(pH 7.8), 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 3% Ficoll, and polydeoxyinosinic-deoxycytidylic acid [poly(dI-dC)] (100 μg/ml). Protein and oligomers were added sequentially at 5-min intervals, incubated for 5 to 10 min at room temperature, and electrophoresed through a 5% nondenaturing polyacrylamide gel in 1× tris-borate EDTA at 200 V. Single-strand oligomers were heat-denatured and snap-cooled on ice to minimize the formation of secondary structures. In competition assays, labeled oligomers were mixed with the indicated molar excesses of unlabeled competitor before heat denaturation. Duplex oligomers were annealed in 50 mM NaCl by heating to 95°C for 2 min and cooling to ambient temperature over a 60-min interval before the binding assay; results for K_A assays were independent of which strand was labeled. In K_A assays, reactions were incubated on ice for 30 to 60 min and gels were run at 4°C; reactions for substrates 1 to 9 did not contain poly(dI-dC). Protein concentration was determined by amino acid analysis and by quantitative comparison to bovine serum albumin standards using SDS–polyacrylamide gel electrophoresis (PAGE) followed by Coomassie staining, which gave comparable results (within 15%). Coomassie gels and gel shifts were quantitated with a scanning densitometer or PhosphorImager, respectively, and the Imagequant software package (Molecular Dynamics).

32. R. J. Wellinger, A. J. Wolf, V. A. Zakian, *Cell* **72**, 51 (1993); R. J. Wellinger, K. Ethier, P. Labrecque, V. A. Zakian, *ibid.* **85**, 423 (1996).

33. We thank H. Zentgraf for monoclonal antibody 13/45/31, A. Salinger and K. Shah for technical assistance, and S. Elledge and S. Plon for critical review of the manuscript. Supported by grants from NIH and Geron Corporation and by an American Cancer Society Junior Faculty Research Award to V.L. T.R.H. is supported by an NIH predoctoral fellowship.

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Visualization of Gene Expression in Living Adult *Drosophila*

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To identify genes involved in the patterning of adult structures, Gal4-UAS (upstream activating site) technology was used to visualize patterns of gene expression directly in living flies. A large number of Gal4 insertion lines were generated and their expression patterns were studied. In addition to identifying several characterized developmental genes, the approach revealed previously unsuspected genetic subdivisions of the thorax, which may control the disposition of pattern elements. The boundary between two of these domains coincides with localized expression of the signaling molecule wingless.

The adult structures of *Drosophila* are subdivided into compartments in which patterning and growth are controlled by the heritable activities of selector genes (*1*) and by signals generated as a result of interactions between cells across compartment boundaries (*2*). However, there are few compartments; each segment is subdivided into anterior and posterior compartments, and only thoracic segments are further subdivided into dorsal and ventral and possibly trunk and appendage compartments (*3*). These compartments define large domains, which ultimately are composed of thousands of cells; little is known about how they are further subdivided genetically. Adult structures are differentiated by "imaginal" cells, which proliferate after the larval patterns are completed (*4*). Whereas a great deal is known about the genetic cascade [see (*5*) for an overall description] leading to generation of larval patterns, much less is known about the genetics of pattern formation in the adult, although progress recently has been made concerning how appendages develop (*2, 6*). Some genes are used during both embryogenesis and imaginal development, but others such as *vestigial* (*vg*), *apterous* (*ap*), *nubbin* (*nub*), *Dorsal wing* (*Dlw*), and *fringe* (*frg*) have their primary role in the adult (*7*), indicating that adult patterns have their own specificity.

To search for specific adult pattern genes, we modified the Gal4 enhancer trap method (*8*) so that we can observe the consequences of Gal4 activity directly in living flies. Our approach uses an upstream activating site (UAS) construct (*9*) containing the cDNA of the *yellow* (y) gene. The gene y is responsible for the normal pigmentation of adult cuticle and bristles all over the body. Flies mutant for *yellow* (y^-) are of yellow color and are clearly distinct from the wild type. The y^- mutations traditionally have been used as gratuitous markers in genetic mosaic experiments and do not affect viability or fertility. Flies that express Gal4 will express the y^+ product under control of the UAS. When expressed in a y^- fly, the body domain in which the y^+ gene is turned on by Gal4 will appear as a patch of y^+ territory on a y^- background, and this y^+ tissue will be detectable under the dissecting microscope.

To determine the developmental period in which the Gal4 product has to be present to produce detectable y^+ rescue, we used a *hsp70-GAL4* construct (*10*) to induce controlled synthesis of the Gal4 product. Single 90-min pulses were given to different batches of *hsp70-GAL4/UAS-y* larvae or pupae grouped according to age with respect to the time of puparium formation, and the emerging adults were inspected for y^+ rescue. Heat shocks given at or after the second half of the third larval period are able to produce y^+ rescue. All genes known to be involved in the patterning of adult structures are expressed at the end of the third larval instar (*2, 6, 7*). Thus, y^+ expression under Gal4 control should be able to detect gene expression not only during late pupal stages when the adult cuticle is being formed but also much earlier when patterning genes are active. Our finding of insertions in several known developmental genes (see below) supports this notion.

We mobilized the transposon *pGawB* (*11*), a Gal4-containing *P* element (*8*), to produce lines that express Gal4 in a manner that reflects the expression pattern of the genes where the transposon inserted. Each insertion was tested indiscriminately in a cross with the *UAS-y* gene construct. Of 1020 insertions, 447 (44%) gave partial or total y^+ rescue in adult flies (see Table 1 for details and different classes of expression). However, the majority of the lines failing to give y^+ rescue correspond to insertions in which the *GAL4* gene is inactive, as more than 90% of them (56 from a sample of 59) also fail to show any embryonic expression when tested with a *UAS-lacZ* construct (*8, 12*). Considering only those lines in which the *pGawB* is active, about 90% of them produce detectable y^+ rescue in adult flies. The implication is that the majority of the *Drosophila* genes are expressed in imaginal cells and are detectable by our method.

We are interested in the lines with y^+ rescue that is restricted to specific and well-delimited body regions (Table 1). These lines contain insertions in genes that may be involved in developmental processes specific to the structures in which they are expressed. Moreover, these lines can provide information about how adult patterns are genetically subdivided. An additional technical advantage of the screen is that it provides a collection of Gal4 driver lines that express Gal4 strongly enough to yield detectable adult expression and therefore are very useful in gene-targeting experiments.

We isolated 27 lines that express y^+ in a region-specific manner. Nine of them were later found to be insertions in known developmental genes (*13*): *Distal-less* (*Dll*), *caudal* (*cad*), *apterous* (*ap*), *optomotor-blind*

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(*omb*), *pannier* (*pnr*), and *teashirt* (*tsh*). The y^+ rescue in *Dll-GAL4* and *cad-GAL4* is illustrated (Fig. 1, A to D). In all the insertions in characterized genes, we compared their expression with *UAS-y* and *UAS-lacZ* with that revealed for these genes by antibody or in situ hybridization experiments. With the partial exception of one of the *cad* lines, which reflected only late zygotic *cad* expression (Fig. 1D), y^+ and *lacZ* expression accurately reflect the overall expression patterns of those genes. Thus, we are confident that in most cases our screen reports genuine gene expression patterns. The 18 remaining lines appear to define previously uncharacterized genes because they yield unreported expression patterns. These lines show specific rescue in wings and halteres, in some antennal segments, and in some

Table 1. Number and class distribution of the *GAL4* insertion lines. Each insertion was tested for y^+ rescue with the *UAS-y* construct. Only 44% of the lines give adult rescue, but we estimate that only 5% (3 out of a sample of 59) of the 573 nonrescue lines show embryonic expression. This result indicates that in most lines the *pGawB* transposon does not respond to local enhancers; only 28 to 30 of them would give a specific pattern; adding these to the 447 that give y^+ rescue amounts to 47% (477/1020) of *GAL4* insertions responding to local enhancers. This is likely to be an underestimate because it does not take into account the possible cases of specific expression in internal adult tissues. This number is somewhat lower than the 61% reported in a previous estimate (*8*). About one-third of the lines give complete rescue; that is, we observe y^+ rescue in the entire body. The strength of the rescue is variable, reflecting the particular level of activity of the resident gene; some lines are darker than normal y^+ flies, whereas in others the pigmentation is intermediate between y^+ and y^-. A high proportion of lines give apparent specificity for bristle rescue, and in at least some cases it appears to reflect specific functions; two of these insertions turned out to be mutant for the gene *javelin*, which affects only bristles (*26*). However, this apparent specificity has to be regarded with caution because in our experience y^+ rescue is easier to detect in bristles than in cuticle. In 12% of the lines only the cuticle was clearly y^+ and the bristles were y^-. No further study of these lines was carried out, but we have observed that the *1J3* line, an insertion in the *hairy* (*h*) locus (*8*), belongs to this class. Because *h* prevents bristle differentiation by acting as a negative regulator of AS-C function (*27*), our finding of its restriction to nonbristle cells in the epidermis is consistent with *h* function. About one-fourth of the lines give heterogeneous rescue with y^+ patches distributed in haphazard fashion in different body regions, making it very difficult to draw a coherent image of the y^+ rescue. Finally, the category of region-specific lines includes insertions in the *Dll*, *pnr*, *tsh*, *ap*, *cad*, and *omb* genes as well as lines defining additional expression patterns such as *em462* and others that yield y^+ rescue in wings and halteres, some antennal segments, and some parts of the abdomen (*13*).

Total number of insertions tested	1020
Number of insertions showing y^+ rescue	447
Complete rescue	143 (32%)
Bristles y^+, cuticle y^-	113 (25%)
Cuticle y^+, bristles y^-	54 (12%)
Heterogeneous rescue	110 (25%)
Body-region–specific rescue	27 (6%)

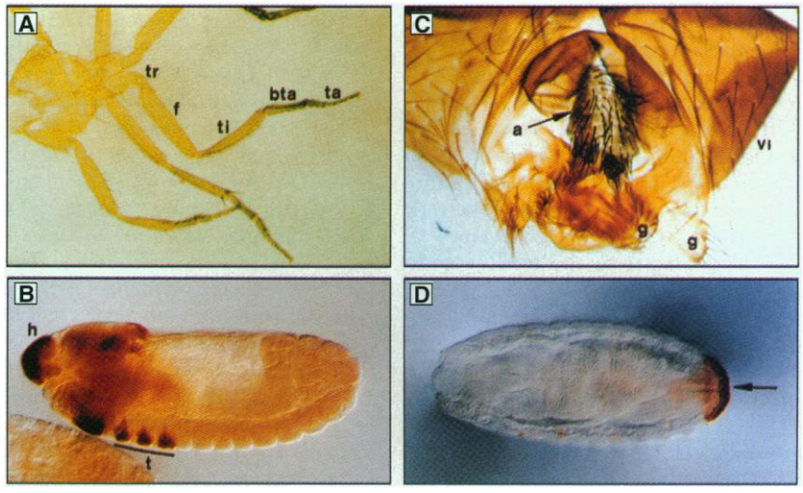
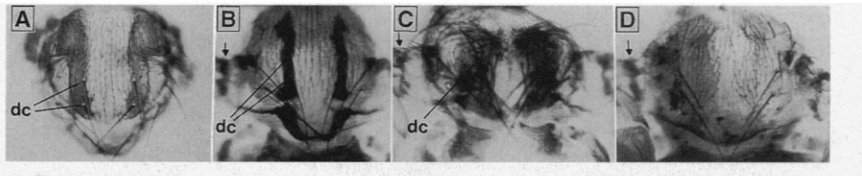


Fig. 1. Two *GAL4* insertions in known developmental genes *Dll* and *cad*. (**A**) Adult legs of genotype *md23/UAS-y* showing y^+ rescue in the tibia (ti), basitarsus (bta), and tarsal (ta) segments but not in more proximal regions such as femur (f) or trochanter (tr), except for a small region at the trochanter-femur joint (not visible at this magnification). The *md23* line is an insertion in the *Dll* locus and is also mutant for *Dll*. *md23/UAS-y* flies also show y^+ rescue along the dorsoventral boundary in the wing and in the distal antennal segments (magnification ×15). (**B**) *lacZ* expression in embryos of the same genotype. Expression in the head (h) and thoracic (t) segments coincides with that described for the *Dll* gene (*4*). We found three insertions in the *Dll* locus (*13*) (magnification ×100). (**C**) Posterior abdominal segments and terminalia of an *em459/UAS-y* male. *em459* is an insertion in the *cad* locus. The VI abdominal segment and genitalia (g) are y^- but the analia (a) is entirely y^+. The rest of the body is y^- (magnification ×60). (**D**) *em459/UAS-lacZ* embryo after retraction of the germ band showing (arrow) characteristic late embryonic expression of *cad* in parasegment 15 (*25*). We found two insertions in the *cad* locus (*13*) (magnification ×100).



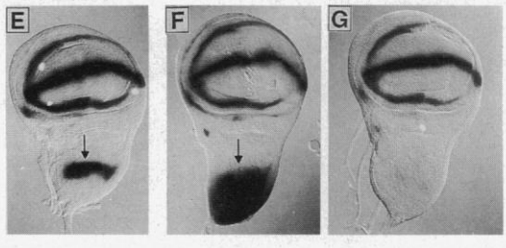


Fig. 2. Relations between *em462* and *pnr* with *wg*. (**A**) Visualization of *em462* expression in a pharate *em462/UAS-lacZ* adult stained with X-Gal. The medial boundary of expression runs close to the dorsocentral (dc) bristles and occupies a mediolateral region but does not reach the more lateral region, which is mainly out of focus. (**B**) Normal *wg* expression as indicated by X-Gal staining of a pharate adult carrying the *wg-lacZ* insertion. The β-galactosidase (β-Gal) product accumulates on a longitudinal stripe defined on one side by the dc bristles. Note that the *wg* domain is included within the *em462* domain shown in (A), but it is smaller. There is also a band of expression in the wing hinge (arrow). (**C**) X-Gal staining of a notum of genotype *wg-lacZ*; pnr^{D1}/pnr^{md237}. There is marked expansion of the *wg* stripe accompanied by an increase in the number of dc bristles. (**D**) X-Gal staining of *wg* expression of the notum of an adult of genotype *wg-lacZ*; pnr^{VX1}/pnr^{VX1} mutant notum. The longitudinal stripe of expression has disappeared, although expression in the hinge (arrow) and the border of the scutellum remains. Note the lack of dc and scutellar bristles. Magnification for (A) through (D), ×35. (**E**) Normal *wg* expression in the wing imaginal disc. The band of expression in the proximal region of the disc corresponds to the notum stripe (arrow). In the zone corresponding to the wing blade, the expression is confined to the proximal region and along the dorsoventral border. (**F**) X-Gal staining of a *wg-lacZ*; pnr^{md237} wing disc showing the expansion of *wg* function toward the medial part of the disc. (**G**) Wing disc of *wg-lacZ*; pnr^{VX1}/pnr^{VX1} genotype stained for β-Gal. The notum band of *wg* expression is lacking, whereas the expression in the wing blade is unaltered. Magnification for (E) through (G), ×60.

parts of the thorax and abdomen (*13*).

The expression of some of these Gal4 insertions in the thorax is of interest because it reveals a genetic subdivision that has not previously been noted. One of these (*md237*) is an insertion at the *pnr* gene; with UAS-*lacZ* it produces an embryonic and imaginal expression indistinguishable from the expression pattern of *pnr* (*14*). *md237*/UAS-y flies show a y^+ rescue marking a dorsal band along the length of the body, from the occipital head region to the end of the abdomen but excluding the terminalia. In the notum, the gene labels a territory extending from the midline to a longitudinal straight line defined by the dorsocentral bristles. Another insertion, *em462*, shows y^+ rescue in a territory adjacent to the *pnr* domain; it is also demarcated by the position of the dorsocentral bristles (Fig. 2A). The *em462* domain extends laterally but does not reach the more lateral region of the notum. The homeobox gene *iroquais* (*iro*) (*15*) apparently affects only this lateral region. We have checked the effect of *iro* viable mutations and found that they remove the macrobristles outside the *em462* domain, suggesting that *iro* defines a distinct, more lateral domain.

The subdivision of the notum by the dorsocentral line may be significant because this same line also appears to demarcate expression of the *wingless* (*wg*) gene, the *Drosophila* homolog of *wnt-1* known to be involved in several major developmental processes (*16*). Expression of *wg* can be visualized by X-Gal staining of pharate *wg-lacZ* adults (*17*). In the notum, it is expressed in a narrow stripe (Fig. 2, B and E), and its medial border is also delimited by the dorsocentral bristles; therefore, it lies at the boundary between the *pnr* and *em462* domains. The *wg* domain is included within the *em462* domain, but the latter extends more laterally (Fig. 2, A and B). Apparently there is no overlap between *pnr* and *wg* in the scutum (the anterior part of the notum), but they overlap in the scutellum.

This arrangement is suggestive of possible interactions between *pnr*, *wg*, and *em462* in the generation of these domains. We have tested the expression of *wg* in several viable combinations of *pnr* alleles. In loss-of-function combinations like pnr^{md237}/pnr^{D1} (*14*), there is a marked expansion of the *wg* stripe toward the medial region (Fig. 2, C and F). This expansion is accompanied by an increase in the number of dorsocentral bristles, which are known to depend on *wg* activity (*17*). In the gain-of-function genotype pnr^{V1}/pnr^{VX1}, in which dorsocentral and scutellar bristles are missing (*14*), the *wg* stripe in the notum is eliminated, whereas the other aspects of *wg* expression in the wing disc remain unaltered (Fig. 2, D and G). These results indicate that *pnr* acts as a negative regulator of *wg* in the notum (*18*) and suggest that some of the effects of *pnr* mutants are produced through an alteration of *wingless* function.

Our results for the notum are summarized and the possible subdivision of the notum into three genetic subdomains (*pnr*, *em462*, and another more lateral one, possibly *iro*) are illustrated in Fig. 3A. Within this arrangement, a critical boundary appears to be the longitudinal line straddling the dorsocentral bristles, which is also defined by *wg* expression. It is possible that *wg* may function in this border in a signaling role across compartments, a role similar to that observed at the embryonic parasegmental border (*19*) or in the dorsoventral border in the wing margin (*17*). Unlike those borders, the dorsocentral line is not a cell lineage border (*1*, *3*, *4*), which raises the question of the mechanism maintaining this boundary and suggests that it is formed not by the activity of selector genes, as are other borders of *wg* activity, but instead may result from an induction mechanism.

What is the significance with respect to the notum patterning of these gene domains demarcated by longitudinal lines? We believe these domains may play a critical and direct role in the spatial arrangement of individual pattern elements. It is significant that pattern elements in the notum tend to be aligned longitudinally. The most obvious case is that of the bristles, which are arranged in straight lines (see also Fig. 3B). This linear arrangement is also obtained when the spatially regulated scute product is replaced by a generalized and uniform (heat shock driven) distribution in notal cells (*20*), suggesting the existence of an underlying patterning mechanism responsible for this arrangement and independent of the achaete scute complex [(AS-C) the genetic system responsible for sensory organ determination in the adult (*21*)]. Another frequent pattern element in dipterans is pigmentation, often disposed in longitudinal bands and used as a diagnostic criterion for the taxonomy of dipteran species (*22*). An example is the longitudinal stripe of pigment (Fig. 3B) in *Zaprionus*, a dipteran close to *Drosophila*. The medial boundary of the pigment band is exactly delimited by the same longitudinal line straddling the dorsocentral bristles that in *Drosophila* demarcates *pnr*, *wg*, and *em462* expressions. In fact, the territory occupied by this stripe resembles the notum domain of *wg* expression in *Drosophila*. In the wing of the lepidopteran *Precis coenia* there is a strong correspondence between the late transcription patterns of *wg* and *Dll* and the differentiation of discrete pattern elements such as rays and eyespots, suggesting that there is a late function of these genes that exerts an immediate control on final patterning events (*23*). Thus, we believe that the longitudinal stripes of expression of *pnr*, *wg*, and *em462* in *Drosophila* may reflect a mechanism for control of the spatial disposition of individual pattern elements and thus may be responsible for establishment of the "pre-pattern" postulated by Stern (*24*) a long time ago.

In conclusion, our approach has revealed previously unsuspected genetic subdivisions that may control the disposition of pattern

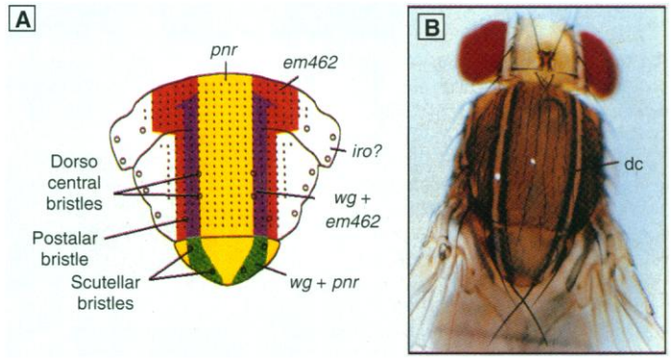


Fig. 3. Correlation between longitudinal borders of gene expression and ordering of pattern elements in the adult notum. (**A**) Tentative subdivision of the notum in *pnr*, *em462*, and *iro* domains. The *pnr* and *em462* domains are based on the expression patterns as visualized by the Gal4 lines, whereas that of *iro* is more speculative and is based on the observation that the region not covered by *pnr* and *em462* appears to match the area affected by *iro* viable mutations. The *wg* stripe is included in the *em462* domain. Small dots indicate the position of microbristles, whereas the larger open dots indicate macrobristles. The lineal arrangement of bristles is emphasized in the scheme. The functional interactions between these domains remain to be analyzed, but our results indicate that *pnr* is involved with control of *wg* expression. Macrobristles marking the limits of the domains are indicated. (**B**) Notum of a *Zaprionus* fly showing two aspects of the pattern defined by longitudinal lines: the arrangement of bristles and a pigmentation band made by two narrow dark stripes separated by an intervening light stripe. As the macrobristle pattern is virtually identical to that of *Drosophila*, we can locate this band of pigment precisely. Note that the border of the dark pigment is limited medially by the dorsocentral bristles (dc). This band resembles the *wg* expression of *Drosophila* along the thorax down to the scutellum (compare with Fig. 2B) (magnification ×25).

elements in the thorax. It may allow a systematic screen for identifying other such subdivisions that may underlie the development of the entire adult pattern.

REFERENCES AND NOTES

1. A. Garcia-Bellido, P. Ripoll, G. Morata, *Nature New Biol.* **245**, 251 (1973); A. Garcia-Bellido, *Genetic Control of Wing Disc Development* (Associated Scientific, New York, 1975), pp. 161–183; G. Morata and P. A. Lawrence, *Nature* **265**, 614 (1975).
2. K. Basler and G. Struhl, *Nature* **368**, 208 (1994); T. Tabata and T. Kornberg, *Cell* **76**, 89 (1994); J. Capdevila and I. Guerrero, *EMBO J.* **13**, 4459 (1994); F. Diaz-Benjumea and S. M. Cohen, *Development* **120**, 1661 (1994).
3. G. Morata and P. A. Lawrence, *Nature* **265**, 211 (1977).
4. S. M. Cohen, in *The Development of* Drosophila melanogaster, M. Bate and A. Martinez-Arias, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1993), pp. 747–841.
5. P. A. Lawrence, *The Making of a Fly* (Blackwell, Oxford, 1992).
6. D. Nellen, R. Burke, G. Struhl, K. Basler, *Cell* **85**, 357 (1996); T. Lecuit *et al.*, *Nature* **381**, 387 (1996).
7. J. A. Williams, S. W. Paddock, K. Vorweck, S. Carroll, *Nature* **368**, 299 (1994); F. Diaz-Benjumea and S. Cohen, *Cell* **75**, 741 (1993); S. T. Tiong, D. Nash, W. Bender, *Development* **121**, 1649 (1995); M. Ng, F. Diaz-Benjumea, S. Cohen, *ibid.*, p. 589; K. D. Irvine and E. Wieschaus, *Cell* **79**, 595 (1994).
8. A. Brand and N. Perrimon, *Development* **118**, 401 (1993).
9. To construct the *UAS-yellow* lines, we first reconstituted a full-length *yellow* cDNA by cloning two Eco RI fragments [P. K. Geyer, C. Spana, V. G. Corces, *EMBO J.* **10**, 2657 (1986)] into a Bluescript M13+ vector. The vector was then digested with Not I and Kpn I, and the resulting fragment was subcloned in pUAST (*8*). The DNA was injected into y^-w^- embryos according to standard procedures. Several *UAS-y* lines were obtained for the three major chromosomes.
10. A. Brand, A. S. Manoukian, N. Perrimon, in *Methods in Cell Biology*, L. S. B. Goldstein and E. A. Fyrberg, Eds. (Academic, New York, 1994), pp. 435–454.
11. To obtain additional insertions, we used the same line carrying the *pGawB* transposon in the first chromosome described in (*8*). This insertion is homozygous lethal and is balanced with a *FM7* chromosome. *pGawB/FM7* females were crossed to y^-w^-; *DrP(Δ2-3)/TM6B* males carrying the source of transposase. The dysgenic F_1 females *pGawB/* y^-w^-; *P(Δ2-3)/+* were then crossed to y^-w^- males. The mobilization of the transposon resulted in y^-w^- (w^+) male progeny. Each of these F_2 males was individually crossed to y^-w^-; *UAS-y/TM3, UAS-y* females and the progeny were inspected under a dissecting microscope for y^+ patches.
12. X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) staining in embryos was performed as described in (*8*), but sometimes we used antibody to β-galactosidase (anti–β-Gal) and standard methods. For better staining of adult cuticle with X-Gal, pharate adults were extracted from the puparium and treated as described in (*16*).
13. M. Calleja, E. Moreno, S. Pelaz, G. Morata, unpublished results.
14. P. Heitzlert, M. Haenlin, P. Ramain, M. Calleja, P. Simpson, *Genetics* **143**, 1271 (1996).
15. C. Dambly-Chaudiere and L. Leyns, *Int. J. Dev. Biol.* **36**, 85 (1992); J. L. Gómez-Skarmeta, R. Diez del Corral, E. de la Calle-Mustienses, D. Ferrés-Marcó, J. Modolell, *Cell* **85**, 95 (1996).
16. R. Nusse and H. E. Varmus, *Cell* **69**, 1073 (1992); E. Siegfried and N. Perrimon, *BioEssays* **16**, 395 (1994).
17. R. G. Phillips and J. R. S. Whittle, *Development* **118**, 427 (1993).
18. The regulation of *wg* expression in the notum by *pnr* also has been observed by M. Haenlin and P. Simpson, personal communication.
19. A. Martinez-Arias, N. A. Baker, P. W. Ingham, *Development* **103**, 157 (1988).
20. I. Rodriguez, R. Hernandez, J. Modolell, M. Ruiz-Gomez, *EMBO J.* **9**, 3583 (1990).
21. S. Campuzano and J. Modolell, *Trends Genet.* **8**, 202 (1992).
22. J. F. McAlpine, Ed., *Manual of Neartic Diptera* (Research Branch Agriculture Canada Monograph 28, Canada Communication Group Publishing, Ottawa, Canada, 1987), vol. 2.
23. S. B. Carroll *et al.*, *Science* **265**, 109 (1994).
24. C. Stern, *Am. Sci.* **43**, 213 (1954).
25. P. M. McDonald and G. Struhl, *Nature* **324**, 537 (1986); M. Mlodzick and W. J. Gehring, *Cell* **48**, 465 (1987).
26. D. L. Lindsley and G. Zimm, *The Genome of* Drosophila melanogaster (Academic Press, San Diego, 1992).
27. P. W. Ingham, S. M. Pinchin, K. Howard, D. Ish-Horowicz, *Genetics* **111**, 463 (1985).

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Cholesterol Modification of Hedgehog Signaling Proteins in Animal Development

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Hedgehog (Hh) proteins comprise a family of secreted signaling molecules essential for patterning a variety of structures in animal embryogenesis. During biosynthesis, Hh undergoes an autocleavage reaction, mediated by its carboxyl-terminal domain, that produces a lipid-modified amino-terminal fragment responsible for all known Hh signaling activity. Here it is reported that cholesterol is the lipophilic moiety covalently attached to the amino-terminal signaling domain during autoprocessing and that the carboxyl-terminal domain acts as an intramolecular cholesterol transferase. This use of cholesterol to modify embryonic signaling proteins may account for some of the effects of perturbed cholesterol biosynthesis on animal development.

The *hedgehog* (*hh*) family of secreted signaling proteins is required for developmental patterning of a wide variety of embryonic structures in insects, vertebrates, and other multicellular organisms (*1–5*). After signal sequence cleavage, the *hh* protein (Hh) precursor (~45 kD) undergoes an autocatalytic internal cleavage to yield an ~20-kD NH_2-terminal domain (Hh-N_p) and an ~25-kD COOH-terminal domain (Hh-C). Whereas Hh-N_p possesses all known signaling activity, Hh-C is responsible for precursor processing, acting by way of an intramolecular mechanism (*6, 7*). In addition to peptide bond cleavage, Hh autoprocessing causes the covalent attachment of a lipophilic adduct to the COOH-terminus of Hh-N_p (*8*). This modification is critical for the spatially restricted tissue localization of the Hh signal; in its absence, the signaling domain exerts an inappropriate influence beyond its site of expression (*8*). Physical and biochemical characterization of this lipophilic adduct indicates that it is not the glycosyl phosphatidylinositol (GPI) anchor (*8*), the only other known lipophilic modification associated with secreted cell surface proteins in eukaryotes (*9, 10*).

In vitro studies of Hh autoprocessing have used a bacterially expressed derivative of the *Drosophila* Hh protein, His_6Hh-C, in which most of the NH_2-terminal signaling domain and the signal sequence are replaced by a hexa-histidine tag. Cleavage of this protein occurs between residues corresponding to Gly^{257} and Cys^{258} (*7*) and likely proceeds through a labile thioester intermediate formed by the cysteine thiol and the glycine carbonyl carbon (*7, 8*). In the presence of high concentrations of thiols or other small molecules with strongly nucleophilic properties at neutral pH, cleavage of the peptide results from nucleophilic attack on the thioester carbonyl, causing displacement of the thiol group and formation of an adduct to Gly^{257} by the attacking nucleophile (*8*) (Fig. 1A). Thus, in reactions with 50 mM dithiothreitol (DTT), in vitro cleavage of His_6Hh-C proceeded to greater than 50% completion within 3 hours at 30°C (Fig 1B). At 1 mM DTT, however, the reaction yielded no visible cleavage product (Fig. 1B).

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