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29. Transient transfection of BLS fibroblasts was carried out in situ on sterile microscope slides with a JOUAN GHT 128/A electropulser (*13*). The cells were transfected in 100 μl of 10 mM sodium phosphate (pH 7.2), 1 mM $MgCl_2$, 250 mM sucrose with 1 μg of plasmid DNA. Five pulses (100 ms, 600 V, 1 Hz, E = 1.5 kV/cm) were delivered, and 2 min later the slides were placed in complete medium and cultivated for 48 hours. Antibody binding [monoclonal antibody (mAb) 2.06] on ethanol-fixed fibroblasts was revealed with biotynilated sheep antibody to mouse Ig (Amersham) followed by avidin–fluorescein isothiocyanate (Southern Biotechnology Associates). Immunofluorescence analysis was carried out with a Leitz Orthoplan optical microscope.
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Pattern Formation and Eyespot Determination in Butterfly Wings

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Butterfly wings display pattern elements of many types and colors. To identify the molecular processes underlying the generation of these patterns, several butterfly cognates of *Drosophila* appendage patterning genes have been cloned and their expression patterns have been analyzed. Butterfly wing patterns are organized by two spatial coordinate systems. One system specifies positional information with respect to the entire wing field and is conserved between fruit flies and butterflies. A second system, superimposed on the general system and involving several of the same genes, operates within each wing subdivision to elaborate discrete pattern elements. Eyespots, which form from discrete developmental organizers, are marked by *Distal-less* gene expression. These circular pattern elements appear to be generated by a process similar to, and perhaps evolved from, proximodistal pattern formation in insect appendages.

Most of the more than 17,000 species of butterflies can be distinguished by their wing color patterns. The diversity of butterfly wing pattern elements has stimulated comparative, theoretical, and experimental study [reviewed in (*1*)]. One pattern element, the eyespot, occurs in varying sizes and numbers on the upper or lower wing surfaces and is used to confuse or warn off predators. This circular pattern of pigmentation is controlled by a patterning focus at the center (*1–3*). In *Precis coenia* (*Nymphalidae*), the best studied model species, cautery of this organizer ablates the eyespot (*2*), whereas transplantation to an ectopic site induces a new eyespot (*3*). Although the pattern-organizing properties of the eyespot focus may be related to other developmental organizers, nothing is known at the molecular level about butterfly wing pattern elements.

Flies diverged from butterflies approximately 200 million years ago and possess wings that are much smaller, have a simpler suborganization, and develop from imaginal discs with a distinct style of growth and morphogenesis. However, the fruit fly *Drosophila melanogaster* is the closest relative of butterflies for which there is detailed developmental and genetic information. Much progress has been made in the characterization of genes involved in *Drosophila* wing organization and patterning [(*4–9*), reviewed in (*10*)]; therefore we examined the potential roles of the butterfly cognates of such *Drosophila* genes in butterfly wing patterning. We cloned and examined the expression of the *Precis coenia* homologs of the *Drosophila wingless* and *decapentaplegic* signaling molecules and the *apterous*, *invected*, *scalloped*, and *Distal-less* transcription factors.

The largest *P. coenia* complementary DNAs (cDNAs) representing the *apterous* (*ap*, 3.2 kb), *scalloped* (*sd*, 3.8 kb), *wingless* (*wg*, 3.0 kb), *decapentaplegic* (*dpp*, 0.9 kb), and *invected* (*inv*, 1.5 kb) genes were isolated and analyzed. Genomic Southern blot analyses demonstrated that the cDNAs represent single copy sequences and are the closest *P. coenia* relatives, at the DNA level, of the respective *D. melanogaster* genes. Conserved regions of these cDNAs were sequenced, and the deduced amino acid translations were aligned with the *D. melanogaster* proteins (Fig. 1). Sequence conservation occurs within and outside (*11*) the *ap* homeodomains, and these residues are also present in an apparent vertebrate *ap* cognate (*12*). The *P. coenia en/inv* gene homolog contains

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residues diagnostic for the *invected* homeodomain (*13*). We have confirmed that this cDNA represents the *P. coenia inv* gene by isolating a *P. coenia en* cognate from an embryonic library (*11*). The *P. coenia wg* protein is similar to *Drosophila wg*, includes residues that are conserved between many *Wnt* genes (*14*), and is distinct from other *P. coenia Wnt* sequences. The region of the *sd* gene shown corresponds to a putative protein interaction domain (*14*), similar to the vertebrate TEFI transcription factor protein that appears to be an *sd* cognate (Fig. 1). In this region, the *P. coenia sd* and vertebrate TEFI are 91% identical, suggesting that there are strong selective constraints on this protein. The *P. coenia dpp* sequence is the closest relative to the *Drosophila dpp* gene (*16*) and is distinct from a second transforming growth factor (TGF)–β homolog we found in *P. coenia* that corresponds to the *Drosophila* 60A gene (*17*).

The *Drosophila* wing imaginal disc is divided into anterior-posterior (A-P) and dorsal-ventral (D-V) compartments by the action of the *engrailed* (Fig. 2F) (*18*) and *apterous* (Fig. 2B) (*7, 9, 19*) homeodomain-containing selector proteins, respectively. The D-V boundary of the developing *Drosophila* second instar disc subsequently acts as a patterning focus for wing formation. Dorsal (ap^+) and ventral (ap^-) cells interact (*9*) and activate the *sd* (Fig. 2H) and *vestigial* pro-wing genes (*7*), whose products are required for formation of the wing (*20, 21*). This D-V boundary also forms the wing edge, or margin, which contains distinct pattern elements and is organized by the *wingless* protein (Fig. 2D) (*5, 22*). The wing perimeter comprises the most distal cells (with respect to the wing blade) and is marked by the expression of the *Distal-less* (*Dll*) gene (Fig. 3A). The global aspects of D-V, A-P, and proximodistal (P-D) positional information revealed by gene expression in butterfly wing discs appear to be similar to those described for *Drosophila*.

Like *Drosophila*, the butterfly wing disc is divided into dorsal and ventral domains by expression of the *ap* gene. The *P. coenia ap* gene is expressed exclusively in dorsal cells of both the hindwing and forewing discs (Fig. 2A and inset) at all stages examined. This pattern is consistent with *ap* functioning in the specification of dorsal cell fate (*9*) and in the regulation of genes involved in wing formation (*7*) and D-V cell interactions (*23*). In addition, because butterfly wing color patterns often differ on their dorsal and ventral surfaces (*1*), D-V positional information may regulate color patterns.

The pattern of *wingless* gene expression also suggests that the D-V organization of the butterfly wing disc is comparable to that of *Drosophila* (Fig. 2D). As in *Drosophila*, where *wg* organizes the patterning of the wing margin (*5, 22*), fifth instar *P. coenia* wing discs express *wg* in cells along the future wing margin (Fig. 2C). The similarity of the third instar *Drosophila* and fifth instar *P. coenia wg* patterns suggests that these stages of wing development may be comparable. The *wg* gene is also expressed in proximal bands that could correspond to banding patterns on the adult forewing (Fig. 2C, arrows).

The anteroposterior organization of the *P. coenia* wing disc is reflected, as in other insect and Arthropod segments and appendages (*24, 25*), by the expression of the *en/inv* selector genes. In the forewing and hindwing discs of *P. coenia*, the *invected* gene is expressed in the posterior 60 to 70% of the disc (Fig. 2E), a slightly larger portion of the disc than in *Drosophila* (*18*) (Fig. 2F). The boundary of *invected* gene expression occurs between the tra-

Fig. 1. Deduced partial amino acid sequences of the *P. coenia apterous, invected, wingless, scalloped,* and *decapentaplegic* proteins. Partial sequences derived from cDNAs (*32*) representing each of the five *P. coenia* genes are aligned with the corresponding portion of the *Drosophila* protein. The identical residues are shaded. For *ap*, the three helices of the homeodomain are overlined. In the sequence, the homeodomain is boxed and the signature regions outside of the homeodomain are bracketed. In the *wg* sequence, widely conserved residues are underlined, and the amino acids used for the design of polymerase chain reaction primers are overlined. For *sd*, the region shown is a putative protein-protein interaction domain. In *dpp*, the conserved cleavage site is indicated (arrow). The numbering system for the *P. coenia* sequences is arbitrary and does not correspond to the beginning of the cDNA clones or to the protein sequence.

chea that will become the R2 and M1 veins in the adult forewing and hindwings (arrow, Fig. 3, E and F). This placement is more anterior than was expected from discontinuities observed in some Lepidopteran wing patterns (*1*) and does not correspond to the boundary of any *P. coenia* adult wing pattern element such as eyespots.

The overall fate map of the *Drosophila* wing disc differs from that of butterfly wing discs in that the former gives rise to the wings (distal structures) and to the body wall (proximal) structures, whereas butterfly discs give rise only to wing tissue. The expression of the butterfly *sd* gene was examined to determine whether the prowing genes are expressed within the wing proper in butterflies. The *scalloped* gene is broadly expressed throughout the butterfly wing disc, in regions including the peripheral tissue (Fig. 2G). This pattern is consistent with the function of *sd* in *Precis* being similar to that in *Drosophila* (*20*).

In spite of the absence of body wall primordia in the butterfly wing disc, we observe that the P-D organization of the wing proper is similar to that in *Drosophila*. The *Dll* homeobox gene is expressed at high levels along the *Drosophila* wing margin,

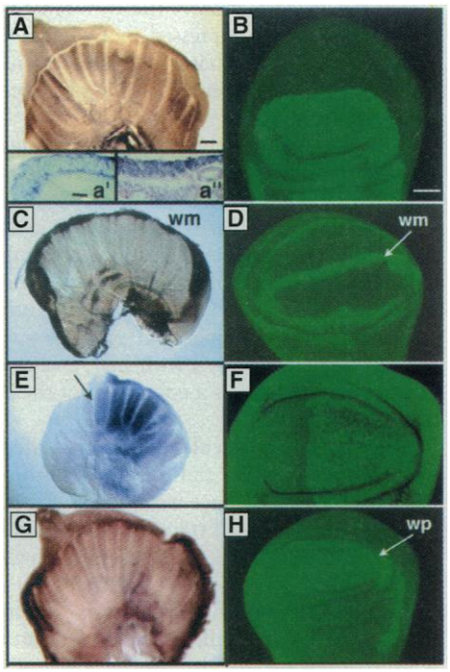

Fig. 2. Conserved features of D-V and A-P compartmentalization and wing patterning in flies and butterflies. (A), (C), (E), and (G) are fifth-insar *P. coenia* wing discs hybridized in situ with specific *P. coenia* probes, while (B), (D), (F), and (H) are *D. melanogaster* discs stained to reveal gene expression patterns as the native protein (F) or as a lacZ pattern of an enhancer trap (B, D, and H). (**A**) Dorsal view of *ap* gene expression, which is restricted to dorsal cells. The adult butterfly wing is formed by tissue proximal to the bordering lacunae (bl), which forms the adult wing D-V boundary (margin). D-V compartmentalization includes both the proximal wing primordia and the peripheral tissue. (**Inset a′**) A cross section of an in situ hybridized disc that demonstrates that (upper) dorsal but not (lower) ventral cells express *ap*. (**Inset a″**) A cross section of a disc as in (a′) counterstained with hematoxylin and eosin to reveal (lower) ventral cells. (**B**) Dorsal restriction of *ap* expression in *Drosophila* includes cells that give rise to body wall and the wing proper. (**C**) The *wg* gene is expressed along the wing margin (wm) and in two proximal bands of cells (arrows) that, on the basis of tracheal landmarks, appear to approximate the position of the outer boundaries of the proximal and distal bands of the central symmetry system of the adult forewing (Fig. 3E). The *wg* gene may function to pattern these forewing bands as well as elements at or near the wing margin. (**D**) The *wg* gene is expressed and required (*22*) along the wing margin (wm) in *Drosophila*. (**E**) Expression of the *inv* gene is restricted to the 65 to 70% posterior of the *P. coenia* hindwing with a boundary between the R_2 and M1 veins (arrow) and extends through the peripheral tissue, indicating that these cells are specified with respect to A-P position, although they die during pupation. Greater transcription in the mid-posterior section of the disc is reproducible. (**F**) Expression of the *en/inv* gene in *D. melanogaster* is restricted to the posterior compartment (*33*). (**G**) *sd* is expressed in all cells of the *P. coenia* wing. (**H**) Expression of the *sd* gene in cells that give rise to the wing proper but not to the body wall in *Drosophila*. Hybridization in situ to butterfly wing discs was performed by a modification of methods established for *Drosophila* wing discs (*34*). Scale bars: (A, C, E, and G) 100 μm; (B, D, F, and H) 50 μm; (a′ and a″) 25 μm.

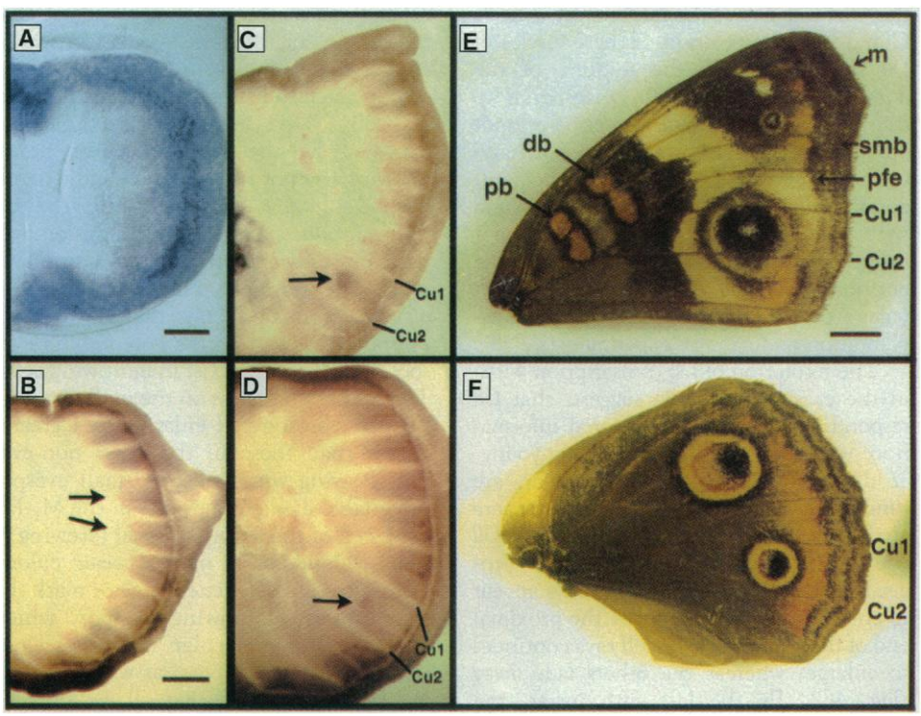

Fig. 3. Two phases of *Dll* expression in the butterfly wing: proximodistal patterning in the wing and wing cell. (**A**) The *Drosophila Dll* gene is expressed in cells along the wing margin in the everting pupal wing disc, which is the distal perimeter of the wing blade. (**B**) In *P. coenia* in the mid-fifth instar, *Dll* is expressed in the distal portion of each wing cell with a pronounced proximal boundary (arrows) and at higher levels down the midline of each wing cell to the proximal boundary. The conservation of the *Dll* pattern suggests some functional constraint on *Dll* in the wing, in spite of the absence of evidence for a genetic requirement in *Drosophila* (*29*). (**C**) In the dorsal forewing imaginal disc, *Dll* resolves into a spot of expression on the midline of the Cu_1-Cu_2 wing cell (arrow). (**D**) On the dorsal hindwing imaginal disc, *Dll* resolves into a spot of expression on the midline of the Cu_1-Cu_2 wing cell (arrow). (**E**) The adult *P. coenia* forewing exhibits two eyespots, a small anterior (in some individuals) and a large posterior element. Other notable pattern elements include the margin (m), submarginal bands (smb), parafocal elements (pfe), and the proximal (pb) and distal (db) bands of the central symmetry system. (**F**) The adult hindwing exhibits two eyespots, a large anterior and a smaller posterior element. The spots of *Dll* expression in (C) and (D) correspond to the posterior eyespots in (E) and (F), respectively. Hybridization in situ to *P. coenia* wing discs was performed as in Fig. 2. Scale bars: (A) 50 μm; (B to D) 200 μm; (E and F) 2 mm.

which corresponds to the distal-most cells of the wing proper (Fig. 3A). The *Dll* gene is also expressed in the distal portion of the butterfly wing disc with a defined proximal boundary (Fig. 3B).

Taken together, the expression pattern of these genes suggests that the butterfly and fruit fly wing fields are organized and regulated in a similar manner (Fig. 5A). Furthermore, the restriction of *apterous* expression to dorsal cells and the conservation of the *sd*, *wg* (at the margin), and *Dll* patterns, all of which require formation of the D-V boundary to be properly expressed in *Drosophila*, strengthens the view that D-V compartmentalization is a fundamental feature of insect wing organization (*7*, *9*, *10*).

The fundamental units of pattern formation in the butterfly wing are the regions bounded by wing veins and the wing margin—a region designated as the wing cells (*1*). Pattern development within each wing cell is independent of that in other wing cells. In most species, the color pattern consists of a serial repetition of the same pattern elements in each wing cell, and the overall wing pattern is a composite of these isolated fields (*1*).

Most of the molecular probes revealed discrete patterns of transcription within each wing cell that have no counterpart in *Drosophila*. Rays of *Dll* transcription extend from the presumptive wing margin down the midline of several wing cells in mid-fifth instar fore- and hindwing discs (Fig. 3B). In the Cu_1-Cu_2 wing cells of the dorsal and ventral forewing and hindwing, *Dll* transcription is resolved into a growing circle at the proximal end of the midline ray (Fig. 3, C and D). The position of these circles of *Dll* transcription in the midline of the dorsal Cu_1-Cu_2 wing cells corresponds to the position of the large posterior eyespot on the adult dorsal forewing (Fig. 3E) and to the small posterior eyespot on the adult dorsal hindwing (Fig. 3F). The position and time of appearance of the forewing *Dll* spot in the fifth larval instar corresponds to the position and time at which the eyespot focus is determined, as established by transplantation and cautery experiments (*2*, *3*).

The resolution of *Dll* transcription within the eyespot primordia suggests that *Dll* responds to a dynamic positional information system within the wing cell. In younger discs, the rays of *Dll* expression are similar in shape and intensity and terminate at the proximal boundary of the global *Dll* pattern (Fig. 4A). As development proceeds, the proximal ends of the rays appear to enlarge (Fig. 4B), but only the proximal end of the Cu_1-Cu_2 wing cell rays continues to enlarge, whereas the others fade away (Fig. 4C). By the late fifth instar, the predominant wing cell subpattern of *Dll* expression is in the position of the future eyespot (Fig. 4D), although we cannot determine what portion of the eyespot is represented by *Dll* expression. Surgical manipulations of the pupal disc suggest that the center (focus) of the eyespot consists of approximately 300 cells, whereas the *Dll* spot in the fifth instar disc consists of about 200 cells (*3*) (Fig. 4D). Because the region occupied by the adult posterior dorsal forewing eyespot is larger than and extends beyond the Cu_1-Cu_2 wing cell, the *Dll* transcription spot in the fifth instar may represent only a portion of the future eyespot. The transient enlargement of the proximal ends of *Dll* rays that do not form eyespots suggests that the potential for eyespot formation may exist in most wing cells. The *Dll* ray that arises on the midline of the M_1-M_2 forewing cell enlarges to a greater degree than those in the other non-eyespot–bearing wing cells. A small eyespot of variable diameter appears in the M_1-M_2 wing cell of the anterior dorsal forewing of some individuals in our *P. coenia* colony (Fig. 3E). The *Dll* gene does not mark the large anterior hindwing eyespot, which suggests that molecular events are not identical in all eyespots, as was suggested by the observations that all eyespots do not respond similarly to ablation or transplantation (*28*).

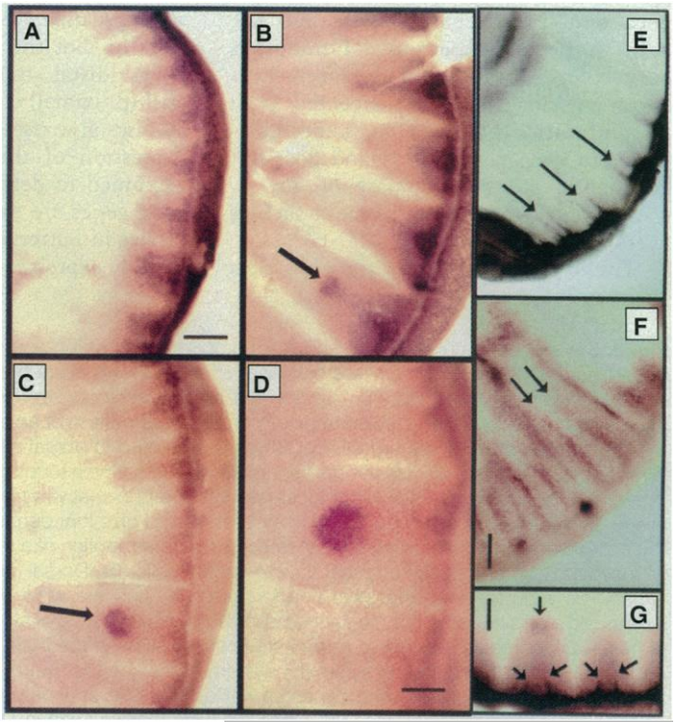

Fig. 4. The ontogeny of *Dll* expression in the eyespot primordia and molecular features of the wing cell coordinate system. (**A** to **C**) Steps in the formation of the circular *Dll* pattern on the dorsal forewing. (A) The *Dll* midline rays initially appear similar. (B) The proximal tips of the midline rays enlarge, with one (Cu_1-Cu_2, arrow) more pronounced than the others. (C) The Cu_1-Cu_2 spot (arrow) continues to enlarge. (**D**) The *Dll* spot in (C) comprises about 200 cells. (**E**) Expression of the *wg* gene in two parallel rays (arrows) that emanate from the wing margin and extend up to about 12 cell diameters proximally into each wing cell. These rays are parallel to and well-spaced from the trachea. (**F**) The *dpp* gene expressed in two rays (arrows) that extend up to approximately 30 to 40 cells proximally into each wing cell. These rays appear to form between the trachea and *wg* rays in (A). (**G**) The relation between *wg* and *Dll* expression in the Cu_1-Cu_2 wing cell revealed by simultaneous in situ hybridization with *wg* and *Dll* probes. The *wg* pattern is restricted to the distal end of the wing cell (bottom arrows) and terminates about 30 cell diameters from the *Dll* spot (top arrow) shown here. Hybridization in situ to *P. coenia* wing discs was performed as in Fig. 2. Scale bars: (A to C and E to G) 100 μm; (D) 50 μm.

In addition to the *Dll* rays and spots, other evidence suggests that a coordinate system operates within each wing cell. For example, *wg* is transcribed in two thin parallel rays that extend proximally into each wing cell from the wing margin and are well-spaced from the trachea (Fig. 4E). The *dpp* gene is also expressed in rays extending proximally from the wing margin, which appear to form just outside of the *wg* rays (Fig. 4F) and extend more proximally in from the wing margin than do the *wg* rays (Fig. 4G). Because *wg* and *dpp* are secreted growth factors and play a role in *Dll* regulation in *Drosophila* embryonic limb primordia (*27*, *28*), one or both proteins may be involved in the formation of the midline ray of *Dll*. However, only *dpp* appears to be expressed closely enough to the *Dll* spot to play a role in its formation. The *wg* rays that emanate from the margin may be involved in the formation of pattern elements along or parallel to the margin, such as the parafocal elements or submarginal bands (Fig. 3E).

These detailed gene expression patterns, including venous stripes (*sd*) (*11*), intervenous rays (*wg*, *dpp*, and *Dll*), spots (*Dll*), and chevrons (*ap*) (*11*), appear to reflect a specialized coordinate system operating within each wing cell that creates

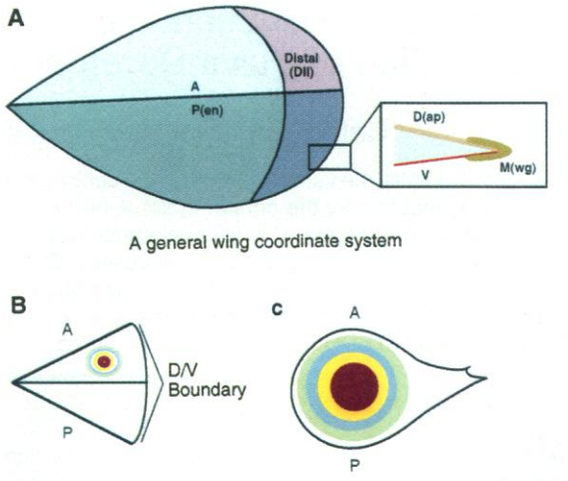

Fig. 5. The global wing and wing cell coordinate systems, and eyespots as proximodistal pattern elements. (**A**) The global wing coordinate system. The insect wing consists of two cell layers, dorsal and ventral, that are directly apposed and sculpted at the margin (M). In *Drosophila*, the wing region is organized at the boundary between cells of dorsal and ventral fate in the growing imaginal disc (*9*). This boundary is demarcated by the expression of the dorsal *ap* (**inset**, gold) homeodomain protein and in mature discs by the expression of the *wg* (**inset**, *wg* at the margin) protein, whereas the entire wing region expresses the *vg* and *sd* (light blue background in wing) regulatory proteins (*7*). Anterior-posterior compartmentalization is demarcated by *en* (turquoise) expression in the posterior compartment, while the P-D organization of the wing proper is reflected by *Dll* (light pink and blue-gray) expression in distal-most cells. These relations hold in the larger and morphologically divergent imaginal wing discs of the Lepidopteran *P. coenia*, which suggests that the function and regulation of the *ap, wg, sd, en*, and *Dll* genes in wing patterning have been conserved. (**B**) An eyespot represented as concentric circles of pigmentation surrounding a focus (clear spot). The expression of *Dll* is within the primordia of the eyespot. (**C**) The circular organization of the third instar *Drosophila* leg imaginal disc represented as concentric rings of cells. The centermost cells are fated to form the most distal structures and express the *Dll* gene.

the wing pattern elements. The rays of *wg* and *dpp* expression indicate that positional information is organized parallel to the wing veins (A-P coordinates), whereas the proximal boundaries of *Dll*, *dpp*, and *wg* expression indicate that positional information is also organized along the P-D dimension. With the exception of the eyespot, no obvious pattern elements on the adult *P. coenia* wing correspond directly to the *wg* and *dpp* rays or to the proximal boundary of *Dll* expression. However, intervenous stripes, venous stripes, chevrons, and proximodistally organized pigmentation zones do appear in numerous butterfly species (*1*). The coordinate system reflected by the various gene expression patterns may be conserved among butterflies and represent a common patterning landscape to which downstream genes (such as those involved in scale formation and pigmentation) respond differently in different species. With molecular probes for particular Lepidopteran gene products now in hand, the conservation or divergence of wing cell patterning between butterfly species may now be addressed.

The *Dll* circular transcription pattern corresponds to the position of *P. coenia* adult eyespots, forms at a time when the eyespot focus (center) has been determined (*3*), and is the first molecular evidence for distinct gene activity in the eyespot field. The *Dll* homeobox gene is involved in pattern formation (*29, 30*) and is the only regulatory gene known to be expressed in a circular pattern in the butterfly wing. Therefore, it is reasonable to postulate a special role for *Dll*, on the basis of known features of *Dll* regulation and function in *Drosophila* [reviewed in (*10*) and (*31*)] in the elaboration of circular patterns in the wing cell. Expression of the *Dll* gene in *Drosophila* embryos (*27, 28*) is related to the formation of the limb primordia and is required for elaboration of the proximodistal axis within the cephalic and ventral thoracic appendages (*29*). The *Dll* gene is activated in a circular pattern within embryonic segments (*28*) and in the growing imaginal discs (Fig. 5C).

Indeed, the eyespot may be a proximodistal element superimposed on the two-dimensional wing surface. That is, the center of the eyespot (the focus) may represent the distal-most positional value (and express *Dll*), and the surrounding rings may represent progressively more proximal positions (Fig. 5B) in a manner analogous to the organization of the *Drosophila* leg imaginal disc (Fig. 5C). In the eyespot field, cell interactions and diffusible factors are constrained to a single cell layer (*1*), and the discrete P-D positional values that are created may be read out as concentric rings of pigmentation. In the imaginal limb disc, the P-D axis is elaborated by growth and morphogenesis of the different limb segments. In both limbs and eyespots, the generation of circular pattern elements and the elaboration of proximodistal positional values from a two-dimensional coordinate system may result from combinations of secreted growth factors (for example, *wg* and *dpp*) operating in one plane and the patterned activation of a regulatory gene (for example, *Dll*) along a new patterning axis. This general principle has also been underscored in comparisons of the outgrowth of *Drosophila* appendages with the elaboration of the *Xenopus* body axis by the Spemann organizer (*9*). The patterning of butterfly wings may then be considered in the same context as these better studied experimental models. The identification of specific proteins that may be involved in the formation or elaboration (or both) of the eyespot suggests that some direct experimental tests of the eyespot determination mechanism and the diversity of butterfly wing patterns may now be addressed at the molecular level.

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32. Libraries of *P. coenia* cDNA were constructed from polyadenylated mRNA isolated from embryos and wing discs. Clones from cDNA for *ap, dpp,* and *sd* were isolated from the embryo library, and *en/inv* clones were isolated from the disc library by cross-hybridization [D. Klessig and J. O. Berry, *Plant Mol. Biol. Rep.* **1**, 12 (1983)] with *Drosophila* probes, whereas a segment of the *wg (wnt-1)* gene was amplified by polymerase chain reaction (PCR) from genomic DNA [A. Sidow, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5098 (1992)], and the resulting 450-bp fragment was used to probe the embryonic cDNA library. The preparation of DNA and subcloning were performed as by J. Sambrook, E. F. Fritsch, and T. Maniatis [*Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, ed. 2, 1989)]. Clones from cDNA were sequenced by the method of F. Sanger, S. Nicklen, and A. R. Coulson [*Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463 (1977)].
33. Photo courtesy of S. Blair.
34. The *P. coenia* butterflies were maintained at 28°C under a 16L:8D photoperiod, and larvae were fed an artificial diet containing *Plantago lanceolata* [R. T. Yamamoto, *J. Econ. Entomol.* **62**I, I427 (1993)]. Fifth-instar larvae were anaesthetized by submersion in distilled water for 30 min, and the imaginal discs were removed under cold phosphate-buffered saline. The preparation of the imaginal discs for in situ hybridization was adapted from J. D. Masucci, R. J. Miltenberger, and F. M. Hoffmann [*Genes Dev.* **4**, 2011 (1990)], the important distinction being the proteinase K treatment. For DNA probes, the discs were treated from 3 to 4 min in proteinase K (100 μg/ml), while for RNA probes, proteinase K (50 μg/ml) was used for 2 to 3 min. Discs were hybridized with 40 ng of digoxigenin-labeled DNA probe (*inv*) or 20 to 50 ng of digoxigenin-labeled RNA probe [J. Jiang, D. Kosman, T. Ip, M. Levine, *Genes Dev.* **5**, 1881 (1991)] in 100 μl of hybridization buffer for 24 hours at 48°C (DNA) or 55°C (RNA). The detection and colorimetric development of digoxigenin-labeled staining was performed as described [J. D. Masucci, R. J. Miltenberger, F. M. Hoffmann (above)] except for the omission of Levamisol. Detailed protocols are available upon request. Localization of *D. melanogaster* gene expression patterns are as follows: The detection of *wg, ap,* and *sd* enhancer trap *lacZ* expression was performed as described (*5*), while *en/inv* gene expression was detected with a monoclonal antibody that recognized both proteins (*25*).
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Changes of Induction and Competence During the Evolution of Vulva Development in Nematodes

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In *Caenorhabditis*, the vulva is formed in the central body region from three of six equivalent cells and is induced by the gonad. In some nematodes, however, the vulva is located in the posterior body region. Vulval development has been analyzed in three such genera. The same precursor cells give rise to the vulva in *Caenorhabditis* and in the posterior vulva species, but in the latter the cells first migrate posteriorly. In two such species, the vulva is not induced by the gonad, but instead relies on intrinsic properties of precursor cells. Thus, evolution of organ position involves changes in induction and competence.

Morphological change during evolution arises from modification of ontogeny, which implies that an understanding of morphologic evolution will require insight into the evolution of development. To elucidate how developmental processes evolve, it is necessary to have detailed knowledge at the cellular and genetic level of a particular aspect of development, as well as the ability to compare the development of a number of species. The invariant development of free-living nematodes provides a useful experimental system for such an analysis at the single cell level. Formation of the vulva, the egg-laying structure of nematodes, is one tractable aspect of development for an evolutionary developmental analysis.

The vulva of *Caenorhabditis elegans* is a derivative of the ventral epidermis, which consists of 12 ectoblasts (Fig. 1A) (*1*). These 12 cells are equally distributed between the pharynx and anus and are named according to their anterior-posterior position (P1–P12). The gonadal anchor cell (AC) induces three of six vulval precursor cells (VPCs) to adopt vulval fates (*2*). The VPCs (P3–8.p) are descendants of the central ectoblasts. They form a so-called equivalence group because all cells have the potential to adopt vulval fates. During wild-type development, only the three cells centered around the AC, P(5–7).p, respond to the inductive signal by generating vulval cells. The cell closest to the AC, P6.p, has the 1° cell fate and generates eight progeny, whereas the two more distal cells, P5.p and P7.p, have the 2° cell fate, and each generates seven progeny. The three remaining VPCs (P3.p, P4.p, and P8.p) do not contribute to formation of the vulva; instead, they generate nonspecialized epidermis, the 3° fate. The VPCs that normally give rise to 3° cells can make 1° or 2° cells and thus regenerate the vulval pattern after ablation with laser microbeam irradiation of more AC-proximal VPCs (*3*). Extensive genetic and molecular analysis indicates that three intercellular signals—an inductive signal, a lateral signal, and a negative signal—are involved in the specification of the precise pattern of VPC types (*4*).

In *Caenorhabditis* and in most other nematode species, the vulva forms at approximately 50% body length (*5*). However, species with the vulva in the posterior body region are present in many nematode taxa (*5, 6*). The family *Rhabditidae* contains several evolutionary lines with posterior vulva–forming species (*7*). Here, we have used three different genera of the subfamily *Rhabditinae* to study vulva development in species that form a posterior vulva (*8*). In *Cruznema tripartitum* and *Mesorhabditis* sp. PS1179, the vulva forms at 80% body length; in *Teratorhabditis palmarum*, the vulva forms at 95% body length, in a position immediately anterior to the anus (Figs. 1 and 2).

In *Caenorhabditis* and in other members of the family *Rhabditidae* with the vulva in the central body region, P(5–7).p generate the vulva (*1, 9*). In *Panagrellus redivivus* of the distinct family *Panagrolaimidae*, in which the vulva forms at 60% body length, P(4–9).p have the potential to generate vulval tissue (*10*). Because a more posterior cell, P9.p, has the potential to participate in vulva formation in *Panagrellus*, it might be expected that the more posterior Pn.p cells would form the vulva in species with posterior vulvae. However, our cell lineage analysis revealed that the central Pn.p cells form vulval tissue in all three species examined (Fig. 1) (*11*). In *Mesorhabditis* and *Teratorhabditis* P(4–8).p are VPCs; in *Cruznema* P(3–8).p are VPCs. In the first larval stage (L1) of all three species, the 12 Pn.p ectoblasts are located in order along the anterior-posterior axis as they are in *Caenorhabditis* (Figs. 1 and 2A). The VPCs migrate posteriorly during the second larval stage (L2), adopting a species-specific position. In *Cruznema*, P(3–8).p lie anterior to P(9–11).p (Fig. 1B). In *Mesorhabditis*, the VPCs lie in the same region as P(9,10).p (Fig. 1C). In *Teratorhabditis*, the VPCs move posterior to P(9–11).p (Fig. 1D) (*12*). P(5–7).p have vulval cell fates in all three species, but

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