Fish and Wildlife Permits (endangered/threatened species and Convention on International Trade in Endangered Species of Wild Fauna and Flora permits US 750138 and US 756611 to N.G.). Funded by the National Institutes of Health, National Geographic Society, European Union (through the Wildlife Conservation Society), NSF, and U.S. Fish and Wildlife Service.

16 February 2001; accepted 4 July 2001

The Ground State of the Ventral Appendage in *Drosophila*

Fernando Casares* and Richard S. Mann†

In *Drosophila melanogaster*, the antennae, legs, genitalia, and analia make up a serially homologous set of ventral appendages that depend on different selector genes for their unique identities. The diversity among these structures implies that there is a common ground state that selector genes modify to generate these different appendage morphologies. Here we show that the ventral appendage that forms in the absence of selector gene activity is leglike but consists of only two segments along its proximo-distal axis: a proximal segment and a distal tarsus. These results raise the possibility that, during evolution, leglike appendages could have developed without selector gene activity.

Selector genes encode transcription factors that specify the identity of segments and appendages in insects and vertebrates (*1*, *2*). The Hox genes are a subset of selector genes that are required for generating morphological differences along the antero-posterior axis of most animals. Studies in the fruit fly, *Drosophila melanogaster*, demonstrate that altering Hox function can cause one body part to be transformed into another. Perhaps in large part because they govern the development of entire body parts, changes in how Hox genes, and selector genes in general, were used during evolution have led to modifications in animal body plans throughout the animal kingdom (*2*, *3*).

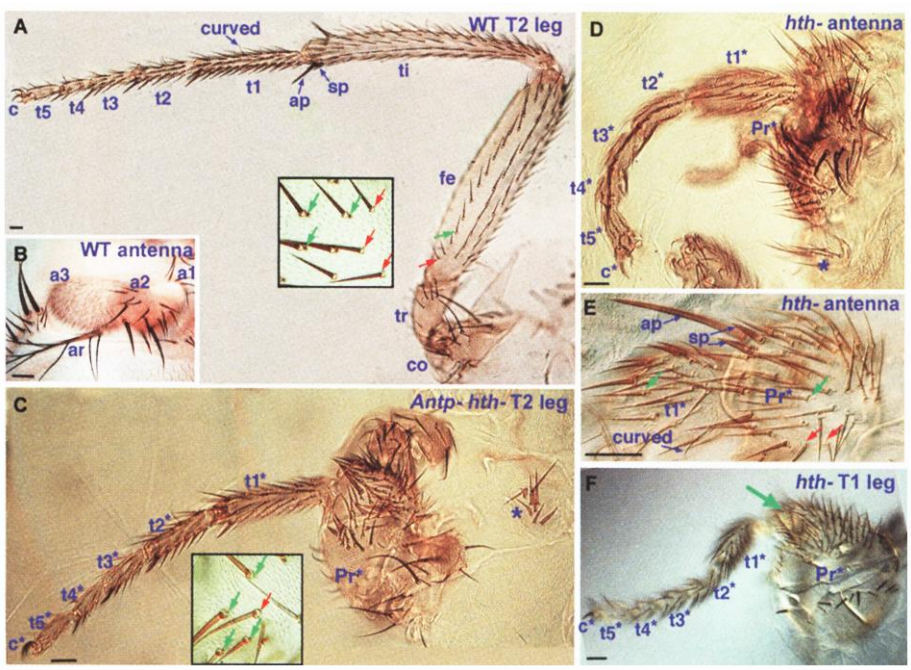

Fig. 1. The ground state ventral appendage is a leglike appendage with two segments. (**A**) A wild-type (WT) T2 leg has five segments from proximal to distal: coxa (co), trocanter (tr), femur (fe), tibia (ti), and tarsus, which is subdivided into tarsal subsegments 1 to 5 (t1 to t5) and a distal claw (c) (*15*). Five bristle types are indicated: bracted (green arrows), unbracted (red arrows), curved, spurs (sp), and apical (ap). The inset shows a closeup of the proximal femur where both bracted and unbracted bristles are present. The inset comes from a different wild-type leg. (**B**) A wild-type antenna consists of four segments, from proximal to distal: antennal segments 1 to 3 (a1 to a3) and arista (ar). (**C**) *Antp*$^-$ *hth*$^-$ T2 leg. Most of this appendage is mutant (*y*$^-$). The recovered tarsal segments (t1* to t5*) and single proximal segment (Pr*) are indicated. The inset shows a region of a similar appendage with bracted and unbracted bristles. The asterisk [also in (D)] indicates a proximal plate with unbracted bristles that is typically associated with the ground state. (**D**) An *hth*$^-$ antenna results in an indistinguishable appendage morphology as seen in (C). Most of this appendage is mutant (*y*$^-$). (**E**) A high-magnification view of part of the t1* and Pr* segments of an *hth*$^-$ *y*$^-$ antenna. The same bristle types are observed in *Antp*$^-$ *hth*$^-$ T2 legs. (**F**) An *hth*$^-$ T1 leg with proximal fusions. Transverse row bristles (arrow), which are indicative of a first leg identity, are observed.

The choice between leg and antenna development in the fruit fly is a classic example of a selector gene function. Eliminating the function of the Hox gene *Antennapedia* (*Antp*) causes the second thoracic (T2) leg to develop as antenna (*4*, *5*). Thus, one role of *Antp* in T2 leg development is to repress genes required for antennal development (*4*). Analogous leg-to-antenna transformations have been observed in the absence of *Antp* function in other arthropods, suggesting that this Hox function is ancient (*6*–*8*). We previously showed that *Antp* is a repressor of *homothorax* (*hth*) in the T2 leg and that *hth* fulfills the criteria for being an antennal selector gene: *hth* function is required for antennal development, and ectopic expression of *hth* in the analia can transform them into antennae [(*9*); see also (*10*)]. Selector genes for the analia and genitalia have also been described (*11*, *12*).

Implicit in the idea that selector genes determine the identities of ventral appendages is that there exists an underlying developmental ground state that selector genes act upon. The ground state appendage would therefore be the structure that forms in the absence of selector gene activity (*13*). What does the ground state appendage look like? What developmental pathways are modified by selector genes to generate different appendage types? And what relation, if any, does the developmental ground state have to the evolutionary predecessor of the arthropod leg?

To begin to address these questions, we studied the morphology of the ventral thoracic appendage that forms in the absence of both Hox and *hth* function. We generated mosaic animals that had large clones of tissue mutant for both *Antp* and *hth* (*14*). The appendages that formed in the T2 segment were leglike throughout their entire proximo-distal (P-D) axis (Fig. 1C). However, these legs were not wild type. Instead of having five distinct segments along the P-D axis (*15*) (Fig. 1A), these appendages had only two: a complete tarsus (with five subsegments and claw) and a single proximal segment, Pr* (Fig. 1C). Because the Pr* has features that derive from several proximal leg segments (see below), it likely results from a fusion of the four proximal-most segments of a wild-type leg.

If the two-segment appendage described here represents the ground state, a nearly identical appendage should be generated when selector genes are removed from other ventral appendages. Consistent with this prediction, when *hth* function is removed from the antenna (where no other identity selector gene is expressed) (*13*), the resulting appendage is indistinguishable from *Antp*$^-$ *hth*$^-$ T2 legs (Fig. 1, C and D) (*14*). The two-segment appendage also forms in first thoracic (T1) segments that are mutant for *Antp*, *hth*, and *Sex combs reduced* (*Scr*), the Hox gene normally required for T1 leg identity (*5*, *14*). The similarity between these structures supports the idea that it represents the developmental ground state.

As in wild-type legs, the ground state appendage exhibits polarity along its P-D axis. The bristles and trichomes usually point distally, and distinct bristle types can be observed at different positions along its P-D axis (Fig. 1, C and E) (*16*). For example, the bristles closest to the body wall usually do not have bracts (small triangular structures at the base of the bristle), whereas the more distal bristles are bracted (Fig. 1, A and C, insets). Distally in Pr* segments, there are usually (>90%) two to three spurs, a thick bristle found in distal tibia (*16*) (Fig. 1, A, C, and E). A single apical bristle, also in distal tibia of T2 legs, is observed in some (~10%) Pr* segments (Fig. 1E). Consistent with these morphological landmarks, *Distal-less* (*Dll*) and *dachshund* (*dac*), which are markers for distal and intermediate positions of the P-D axis in legs (*17*–*19*), are expressed normally in the imaginal discs that give rise to these appendages. Thus, the underlying positional information along the P-D axis is intact in the absence of selector gene function.

Our observation that simultaneously removing both *Antp* and *hth* from the T2 appendage results in a leglike structure is consistent with the idea that *Antp* functions primarily by repressing *hth*. If the only role of *Antp* is to repress *hth*, *Antp*$^-$ *hth*$^-$ legs should be identical to *hth*$^-$ legs. We tested this prediction by examining *Antp*$^+$ *hth*$^-$ legs. In T2, these legs are indistinguishable from *Antp*$^-$ *hth*$^-$ legs (*14*). In fact, removing *hth* function from the first (T1) or third (T3) thoracic legs also results in a similar overall morphology: a single proximal segment similar to Pr* and a complete tarsus (Fig. 1F)

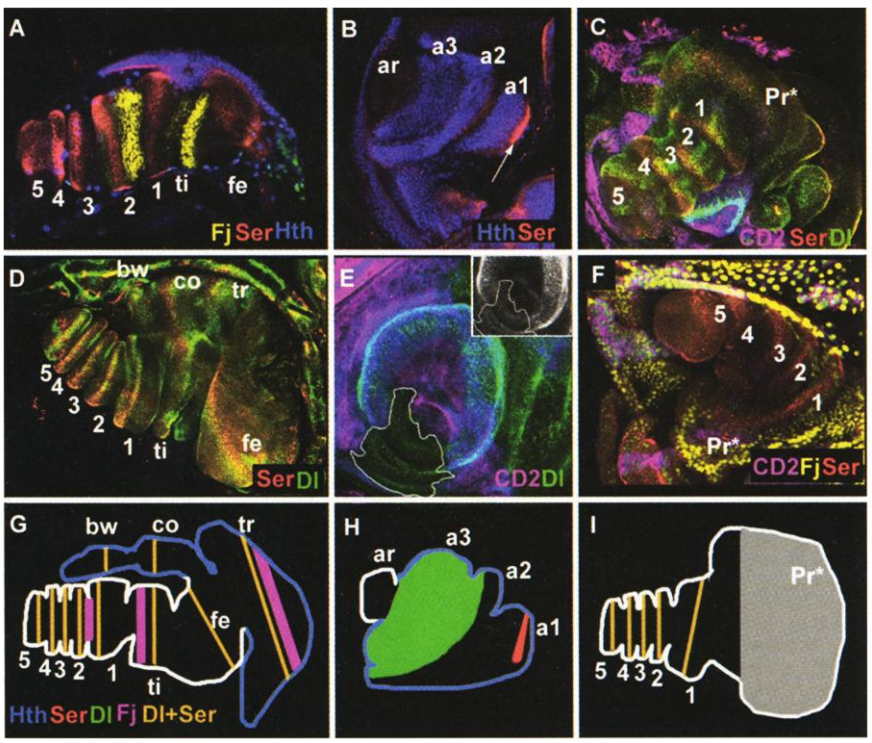

Fig. 2. Dl and Ser are expressed differentially in leg, antenna, and ground state. (**A**) Wild-type pupal leg disc stained for Ser (red), Hth (blue), and Fj (yellow). Tarsal segments are numbered and the ti and fe are indicated. (**B**) Wild-type pupal antenna stained for Ser (red) and Hth (blue). Ser is only detected in a small region of a1 (arrow). (**C**) *Antp*$^-$ *hth*$^-$ T2 leg (ground state) stained for Ser (red) and Dl (green). Absence of CD2 (purple) staining marks mutant tissue. Tarsal segments and Pr* are indicated. (**D**) Wild-type pupal leg stained for Ser (red) and Dl (green). Peak levels of Ser and Dl are seen in rings proximal to the joints that separate the leg segments: bw (body wall), co, tr, fe, ti, and tarsal segments t1 to t5. (**E**) A third instar antennal disc with a *hth*$^-$ clone (outlined with white line, marked by the absence of CD2 staining) stained for Dl (green; single channel in inset) and CD2 (purple). (**F**) *hth*$^-$ pupal antenna stained for Ser (red), Fj (yellow), and CD2 (purple). Tarsal segments and Pr* are indicated. (**G** to **I**) Schematic drawings of pupal leg (G), antenna (H), and ground state (I) summarizing the expression of Hth (blue outline), Ser, Dl, and Fj. The Pr* (I), where we observe weak expression of multiple markers, is indicated in gray.

Department of Biochemistry and Molecular Biophysics, Columbia University, 701 West 168 Street, New York, NY 10032, USA.

*Present address: Instituto de Biologia Molecular e Celular, Rua do Campo Alegre 823, Porto-4150, Portugal.

†To whom correspondence should be addressed. E-mail: rsm10@columbia.edu

(*14*). The only apparent difference between *hth*$^-$ first, second, and third legs is that these appendages retain leg type–specific bristle patterns such as transverse row bristles in T1 (Fig. 1F, arrow). This is expected because *hth*$^-$ legs still express Hox genes (in this example, *Scr*), which specify leg type–specific bristle patterns (*5*).

These results demonstrate that for ventral appendage development, *hth* is epistatic to *Antp*; that is, in the absence of *hth*, *Antp*$^+$ activity is irrelevant. This conclusion is consistent with the idea that *Antp*'s only function in the ventral appendage is to repress *hth*. However, two observations suggest that *Antp* has additional functions. The first is that the coxa of the T2 leg and first antennal segment both express *hth* but have different morphologies that depend on *Antp*$^+$ (*4*, *20*). The second is that *Antp*$^-$ *hth*$^+$ T1 or T3 legs occasionally exhibit fusions between the femur and tibia in the absence of *hth* derepression (*4*, *5*, *19*, *21*). Although these phenotypes are less severe than those observed in *hth*$^-$ legs, they suggest that *Antp*$^+$ contributes to the growth and segmentation of the proximal leg and that these functions are not mediated by repressing *hth*.

One of the most striking features of the putative ground state appendage is the absence of segmentation in Pr*. In wild-type legs, the joints that form between leg segments require the spatially restricted activation of the Notch pathway (*22*–*24*). Thus, the lack of segmentation in the ground state could be due to a difference in Notch signaling. To test this, we examined the expression patterns of Notch, *fringe* [a modifier of the Notch pathway (*25*)], and the two Notch ligands, Delta (Dl) and Serrate (Ser), in legs, antennae, and ground state appendages. No differences between the patterns of Notch or *fringe* expression were detected between these three appendage types. In contrast, the patterns of Dl and Ser expression were very different in legs, antennae, and ground state appendages. In pupal leg discs (when Dl and Ser expression along the P-D axis can be more easily visualized), peak levels of these proteins were detected in rings proximal to the joints that separate each leg segment (Fig. 2, A, D, and G) (*19*, *22*–*24*). In antennal discs, Ser was detected in a small arc of cells in the first antennal segment (Fig. 2B), and Dl was observed in a broad domain of the P-D axis, including cells that will give rise to both the third and second antennal segments (Fig. 2, E and H) (*19*). In *hth*$^-$ antennae or *Antp*$^-$ *hth*$^-$ T2 legs, coexpression of Ser and Dl was observed in the six distal-most tarsal rings (Fig. 2, C, E, F, and I) (*19*). In Pr*, Ser and Dl were detected at low levels but did not show a consistent pattern. Thus, the lack of segmentation in Pr* correlates with a lack of Dl and Ser expression in rings.

We also examined the expression of two targets of Notch signaling in the leg: *four-jointed* (*fj*) and *oddskipped* (*odd*) (*23*, *26*, *27*). *fj* is expressed in three rings along the P-D axis of legs but is only weakly expressed in the antenna (Fig. 2, A, G, and H) (*19*). In the ground state, *fj* is derepressed in most cells in the Pr* but was not detected in the tarsus (Fig. 2, F and I). In wild-type leg discs, *odd* is expressed in six concentric rings along the P-D axis (Fig. 3, A and B) (*19*). In wild-type antennal discs, *odd* is expressed in only two rings, consistent with the fewer number of joints in wild-type antennae (Fig. 3, D and E). In the ground state, the distal-most *odd* ring is recovered, and, proximally, most cells weakly express this gene (Fig. 3, C and F). These data support the idea that the activity of the Notch pathway is different among these appendages.

In addition to defining the developmental ground state for the ventral appendage, these results clarify the roles of *hth* and *Antp* in leg development. The main points can be summarized as follows (Fig. 4): (i) An apparently normal tarsus can develop in the absence of all Hox and *hth* function. (ii) In the distal and medial leg, *Antp*'s only function is to repress *hth*. (iii) In the proximal leg, *hth* is required for growth and segmentation in all three thoracic legs. (iv) *Antp* also contributes to proximal leg development where it does not function by repressing *hth*. (v) Selector genes modulate Notch signaling in the ventral appendage by altering the expression of the Notch ligands, Dl and Ser. As Notch signaling is required for both joint formation and growth of the leg, controlling Notch ligand

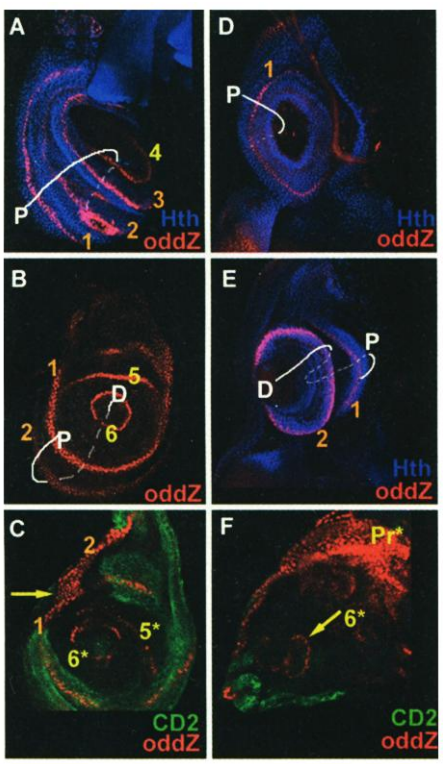

Fig. 3. The Notch target *odd* is expressed differently in leg, antenna, and ground state imaginal discs. (**A** and **B**) Wild-type leg discs stained for odd-lacZ (red); (A) is also stained for Hth (blue). Six odd rings are numbered from proximal to distal, 1 to 6, and the P-D axis is indicated. Rings 1 to 3 overlap with Hth. (**C**) A T2 leg disc with an *Scr*$^-$ *Antp*$^-$ *hth*$^-$ clone, marked by the absence of CD2 staining, stained for odd-lacZ (red). Additional odd-Z staining can be seen between rings 1 and 2 because of proximal fusions (arrow). Rings 5 and 6 are observed. (**D** and **E**) Wild-type antenna discs stained for odd-lacZ (red); (D) is also stained for Hth (blue). Two odd rings are numbered from proximal to distal and the P-D axis is indicated. (**F**) Everting *hth*$^-$ antennal disc stained for odd-lacZ (absence of CD2 marks the mutant tissue; most of this appendage is mutant). The sixth odd ring is recovered (arrow) in the ground state.

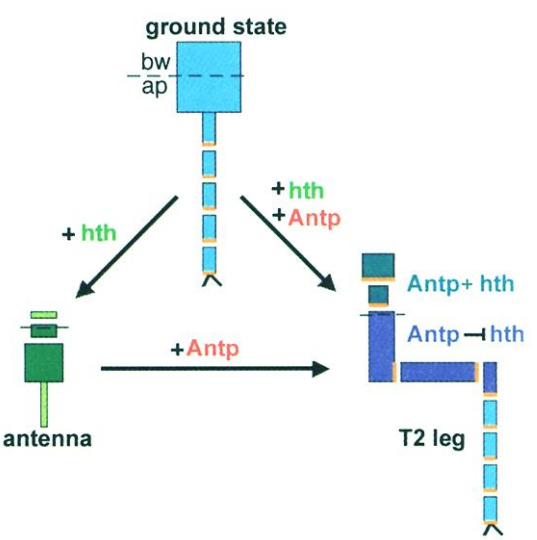

Fig. 4. The relationship between the ground state, the antenna, and the T2 leg. The ground state appendage has a single Pr* segment (light blue box) and five tarsal subsegments (smaller blue boxes). Addition of *hth* generates antennae, which have three segments and the arista (green boxes). Addition of *Antp* to the antenna results in a T2 leg, which have five tarsal subsegments and four proximal segments. The tarsus of the leg is similar to the tarsus of the ground state. In the medial leg (darker blue boxes), *Antp* is required to repress *hth*. In the proximal leg (blue-green boxes), *Antp* and *hth* are both required for its proper specification. Dl and Ser expression domains are indicated by orange bars proximal to each leg joint. Dl expression in the antenna is shown by the darker green color. The delineation between the appendage (ap), proper, and the body wall (bw) is based on the proximal limit of the Dac and Dll expression patterns and is indicated by the dashed lines.

expression provides a plausible mechanism for varying the size and number of segments among the different ventral appendages.

Finally, these results raise the possibility that there may be a relationship between the developmental ground state appendage defined here and the ancestral leg (evolutionary ground state) that predates the arthropods. Although all arthropods have highly segmented legs, it is likely that the predecessor to the arthropods had simpler, unsegmented legs (*28*, *29*). Because the developmental ground state appendage is a simple leglike appendage, it is plausible that at some time during evolution, legs developed without Hox or *hth* inputs. If nonarthropod phyla are surveyed for Hox and *hth* expression patterns, it may be possible to find an extant example of such an appendage.

References and Notes

1. R. S. Mann, G. Morata, *Annu. Rev. Cell Dev. Biol.* **16**, 243 (2000).
2. S. D. Weatherbee, S. B. Carroll, *Cell* **97**, 283 (1999).
3. G. Gellon, W. McGinnis, *Bioessays* **20**, 116 (1998).
4. G. Struhl, *Nature* **292**, 635 (1981).
5. ———, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7380 (1982).
6. R. W. Beeman, J. J. Stuart, M. S. Haas, R. E. Denell, *Dev. Biol.* **133**, 196 (1989).
7. S. Brown *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 4510 (2000).
8. C. L. Hughes, T. C. Kaufman, *Development* **127**, 3683 (2000).
9. F. Casares, R. S. Mann, *Nature* **392**, 723 (1998).
10. P. D. Dong, J. Chu, G. Panganiban, *Development* **127**, 209 (2000).
11. B. Estrada, E. Sanchez-Herrero, *Development* **128**, 331 (2001).
12. E. Moreno, G. Morata, *Nature* **400**, 873 (1999).
13. Because *Distal-less* (*Dll*) is a selector gene for all ventral appendages [N. Gorfinkiel, G. Morata, I. Guerrero, *Genes Dev.* **11**, 2259 (1997)], it is not required for differences in appendage identity. Similarly, *spinless* (*ss*) is required for both distal antennal and distal leg fates, where it is required for tarsal segmentation [D. M. Duncan, E. A. Burgess, I. Duncan, *Genes Dev.* **12**, 1290 (1998)]. Thus, *ss* and *Dll* are required for the ground state, and their activities are apparently modified by the identity selector genes.
14. Clones were generated by flp-mediated mitotic recombination: $Antp^{-}$ clones: *y w*; *FRT82B* $Antp^{Ns+RC3}$/TM6B; $Antp^{-}$ hth^{-} clones: *y w*; *FRT82B* Scr^{C1} $Antp^{Ns+RC3}$ hth^{P2}/TM2; and hth^{-} clones: *y w*; *FRT82B* hth^{P2}/TM2. Males of these genotypes were crossed to *y hs-FLP*; *FRT82B hs-CD2*, *y+ M*/TM2 females, and the progeny were heat-shocked for 30 min at 37°C, at 0 to 24, 24 to 48, 48 to 72, or 72 to 96 hours after egg laying (AEL). The Minute technique [G. Morata, P. Ripoll, *Dev. Biol.* **42**, 211 (1975)] was used to produce large clones. T1 legs require two Hox genes, *Scr* and *Antp*, whereas T2 legs only require *Antp* (5). Thus, Scr^{C1} $Antp^{Ns+RC3}$ hth^{P2} clones generated the two-segment appendage in both T1 and T2. Clones induced 0 to 24 or 24 to 48 AEL resulted in ground state appendages in the thorax. Clones induced 0 to 24, 24 to 48, 48 to 72, or 72 to 96 hours AEL resulted in ground state appendages in the head. Two-segment appendages were observed in all three legs at similar frequencies in hth^{-} clones.
15. R. E. Snodgrass, *Principles of Insect Morphology* (McGraw-Hill, New York, 1935), p. 83.
16. A. Hannah-Alava, *J. Morphol.* **103**, 281 (1958).
17. M. Abu-Shaar, R. S. Mann, *Development* **125**, 3821 (1998).
18. J. Wu, S. M. Cohen, *Development* **126**, 109 (1999).
19. Supplementary material is available on *Science* online at www.sciencemag.org/cgi/content/full/293/5534/1477/DC1.
20. W. Gehring, *Arch. Klaus-Stift. Vererb. Forsch.* **41**, 44 (1966).
21. About 20% of $Antp^{-}$ T1 or T3 legs had fusions between the femur and tibia; the remainder appeared wild type. The low penetrance of this phenotype may be due to partial redundancy with *Scr* and *Ubx*. Where fusions were observed, the leg segments were also ~50% smaller than their normal size. About 20 $Antp^{-}$ T1 or T3 leg discs were stained with an antibody to Hth, and no evidence for derepression was observed.
22. J. F. de Celis, D. M. Tyler, J. de Celis, S. J. Bray, *Development* **125**, 4617 (1998).
23. C. Rauskolb, K. D. Irvine, *Dev. Biol.* **210**, 339 (1999).
24. S. A. Bishop, T. Klein, A. M. Arias, J. P. Couso, *Development* **126**, 2993 (1999).
25. V. M. Panin, V. Papayannopoulos, R. Wilson, K. D. Irvine, *Nature* **387**, 908 (1997).
26. J. L. Villano, F. N. Katz, *Development* **121**, 2767 (1995).
27. Imaginal discs were dissected from third instar larvae or early pupae and prepared for confocal microscopy with standard methods. The antibodies used were mouse antibody to CD2 (Serotec), guinea pig antibody to Hth (*9*), rat antibody to Ser (*23*), guinea pig antibody to Dl (*22*), rabbit antibody to β-galactosidase (Cappell), mouse antibody to Dac (*17*), and mouse antibody to Dll (*28*). Fluorescein isothiocyanate, Texas Red, or Cy5-conjugated secondary antibodies were used. To identify the mutant tissue, we heat shocked larvae or pupae for 30 min at 37°C to induce CD2 expression; they were then recovered for 30 min at room temperature just before dissection. Expression of *odd*, *fng*, and *fj* was accomplished by examining *lacZ* expression from the enhancer traps *odd-lacZ* (odd^{01863}), *fng-lacZ* (*23*), and *fj-lacZ* (*26*).
28. J. K. Grenier, T. L. Garber, R. Warren, P. M. Whitington, S. Carroll, *Curr. Biol.* **7**, 547 (1997).
29. B. H. Boudreaux, *Arthropod Phylogeny, with Special Reference to Insects* (Krieger, Malabar, FL, 1987).

30. We thank T. Jessell, L. Johnston, B. Konforti, E. Laufer, G. Struhl, and members of the Mann and Struhl labs for suggestions and comments on the manuscript. We also thank J. de Celis, K. Irvine, F. Katz, G. Struhl, and the Bloomington Stock Center for fly stocks and antibodies. Supported by grants from the NIH (to R.S.M), the Human Frontier Science Program (to R.S.M. and F.C.), and the Leukemia and Lymphoma Society (to F.C.). R.S.M. is a Scholar of the Leukemia and Lymphoma Society.

15 May 2001; accepted 2 July 2001

Pollen Tube Attraction by the Synergid Cell

Tetsuya Higashiyama,[1]* Shizu Yabe,[1] Narie Sasaki,[2] Yoshiki Nishimura,[1] Shin-ya Miyagishima,[1] Haruko Kuroiwa,[1] Tsuneyoshi Kuroiwa[1]

In flowering plants, guidance of the pollen tube to the embryo sac (the haploid female gametophyte) is critical for successful fertilization. The target embryo sac may attract the pollen tube as the final step of guidance in the pistil. We show by laser cell ablation that two synergid cells adjacent to the egg cell attract the pollen tube. A single synergid cell was sufficient to generate an attraction signal, and two cells enhanced it. After fertilization, the embryo sac no longer attracts the pollen tube, despite the persistence of one synergid cell. This cessation of attraction might be involved in blocking polyspermy.

During fertilization in flowering plants, the pollen tube (the male gametophyte) grows to the embryo sac (the female gametophyte) in the pistil and delivers immotile male gametes. As with axons in the developing nervous system, directional growth of the pollen tube cell is controlled by complex interactions with the female reproductive system along its path (*1–4*). Lipids on the stigma (*5*, *6*), arabinogalactan proteins in the style (*7–9*), and adhesion of the pollen tube to surrounding tissues (*10*) all appear to participate in this control. The female sporophytic tissues alone cannot guide the pollen tube to the embryo sac within the ovule; guidance by the target embryo sac itself is required (*11–13*). In *Torenia fournieri*, the pollen tube is attracted directly to the embryo sac in vitro (*14*), suggesting that the embryo sac produces a diffusible signal. It has been proposed that it is the synergid cell adjacent to the egg cell that attracts the pollen tube, because of its location, appearance, and histochemical properties (*15–17*). Here we used the *T. fournieri* in vitro system to try to identify the cell responsible for the attraction of the pollen tube to the embryo sac.

Torenia fournieri has a naked embryo sac that protrudes from the micropyle of the ovule (*18*). In general, the embryo sacs of flowering plants are enclosed within thick layers of ovular tissues. When pollen tubes that grew through a cut style were cocultivated with ovules (Fig. 1A), some of the pollen tubes were attracted to the micropylar end of the embryo sac (Fig. 1, B and C) (*14*). Pollen tubes barely collide with the micropylar end of the embryo sac before attraction takes place (*14*). Continuous observation of the pollen tube after arrival but before entrance into the embryo sac showed that the tip

[1]Department of Biological Sciences, Graduate School of Science, University of Tokyo, Hongo, Tokyo 113–0033, Japan. [2]Department of Biology, Ochanomizu University, Tokyo 112–8610, Japan.

*To whom correspondence should be addressed. E-mail: higashi@biol.s.u-tokyo.ac.jp