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Hedgehog Is a Signaling Protein with a Key Role in Patterning Drosophila Imaginal Discs

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Summary

The segment polarity genes *hedgehog* and *engrailed* are expressed in identical posterior-compartment-specific patterns in both Drosophila embryos and imaginal discs. We show here that the hedgehog protein is secreted, and it can cross embryo parasegment borders and the anterior-posterior compartment border of imaginal discs to neighboring cells that express neither *engrailed* nor *hedgehog*. In these cells, it is localized in discrete punctate structures that are sequestered within the polarized epithelium. Analysis of animals that have expressed *hedgehog* ectopically, or of a mutant that expresses *hedgehog* abnormally in the anterior compartment of the wing disc, indicates that *hedgehog* is involved in regulating *patched*. In the embryo, *hedgehog* regulation of *patched* apparently facilitates *patched* and *wingless* expression. In the discs, *hedgehog* regulation of *patched* and other genes in the anterior compartment helps to establish the proximodistal axis. We propose that the cell-cell communication mediated by *hedgehog* links the special properties of compartment borders with specification of the proximodistal axis in imaginal development.

Introduction

The principal axes of the early Drosophila embryo are established by a positional information system encoded by protein gradients (reviewed by St. Johnston and Nüsslein-Volhard, 1992). Two such gradients have been identified that establish the anterior-posterior (A-P) axis. bicoid protein diffuses away from the anterior pole to form a monotonic gradient, and the concentration of bicoid protein at various positions along the A-P axis determines where its regulatory targets are expressed. An analogous gradient of nanos protein forms from the posterior pole. Although our understanding of the gradient that organizes the dorsal-ventral (D-V) axis is less complete, it is thought that the D-V axis is created by an extracellular ligand whose active form is generated along the ventral midline and then diffuses dorsally to generate a monotonic gradient.

Protein gradients continue to encode positional information throughout the period of syncytial nuclear divisions of the early Drosophila embryo. The broad gradients of bicoid and nanos protein that form along the length of the embryo activate a cascade of cross-regulating genes (the gap genes) during the syncytial blastoderm stages. The proteins encoded by these gap genes are themselves thought to form short-range morphogenetic gradients that help both to refine their respective expression domains and to activate the pair-rule genes in appropriate relative positions (reviewed by Hoch and Jäckle, 1993). These gap and pair-rule proteins are transcription factors whose diffusion is enabled by the syncytial nature of the early Drosophila embryo.

Such nuclear proteins are no longer free to diffuse from their sources of synthesis after the embryo cellularizes, and it seems likely that the mechanisms involved in patterning within the developmental fields of cellularized embryos incorporate novel ways to define relative position. Along the D-V axis, the graded distribution of a TGFβ-related ligand (the product of the *decapentaplegic [dpp]* gene) specifies the pattern of the dorsal 40% of the embryo (Ferguson and Anderson, 1992; Wharton et al., 1993). In contrast, patterning along the A-P axis involves a set of so called segment polarity genes that are responsible for organizing pattern within segmental (or parasegmental) intervals. These genes assume control of the A-P patterning process as cellularization of the embryo is completed and gastrulation commences. In contrast to the gap and pair-rule gene products, all of which are transcription factors that function in nuclei in a syncytium, the segment polarity gene products are diverse in character and mediate communication between the newly formed cells of the embryo. Some of the segment polarity gene products are likely to be nuclear transcription factors (e.g., engrailed [DiNardo et al., 1985; Poole et al., 1985]), while others are protein kinases, membrane associated proteins (e.g., patched [Hooper and Scott, 1989; Nakano et al., 1989]), or are secreted factors (e.g., wingless [González et al., 1991; van den Heuvel et al., 1989] and hedgehog [Lee et al., 1992; Taylor et al., 1993; see below]). Our understanding of their roles and relationships is rudimentary at this time.

Cross-regulatory interactions among the segment polarity gene products ultimately determine their patterns of expression in the embryo, and their expression in restricted domains provides instructions to specify and pattern the cuticular structures along the A-P axis of the first instar larval cuticle. For example, *wingless* will specify naked cuticle and *engrailed* the first denticle row in thoracic and abdominal segments of first instar larva (Dougan and DiNardo, 1992; Bejsovec and Wieschaus, 1993). During gastrulation, *wingless* and *engrailed* become dependent upon each other for their continued and stable expression, and both the *wingless* and *engrailed* signaling pathways involve several other segment polarity genes. *Wingless* is expressed in the row of cells just anterior to the parasegment border, and encodes a secreted signaling protein. The neighboring more posterior row of cells (the most anterior cells of each parasegment) requires the wingless signal to maintain expression of *engrailed* (Bejsovec and Martinez, 1991; DiNardo et al., 1988; Heemskerk et al., 1991; Martinez Arias et al., 1988). *Engrailed* encodes a homeodomain protein (Fjose et al., 1985; Poole et al., 1985) that, in turn, positively regulates *hedgehog*, a gene whose pat-

tern of expression coincides precisely with *engrailed*. *Hedgehog* encodes a putative extracellular protein that is either membrane-associated or secreted (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992). Although *hedgehog* activity is needed to stabilize *wingless* expression (Hidalgo, 1991; Ingham and Hidalgo, 1993), and although it has been proposed that *hedgehog* acts to antagonize a repressor of *wingless* (Ingham et al., 1991), the mechanism by which *hedgehog* might effect such regulation is unclear. *Patched*, which encodes an integral membrane protein (Hooper and Scott, 1989; Nakano et al., 1989) and is expressed most abundantly in the *wingless*-expressing cells, is certainly involved in these cross-regulatory interactions. The patterns of expression of *wingless*, *engrailed*, and *hedgehog* expand in *patched* mutants, and *patched* expression depends upon *hedgehog* expression in adjacent cells (Hidalgo and Ingham, 1990).

Patterning in the adult epidermis entails the specification of three major axes: an A–P axis, a D–V axis and, in the appendages, a proximodistal axis that is orthogonal to the first two. Although most of the segment polarity genes are involved in pattern formation in both embryos and imaginal discs, their roles and cross-regulatory relationships in embryonic and imaginal development differ in some respects. *engrailed* and *patched* help to establish pattern regulation with respect to the A–P axis at both stages. In discs, *engrailed* is required in the posterior compartment cells to maintain the compartment border and to specify the posterior character of pattern elements in the posterior compartment (Lawrence and Morata, 1976). *patched* is required in the wing anterior compartment, where the consequences of loss of *patched* function increase in severity with increasing distance from the A–P compartment border (Phillips et al., 1990). However, neither the expression nor the function of *engrailed* and *patched* is dependent upon *wingless*. Rather, *wingless* is believed to play a key role in organizing pattern along the D–V axis, at least in the leg disc (Struhl and Basler, 1993), and, in association with *dpp* and a homeobox gene *aristaless*, in generating the proximodistal axis (Campbell et al., 1993).

Imaginal discs are composed of anterior and posterior developmental compartments, and it would seem to be more than coincidence that several of the genes with roles in establishing the primary disc axes and in pattern specification in the discs are expressed in patterns that correspond to the compartmental organization: *engrailed* and *hedgehog* in all posterior compartment cells, *patched* in all anterior compartment cells but most prominently along the A–P compartment border (Phillips et al., 1990), *dpp* along the A–P compartment border (Raftery et al., 1991) and, in the wing disc, *wingless* both around the perimeter of the wing blade primordium and in a stripe along the D–V wing margin that transects the A–P compartment border. The compartment border has been shown to be an important influence in the growth and patterning of discs (Lawrence and Morata, 1976), and theoretical models in which the compartment border acts as an organizing center within the disc primordium have been proposed (Meinhardt, 1983). Yet, no direct link between the compartments, the genes that establish and maintain them, and the primary axes has been established.

In the course of a study of the properties of the hedgehog (HH) protein and of the role of *hedgehog* in embryonic and imaginal patterning, we found that *hedgehog* is involved in regulating *patched* in both embryos and discs. Moreover, through its role in regulating *patched* and other genes that are expressed along the A–P compartment border (e.g. *patched* and *dpp*), *hedgehog* apparently establishes the proximodistal axis in discs. This observation suggests a mechanism whereby the compartment boundaries, which are set up initially to subdivide the embryo into metameric units, are used subsequently to orient the proximodistal development of appendages.

Results

The HH Protein Is Processed Posttranslationally

The hh cDNA we previously characterized can putatively encode a 471 residue protein with a probable transmembrane domain (residues 63–81) (Lee et al., 1992; Tabata et al., 1992). This protein sequence also contains a possible site for proteolytic cleavage C-terminal to the transmembrane domain (Lee et al., 1992). Based upon comparisons with the sequences of several membrane proteins known to have internal signal sequences, Lee et al. proposed that the orientation of HH protein is N-in/C-out, and presented evidence that HH protein synthesized in vitro can be translocated into microsomes and processed (Lee et al., 1992). We have also observed that HH protein produced by cell-free translation can be translocated into dog pancreas microsomes and cleaved, although the efficiency of processing was poor (less than 25%; data not shown).

To determine the distribution and form of HH protein in vivo, we raised a serum antibody against a peptide containing the HH protein sequence C-terminal to the putative transmembrane domain. The antibody (HH-Ab) was used to probe Western blots of embryos, imaginal discs, and cultured Drosophila cells. The antibody recognized several protein species specific to extracts that had been prepared from these tissues after induction of a *hedgehog* transgene (Figure 1). A protein migrating with the same apparent mobility as the translation product of the entire *hedgehog* open reading frame (M_r 52,147) was observed in extracts from imaginal discs. The other three protein moieties common to these extracts migrated with a smaller apparent molecular weight (43, 28, and 20 kDa), suggesting that proteolytic processing reduces the size of the HH peptide in these cells. The largest of the three proteolytic fragments (43 kD) had an electrophoretic mobility similar to that of a bacterially-expressed peptide containing the hedgehog sequences C-terminal to the transmembrane domain. Since the molecular weight of HH protein that had been translated in vitro in the presence of a microsome fraction was not altered by treatment with endoglycosidase (Lee et al., 1992), glycosylation does not apparently contribute to the electrophoretic mobility of HH protein. Although we do not know which of the three forms of HH protein is the functional moiety in vivo, or whether

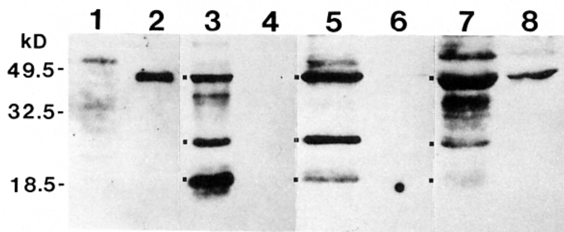

Figure 1. Immunoblot Analysis of Hedgehog Protein in Schneider cells, Imaginal discs, and Embryos

HH protein translated in vitro in rabbit reticulocyte lysate (lane 1) and an *E. coli*-produced polypeptide composed of residues 90–471 of HH protein and six additional amino acids (lane 2) serve as markers for full-length HH protein (52.1 kD) and the product of cleavage at the transmembrane domain (43.6 kD), respectively. Extracts of the following tissues were resolved on 10% SDS-PAGE and probed with HH-Ab: Schneider cells transfected with the full-size *hedgehog* cDNA driven by an actin promoter (lane 3); Schneider cells transfected with a vector not carrying the *hedgehog* cDNA (lane 4); imaginal discs of heat-treated third instar larvae of a fly strain (HS-HH) carrying the HS-hedgehog construct (lane 5); imaginal discs of nonheat-treated third instar HS-HH larvae (lane 6); heat-treated 2–6 hr HS-HH embryos (lane 7); and 2–6 hr HS-HH embryos without heat treatment. Three protein moieties common to these extracts are depicted with dots (lanes 3, 5, and 7). The position of migration of molecular weight markers is shown on the left side.

one or several of the forms might have been generated during the isolation procedure, the results of this Western blot analysis are consistent with the proposal that HH protein is cleaved at or near the transmembrane domain.

HH Protein Distribution in Embryos

HH-Ab was used to immunostain whole-mount embryos. HH protein was found in stripes that are similar in shape and distribution to the stripes of *hedgehog* RNA at these stages. However, in contrast to the pattern of *hedgehog* RNA, the stripes of HH protein were less well-defined and broader (Figures 2A, 2B, and 2D). Since we had previously determined that *hedgehog* RNA is synthesized in precisely the same cells that express *engrailed* (Tabata et al., 1992), embryos were stained simultaneously with anti-engrailed antibody to establish the relative distribution of HH and engrailed proteins. HH protein was detected in domains that include the engrailed protein-containing cells; in addition, the HH protein was found at least one cell diameter away (Figures 2C and 2E). Engrailed protein is localized to nuclei. HH protein was found predominantly in the basal half of the ectodermal cell layer, although a small amount was present on the apical surface (Figure 2C). Some HH-Ab staining was observed in apparent association with cellular membranes as heavily stained punctate structures. These punctate structures were usually shared by two or three cells (Figures 2B and 2D). These observations are consistent with the biased localization to basal regions and accumulation in punctate structures previously reported by Taylor et al. (1993).

To further characterize the apparent movement of HH protein from the cells in which the *hedgehog* gene is expressed, embryos were treated with monensin, a drug that inhibits protein secretion by interfering with protein traffic in the Golgi (Tartakoff, 1983). Administration of monensin to permeabilized embryos altered the distribution of HH protein. HH-Ab staining was more intense and the stripes were more sharply delineated than in untreated embryos. Moreover, staining was more evenly distributed and was almost entirely restricted to the engrailed protein–expressing cells (Figures 2F, 2G, 2H, 2I, and 2J). The most intensely stained structures were intracellular (Figure 2I), not the cellular membrane-associated punctate structures characteristic of untreated embryos. These results suggest that the HH protein is processed in the Golgi complex, sorted to discrete intracellular structures, and then secreted. The biased basal localization and accumulation in punctate structures are presumably the consequence of secretion and transport.

The distribution of HH protein is also dependent upon the endocytic pathway. Endocytosis can be arrested reversibly in Drosophila with appropriate temperature shifts of the temperature-sensitive allele of the *shibire (shi)* gene, shi^{ts1} (Grigliatti et al., 1973; Kosaka and Ikeda, 1983; Poodry and Edgar, 1979). *shi* encodes a Drosophila homologue of dynamin (Chen et al., 1991; van der Bliek and Meyerowitz, 1991) that is thought to provide the motor for vesicular transport during endocytosis. shi^{ts1} embryos that had been incubated at the restrictive temperature had an abnormal distribution of HH protein. Staining with HH-Ab revealed the stripes of HH protein to be less dispersed than in untreated embryos. In addition, many stained punctate structures were found in the apical region, and more HH protein was associated with the cellular membrane than in wild-type embryos (Figures 2K–2O). These observations suggest that HH protein is normally transported by a mechanism that is dependent upon the endocytic pathway. The apical localization of HH protein in mutant embryos suggests that HH protein normally binds to cells along their apical surface, and is in the apical region only transiently; the block in endocytosis apparently interferes with its redistribution to or concentration in the basal region.

To evaluate the possibility that the patched protein might be involved in transporting HH protein, the pattern of HH-Ab staining was determined in *patched* mutant embryos. Patched protein is an integral membrane protein whose peptide chain is thought to cross the membrane multiple times (Hooper and Scott, 1989; Nakano et al., 1989), and functional interactions between patched and HH proteins have been proposed (Ingham et al., 1991). In embryos of the germband extended stage, patched protein is distributed uniformly around the periphery of cells that do not express *engrailed* or *hedgehog* (Taylor et al., 1993). In *patched* mutant embryos, the stripes of HH protein appear to be more intense and broader than in wild-type embryos (Figures 2P–2T; Taylor et al., 1993). Although ectopic stripes of *engrailed* and *hedgehog* expression form in *patched* mutants (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992), their weak and discontinuous character (Figures 2R and 2T) would make them an unlikely source for the elevated levels of HH protein observed in *patched* mutants. Rather, the increased subcellular concentration of HH protein in the basal region (Figure 2R) indicates that patched function affects the distribution of

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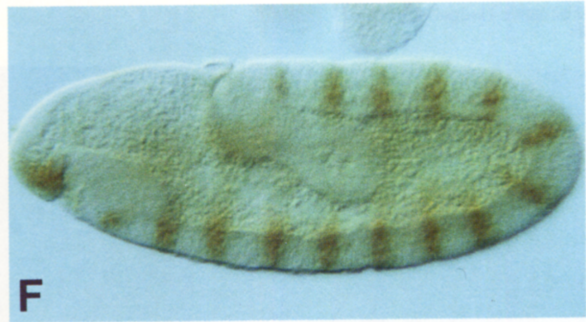
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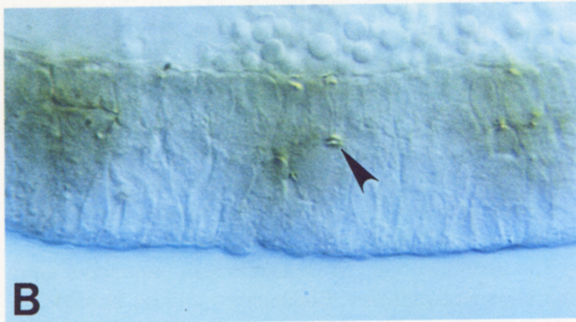
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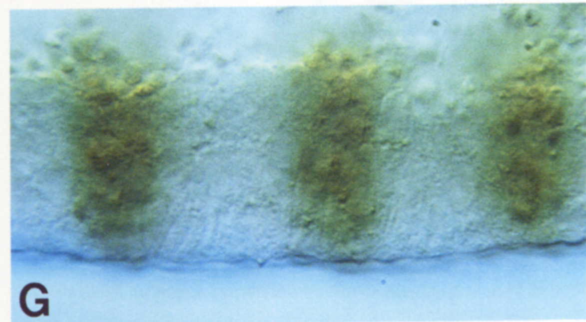
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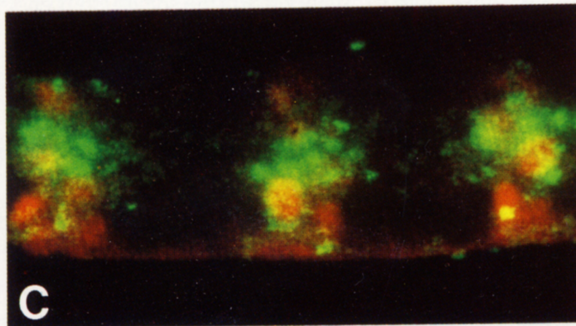
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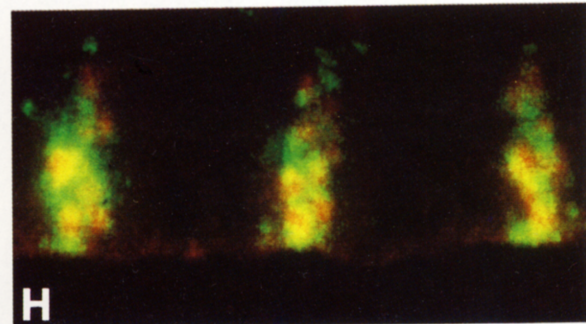
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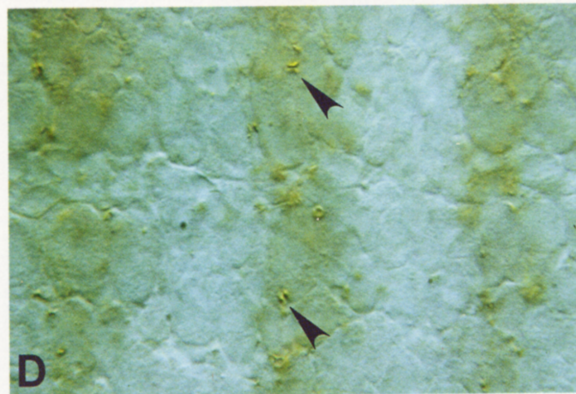
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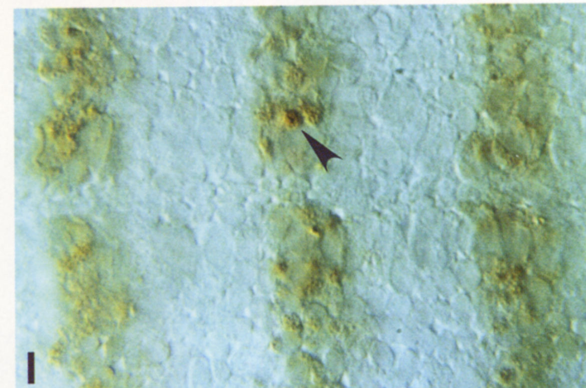
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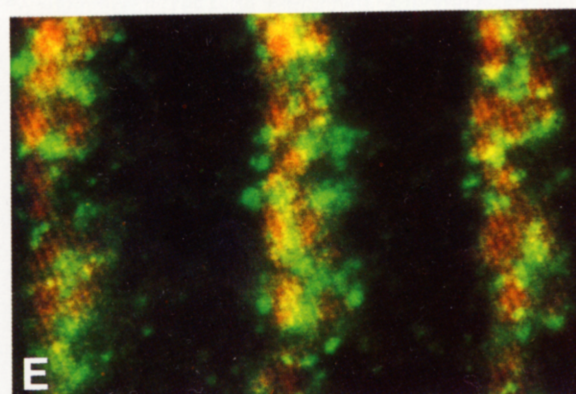
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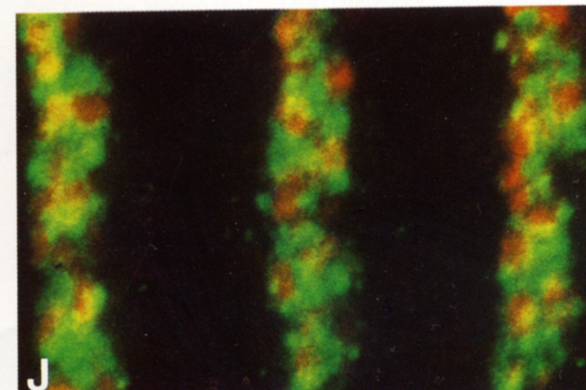
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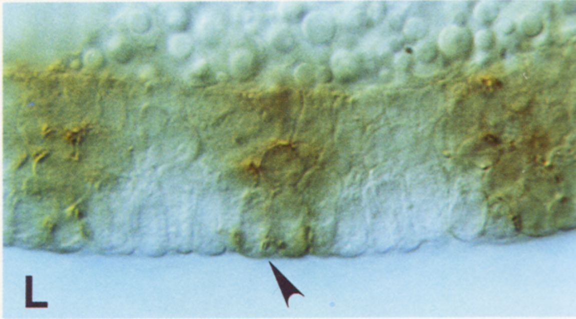
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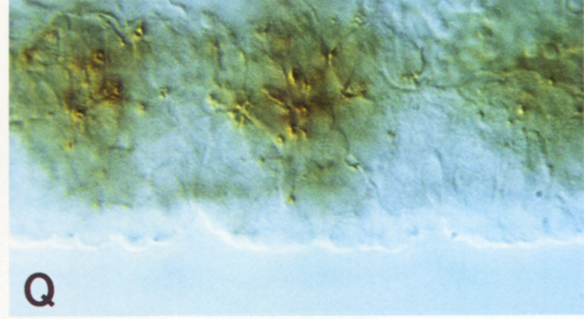
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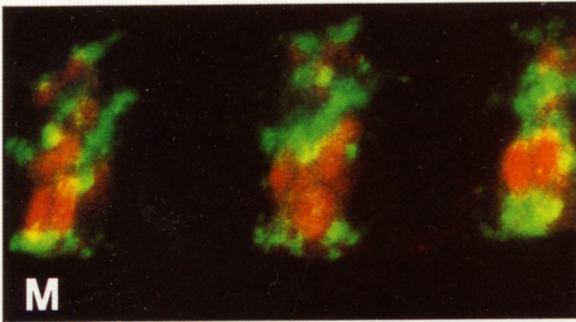
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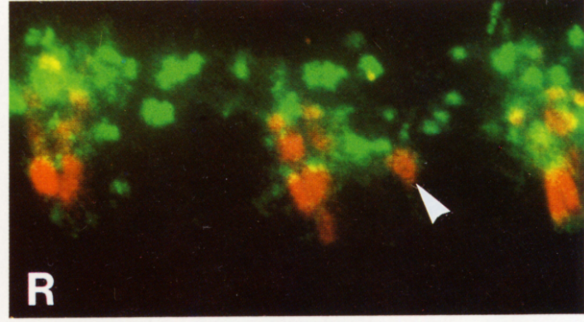
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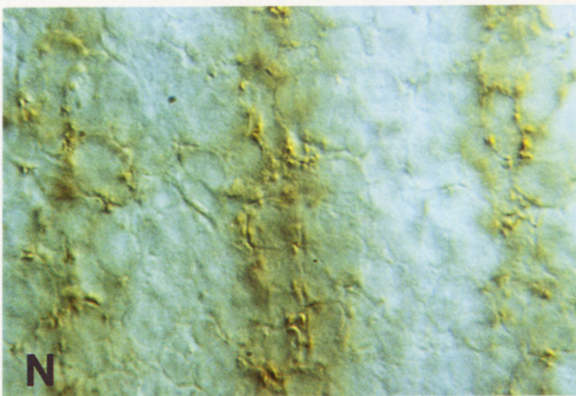
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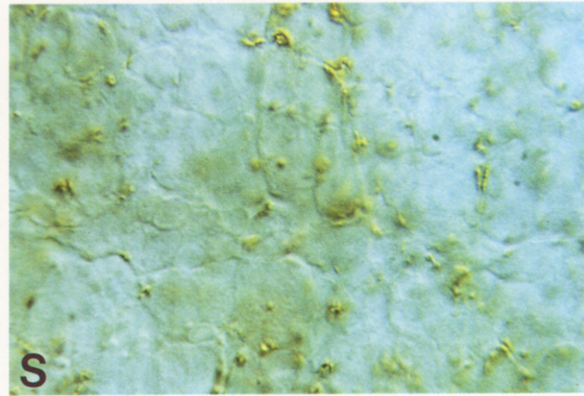
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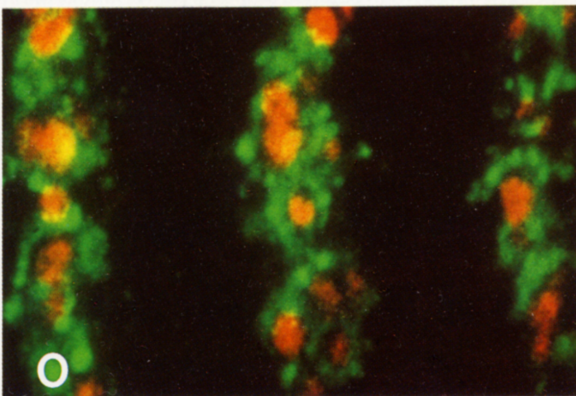
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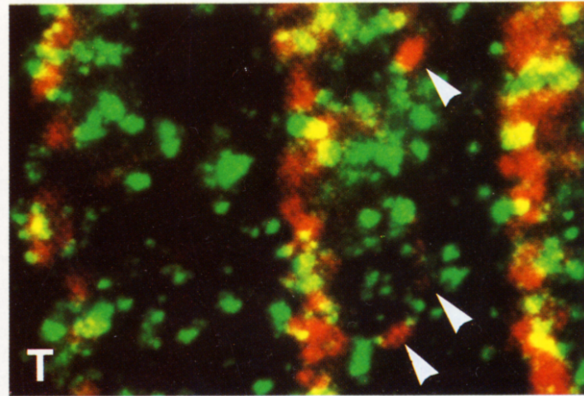
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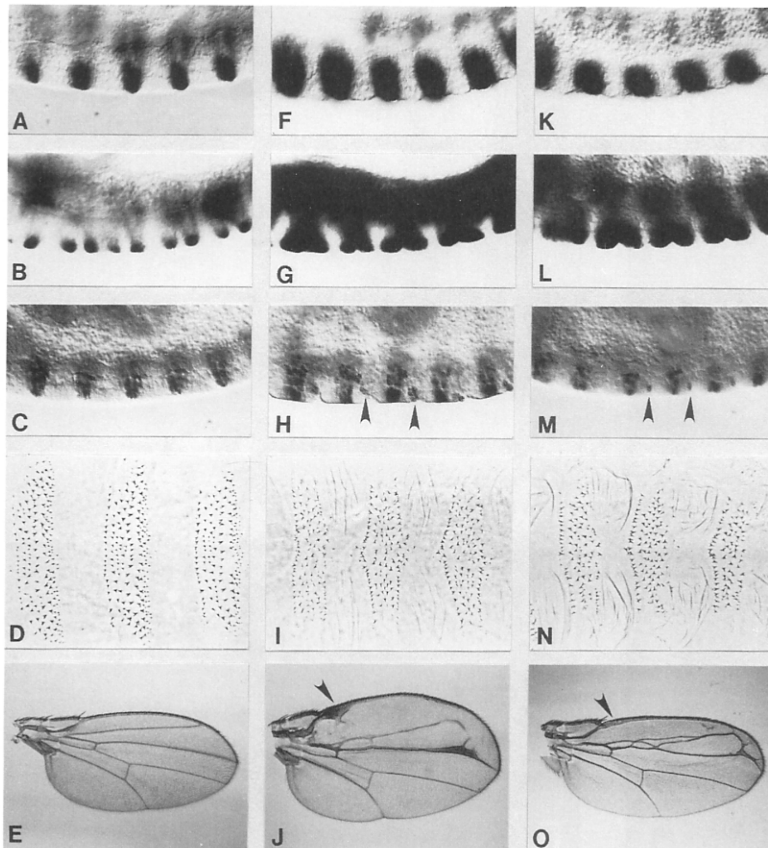

Figure 3. Comparisons of Wild-Type, *patched*, and HS-HH Phenotypes

Shown are expression patterns of *wingless* (A, F, and K) and *patched* (B, G, and L) and distribution of engrailed protein (C, H, and M) in wild-type (A, B, and C), *patched* (F, G, and H), and HS-HH (K, L, and M) embryos. These are lateral views of the thoracic and abdominal segments of stage 11 embryos. Arrowheads in (H) and (M) mark ectopic *engrailed* expression and ectopic grooves. Panels (D), (I), and (N) show the denticle belts of segments A5, A6, and A7 of wild-type, *patched*, and HS-HH first instar larvae, respectively. The panels at the bottom show adult wings of wild-type (E), ptc^{G20}/ptc^{IN} (J), and HS-HH (O) flies.

HH, and suggests that patched protein may block redistribution of HH protein to the basal region. We do not know which form of the HH protein is functionally important: the protein present in the apical region, the protein associated with the membrane-associated structures in the basal region, or both. However, if HH protein in the apical region is functionally significant, then there is an opportunity for the HH and patched proteins to interact directly.

Ubiquitous Expression of the HH Protein

To characterize the role of *hedgehog* in embryonic and imaginal disc development and to evaluate the importance of the normal pattern of *hedgehog* expression, we constructed a strain (HS-HH) with a transgene consisting of the *hedgehog* cDNA under the control of the HSP70 promoter. In transgenic animals, heat shocks induced *hedgehog* expression that was ubiquitous, if not entirely uniform. We examined the patterns of *wingless*, *patched*, and *engrailed* expression in transgenic embryos that had been subjected to multiple heat shocks in early gastrulation. We also examined the denticle belts of first instar larvae that developed from such heat-shocked embryos. Of the heat-shocked embryos, eighty to ninenty percent died before hatching, while generating cuticle patterns with abnormal denticle belts (Figure 3N). In a similar percentage of embryos, ubiquitous *hedgehog* expression caused the stripes of *wingless* RNA to widen (Figure 3K), ectopic stripes of engrailed protein to form (Figure 3M), and *patched* expression to persist in all the anterior compartment cells (Figure 3L), at a stage when *patched* stripes would normally shrink to a width of 1 cell. In addition, ectopic deep grooves just posterior to the ectopic *engrailed* stripes formed (Fig-

Figure 2. Distribution of the Hedgehog Protein in Wild-Type, Monensin-Treated, and Mutant Embryos

Lateral and ventral views of HH protein distribution at stage 10 are shown of wild-type (A–E), monensin-treated (F–J), shi^{ts1} (K–O), and *patched* (P–T) embryos. High magnification lateral (B, C, G, H, L, M, Q, and R) and ventral (D, E, I, J, N, O, S, and T) views are shown. In addition, panels (A), (F), (K), and (P) show low magnification lateral views. Localization of the HH protein was visualized with the HRP-conjugated antibody and Nomarski optics (A, B, D, F, G, I, K, L, N, P, Q, and S). Comparison of the distributions of HH (green) and engrailed (red) proteins was achieved with fluorescence-conjugated antibody staining examined by confocal microscopy (C, E, H, J, M, O, R, and T). The yellow regions are regions of overlap between the proteins. The arrowheads in (B) and (D) show the typical punctate accumulation of the HH protein, while the arrowheads in (I) show accumulation in intracellular structures in the monensin-treated embryos. The arrowhead in (L) depicts the typical punctate accumulation of HH protein near the apical surface of the ectoderm in the shi^{ts1} embryos. Ectopic *engrailed* expression in *patched* embryos is indicated by the arrowheads in (R) and (T). In this and subsequent figures, embryos are oriented anterior to the left and dorsal up (for lateral view).

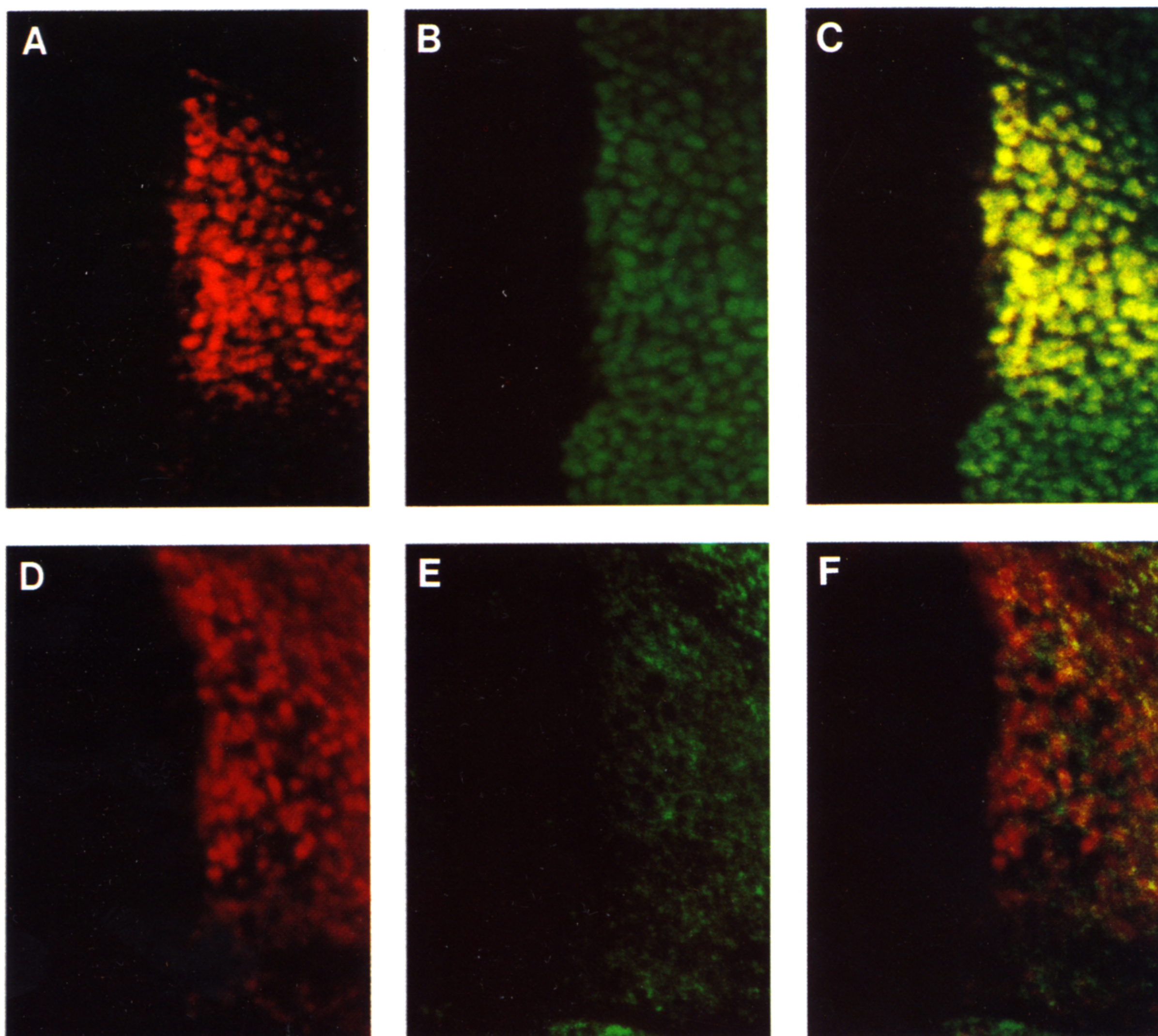

Figure 4. *hedgehog* Expression in Wing Imaginal Discs

The domain of *hedgehog* expression was located in imaginal discs of early to mid third instar larvae by immunostaining the *hedgehog* enhancer trap line P30 with anti-β-galactosidase antibody (A, B, and C) or by immunostaining the wild type with HH-Ab (D, E, and F). In both cases, the domain of *hedgehog* expression (B and E) is the same as the domain of *engrailed* expression (A and D). Merged images are shown in (C) and (F). Since *engrailed* expression is strong in the wing pouch and weak in the notum, the wing pouch region is shown here. All micrographs were taken on a confocal microscope. In this and subsequent figures, imaginal discs are oriented anterior to the left.

ure 3M). All aspects of these phenotypes are similar to the *patched* mutant phenotype (Ingham et al., 1991), and suggest that ubiquitous expression of HH protein produces a phenocopy of *patched*.

To study the role of *hedgehog* in imaginal development, the HS-HH strain was subjected to multiple heat shocks during the third larval instar. Whereas wild-type flies were normal in appearance, the phenotypes that were generated by this treatment of HS-HH flies were both consistent and remarkably specific in the adult wing. Although development of the posterior compartment of the wing was unaffected by the heat shock regimen, wing veins in the anterior compartment of approximately 20% of the flies were disorganized (Figure 3O). Vein 1 was broadened at the base (arrow head), vein 2 did not form completely, and vein 3 had a plexate appearance. The shape of the wings showed a subtle expansion of the anterior–dorsal wing blade. This wing phenotype is reminiscent of wing abnormalities in the anterior compartments of viable *patched* heteroallelic combinations (Figure 3J; Phillips et al., 1990).

HH Protein in the Wing Imaginal Disc

In wild-type third instar imaginal discs, *hedgehog* is expressed specifically in the cells of the posterior compartments (Lee et al., 1992; Tabata et al., 1992). For example, wing imaginal discs from a strain carrying a *hedgehog* enhancer trap insertion contain both engrailed protein and β-galactosidase in their posterior compartment cells, and staining these discs with antibodies directed against engrailed protein and β-galactosidase revealed that both pro-

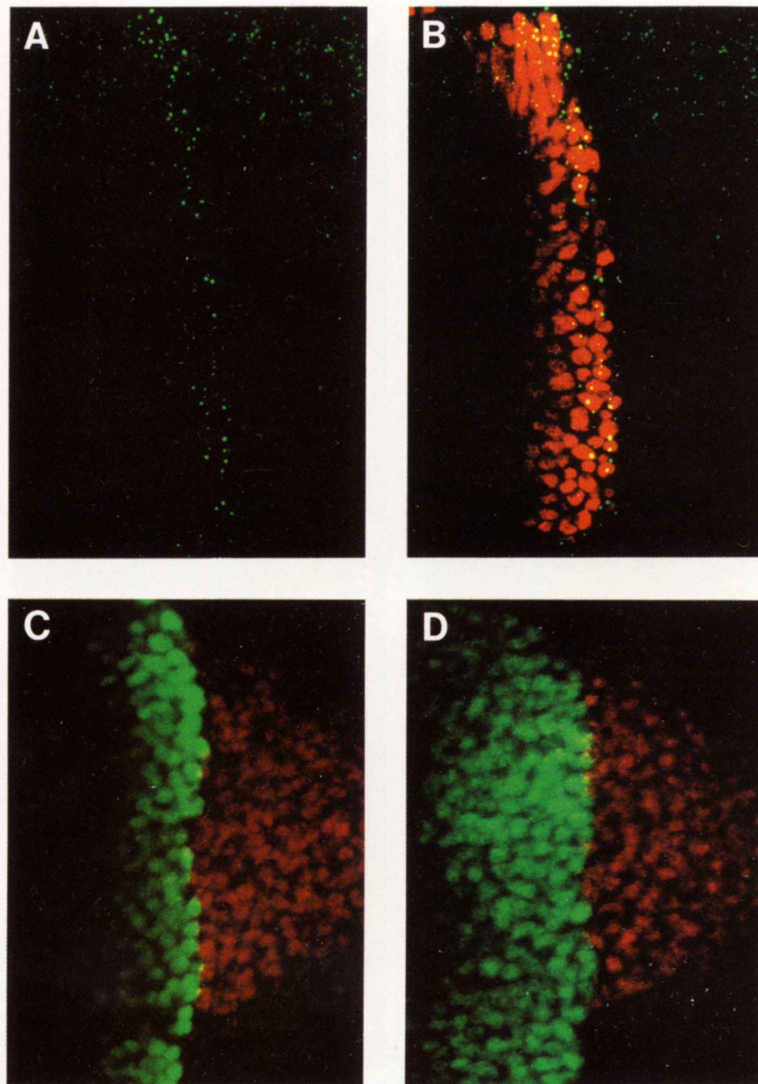

Figure 5. HH Protein Accumulation and *patched* and *decapentaplegic* Gene Expression Meet Along the A–P Compartment Border of Wing Imaginal Discs

HH protein accumulation (green) in wing imaginal discs was revealed as dots in the anterior compartment (A) along the A–P border. The merged image in (B) shows that these dots overlap the domain of *patched* expression (red) that was visualized by immunostaining the *patched* enhancer trap line. Weak HH signal that can be detected in the posterior compartment is not seen in this figure because it was in a different focal plane. Imaginal disc expression patterns of *patched* and *dpp* were localized by immunostaining discs of early to mid third instar larvae from *patched* and *dpp* enhancer trap lines. Both *patched* (C) and *dpp* (D) are restricted to the anterior compartment (green staining), and both abut the *engrailed* expression domain (red).

teins are present in identical patterns (Figures 4A–4C). As described above, synthesis of HH protein in the anterior compartment cells of the wing imaginal disc can perturb development of the anterior wing. However, when wing imaginal discs were probed with HH-Ab, we detected HH protein in both anterior and posterior compartment cells.

Staining wing imaginal discs with HH-Ab revealed different distributions of cross-reacting material at different levels within the disc epithelium. Near the apical surface of the wing pouch, staining was diffuse and was limited to the posterior compartment cells that also contained engrailed protein (Figures 4D–4F). However, at the extreme apical surface, dots of intense staining were seen (Figure 5A). These dots are reminiscent of the punctate structures revealed by HH-Ab in embryos, but the dots seen in the imaginal discs were smaller. The dots in the discs was largely confined to a stripe at the anterior–posterior compartment border (Figure 5A). The relative position of this stripe of dots in the wing discs was established by staining with a combination of probes. Wing imaginal discs from a strain carrying a *patched* enhancer trap insertion were stained with antibodies directed against engrailed protein and β-galactosidase. *patched*-dependent lacZ expression was present in a stripe in the anterior compartment that is aligned precisely at the anterior–posterior compartment border, and that complements but does not overlap the pattern of engrailed protein (Figure 5C). Discs from this strain that were stained with both HH-Ab and anti-β-galactosidase antibody revealed that the stripe of punctate HH staining and the stripe of *patched*-dependent expression overlap directly (Figure 5B). This observation suggests that HH protein secreted from the posterior compartment cells is not confined by the compartment border, but can cross the border to interact with neighboring anterior compartment cells.

hedgehog Function in Imaginal Development

The distribution of HH protein in the anterior compartment is apparently critical to normal wing development, since ubiquitous synthesis in the anterior compartment is deleterious (see above). In addition, the presence of HH protein along the anterior wing margin leads to abnormal growth.

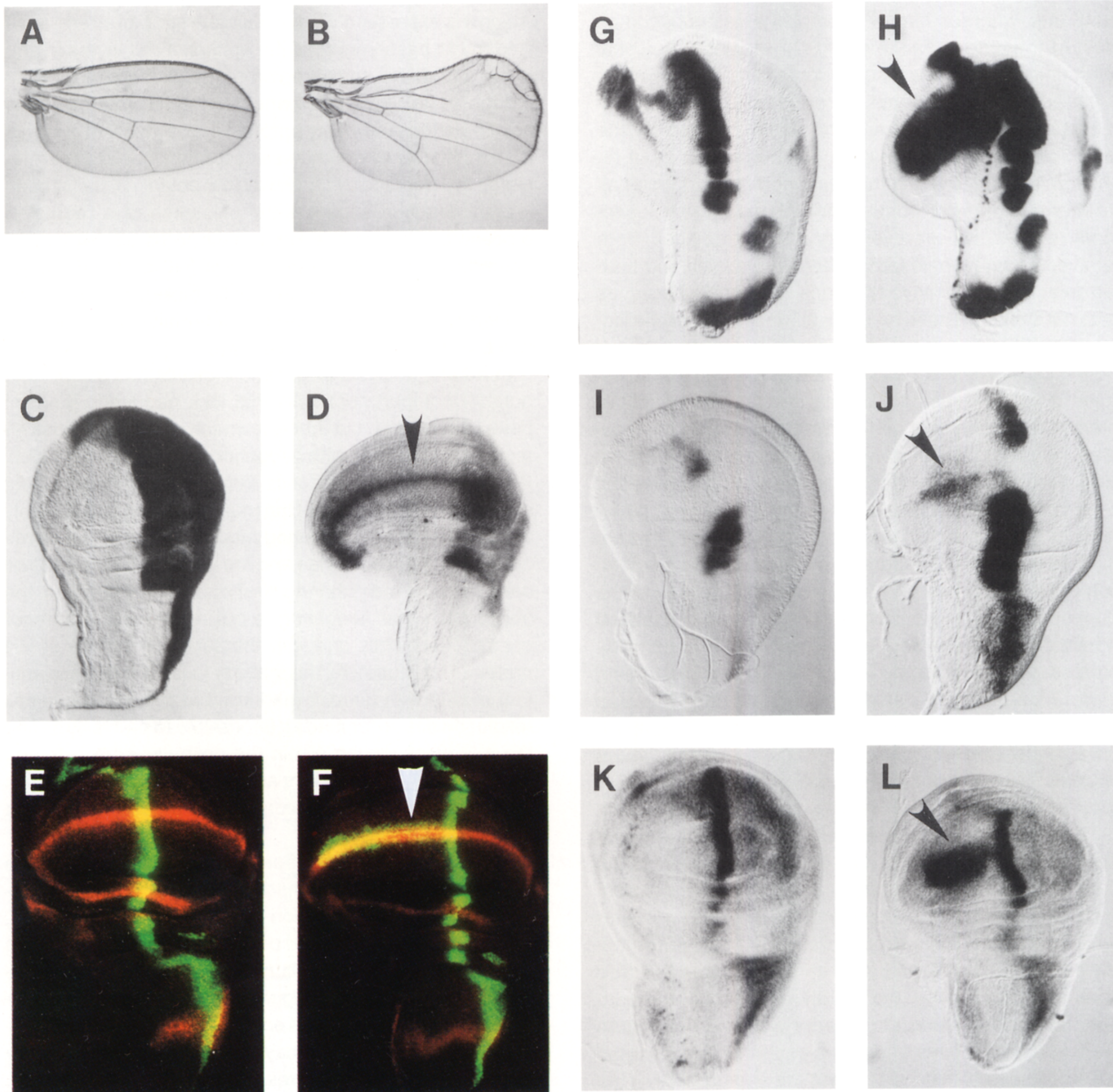

Figure 6. Comparisons of Wild-Type and *Moonrat* Wing Imaginal Discs

Adult wings are shown for wild type (A) and *Moonrat* (B). Gene expression in imaginal discs of third instar larvae is compared between wild type (C, E, G, I, and K) and *Mrt* (D, F, H, J, and L). In (C) and (D), *hedgehog* expression was visualized by in situ hybridization. (E) and (F) show *wingless* and *patched* expression patterns by double staining for the WG protein (red) and β-galactosidase (green) in the *patched* enhancer trap line. The remaining panels show X-Gal staining of the folowing enhancer trap lines in wild-type and *Mrt* backgrounds: (G) and (H), *dpp*; (I) and (J), *LF06*; (K) and (L), P1531. Ectopic gene expression along the D-V border in the *Mrt* discs is indicated by the arrowheads in (D), (F), (H), (J), and (L).

This conclusion is based upon analysis of the *Moonrat (Mrt)* mutant. *Mrt* flies have deformed appendages, most notably their wings, in which a network of extra veins form in the anterior compartment and wing blades develop abnormal shapes that are characterized by expansion along the anterodistal edge (Figure 6B). Expansion is greatest in the more distal regions, and at the wing margin the regions of expansion are invariably associated with bristles characteristic of the distal wing, not of the medial triple row (data not shown). *Mrt* wing discs are similarly deformed, and the anterior portion of the wing blade primordium is significantly enlarged. In these mutant discs, *hedgehog* expression was also abnormal. Whereas *hedgehog* expression in the posterior compartment was unaffected by the *Mrt* mutation, *hedgehog* expression in the anterior compartment, which is normally nonexistent, was present along the wing margin in the anterior compartment (Figure 6D). HH protein in these ecotpic locations was present in concentrated dots at the extreme apical surface of the wing disc epithelium (data not shown).

Two lines of evidence suggest that the *Mrt* mutation affects the *hedgehog* gene directly, and that *Mrt* is a dominant *hedgehog* allele. First, J. Kennison, who isolated *Mrt*, mapped it meiotically to a location near *hedgehog* and

found that, whereas *Mrt* fully complements *hedgehog* alleles, *Mrt* revertants fail to do so (J. Kennison, personal communication). These results suggest that *Mrt* is an allele of the *hedgehog* locus, but, unfortunately, these *Mrt* revertants no longer exist, and the parental strain for the *Mrt* chromosome is unavailable (although the size of restriction fragments within the *hedgehog* transcription unit are altered in the *Mrt* chromosome). We therefore independently isolated two *Mrt* revertants after X irradiation. Both behaved as embryonic lethal *hedgehog* alleles and both had deletions that removed portions of the *hedgehog* locus (data not shown). These results confirm the earlier characterization of *Mrt* as a dominant *hedgehog* allele.

If *Mrt* causes misexpression of *hedgehog* and the consequent overgrowth along the wing margin, it is of special interest to identify other genes that might also be misregulated. *wingless*, which is normally expressed along the wing margin, along the perimeter of the wing pouch, and in part of the notum, and which is thought to be a target of *hedgehog* signaling in embryonic development, was not affected in *Mrt* discs (Figure 6F). In contrast, the expression of several genes that are normally expressed at the anterior–posterior compartment border were changed dramatically. An example is *patched*, which is normally expressed predominantly along the A–P compartment border. A *Mrt* mutant carrying a *patched* enhancer trap expressed β-galactosidase, both along the A–P compartment border and along the anterior wing margin where *wingless* is expressed (Figure 6F). Another example is *dpp*, which encodes a TGFβ-related ligand (Padgett et al., 1987), and which is normally expressed in the anterior compartment of the wing disc only in the region of the compartment border (Raftery et al., 1991). Like *patched*, its domain of expression complements, but does not overlap with *engrailed* expressing cells (Figure 5D). A *Mrt* mutant carrying a *decapentaplegic* enhancer trap expressed β-galactosidase along the anterior–posterior compartment border and along the anterior wing margin (Figure 6H). *LF06* is the designation for a gene that is expressed in a stripe several cells wide along the anterior side of the anterior–posterior compartment border, and its putative coding sequence suggests that its protein product is secreted (S. Eaton and T.B. K., unpublished data). A *Mrt* mutant carrying a LF06 enhancer trap expressed β-galactosidase both along the anterior–posterior compartment border and along the anterior wing margin (Figure 6J). Similar observations were obtained for *aristaless* (data not shown) and for another enhancer trap strain, P1531, which has an insert at polytene region 42 E/F (K. Johe, S. Eaton, B. Yoshinaga, and T.B.K., unpublished data), and whose expression is also border-specific (Figure 6L).

Discussion

The Activity and Targets of HH Protein

Using a serum antibody directed against the putative HH protein, we found that much of the HH protein in embryos and imaginal discs is concentrated in discrete membrane-associated structures at some distance from the cells in which it is expressed. These punctate structures are localized to the basal region of the epithelial cell layers in embryos and to the apical region of the disc epithelium. Although we do not yet know whether the HH protein in these accumulations represents protein that is involved in signal transduction or whether these accumulations are intermediates in a degradation pathway, the polarity of the subcellular distribution suggests the existence of a regulated process that transports HH protein to its site of action. Therefore, although these findings fail to identify either the active form of HH protein or its molecular target, our immediate interest has been to determine what information the HH protein signal conveys.

In adults, analysis of genetic mosaics has shown that anterior compartment cells that lack hedgehog function develop normally, but that elsewhere, nonautonomous effects are associated with hedgehog clones (Mohler, 1988). Posterior compartment cells that are genetically mutant and tissue in the vicinity of the mutant cells can be severely affected by loss of hedgehog function. The normal development of anterior clones and the dominant effect mutant cells have on neighboring tissue is consistent with the observation that *hedgehog* is not expressed in anterior compartment cells and with the secretory nature of HH protein. Unfortunately, the inability to label *hedgehog* mutant clones with genetic markers that provide single-cell resolution has so far made it impossible to precisely define the role of *hedgehog* within the posterior compartment. Nevertheless, an important aspect of *hedgehog* activity is its influence on anterior compartment cells that do not express the gene.

In embryos, *hedgehog* appears to suppress the negative regulatory effect that *patched* has, both on its own expression and on the expression of *wingless* (Ingham et al., 1991). The presence of HH protein in *patched*-expressing cells is consistent with this model, as is the relationship between *patched* and *hedgehog* stripes. *patched* expression is initially robust in the *engrailed* (and *hedgehog*) non-expressing cells, but it decays in cells that do not directly contact the *hedgehog*-expressing cells (Hooper and Scott, 1989; Nakano et al., 1989). The eventual pattern that matures (*hedgehog* stripes contacted on both sides by *patched* stripes) indicates that the influence of HH protein is symmetrical. Consistent with these patterns, our HH-Ab staining revealed no asymmetry in the distribution of HH protein around the domain of *hedgehog* expression. We conclude that the parasegment border did not impede HH protein movement.

The postulated inhibitory influence of HH protein on *patched* activity was also verified by the phenotypes of transgenic animals that had received a pulse of ubiquitous *hedgehog* expression with a heat shock regimen. To a remarkable extent, both embryos and adults phenocopied *patched* mutants. The similarities between the heat shock phentoypes and *patched* mutants was particularly striking in the wings, where abnormalities developed only in anterior compartments. For *patched*, the compartment specificity of its mutant phenotype is consistent with its anterior compartment-specific domain of expression. For *hedge-*

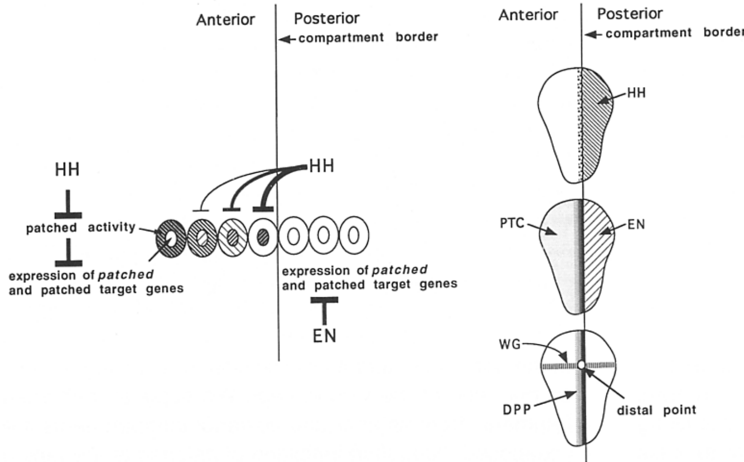

Figure 7. Proposed Mechanism by which *hedgehog* Relates the Anterior–Posterior Axis to the Proximodistal Axis

Diagrammed on the left are the relevant genetic interactions and, on the right, patterns of the protein distribution in the wing imaginal disc. *patched*, which is expressed throughout the anterior compartment, is thought to repress the expression of several target genes. *hedgehog* and *engrailed* are expressed in the posterior compartment. Secreted HH protein antagonizes the *patched* repressive activity in the anterior compartment, while engrailed protein directly represses the transcription of *patched* and other downstream target genes in the posterior compartment. Because HH protein is secreted from the posterior compartment anteriorly across the A–P border, the *patched* repressive activity is antagonized there in a graded fashion. In the cells closest to the border, the downstream genes are most completely released from *patched* repression, whereas in the more anterior cells, *hedgehog* derepression is only partial. This results in a sharply defined border of expression at the posterior edge, but a gradual decrease in expression of the target genes anteriorly. Cells that also express *wingless* and *dpp* are fated to become the distal point (distal most structure of adult wings) (bottom right).

hog, the implication is that posterior compartment cells are unaffected by elevated levels of HH protein, and that anterior compartment cells as far away from the compartment border as the anterior margin can respond to HH protein.

Developmental Compartments, Compartment Borders, and Axis Specification

Developmental compartments are spatially-defined groups of cells that are united by their commitment to a common lineage (Garcia-Bellido et al., 1973). Developmental compartments are units within which growth and pattern are regulated; they are what was classically termed developmental fields. Imaginal primordia are composed of two such compartments (anterior and posterior) that are juxtaposed along their common compartment border. These compartment borders likely correspond to the parasegment borders in the embryo, since the imaginal primordia are thought to develop from groups of cells chosen from either side of the parasegment border. Both borders are absolute lineage restrictions and both partially define limits to the domain of expression of patterning genes, such as *engrailed, hedgehog, cubitus-interruptus-Dominant (ci-D)* and *patched*. What is a compartment border and what does it do? Although it can have a physical presence (a double row of aligned cells has been observed at the A–P border in the wing and leg discs [D. Fristrom, personal communication; Blair, 1992]) we know little of its structure or its properties. It is not, apparently, a barrier to passage for HH protein.

We argue above and report here that the domains of expression of *patched, dpp, LF06*, and P1531 align precisely with *engrailed* at the compartment border. Since this is at variance with several previous publications (Blair, 1992; Phillips et al., 1990; Raftery et al., 1991), a clarification is warranted. Whereas some anterior compartment cells near the A–P border express *engrailed* in late third instar discs, *engrailed* expression and the posterior lineage compartment coincide precisely in discs from younger larvae (Blair, 1992). We have confirmed these observations and the results reported here describe expression patterns in early third instar discs. Although we do not understand what leads to expansion of the domain of *engrailed* expression in older larvae, we believe it to be without functional significance: neither anterior nor posterior clones cross the compartment border, and cells on the anterior side of border have no requirement for *engrailed* function (Kornberg, 1981; Lawrence and Morata, 1976; Morata and Lawrence, 1975).

We propose that the stable expression of *engrailed* on only one side of the compartment border creates an initial asymmetry, and that in the imaginal discs this asymmetry has several possible consequences. One interpretation might be that the induction of *patched, dpp, aristaless, LF06*, and P1531 expression by *hedgehog* in the anterior compartments of *Mrt* wings is indicative of an ectopic A–P compartment border, and that the overgrowth phenotype of this mutant is a consequence of a new patterning influence. A second possible interpretation is that the asymmetry at the A–P compartment border is normally elaborated by a series of genetic interactions to define an orthogonal proximodistal axis, and that an ectopic proximodistal axis forms in the *Mrt* wings. We base this statement on the following model (Figure 7). In posterior compartment cells where *engrailed* and *hedgehog* are specifically expressed, *engrailed* represses a number of genes, including *patched, ci-D, dpp, LF06*, and P1531. HH protein, in turn, diffuses across the compartment border, where it inhibits *patched* activity in the anterior compartment. Its inhibitory activity is greatest near the A–P compartment border where its concentration is highest and where the normal development of clones of *patched* mutant cells indicates that *patched* function is not required (Phillips et al., 1990). Since *patched* is expressed ubiqui-

tously in the anterior compartment and is thought to indirectly repress its own expression (Ingham et al., 1991), the paradoxical consequence of HH protein inhibition of patched activity is to enhance *patched* expression on the anterior side of the A–P border. In addition, the expression of genes such as *dpp*, *LF06*, and P1531, whose expression is thought to be negatively regulated by *patched*, is also elevated in the region of high HH protein concentration. The patterns of expression of these genes in a spatially well-defined band is ultimately dependent upon the posterior compartment-specific pattern of *hedgehog* expression.

Although the function of *LF06* and P1531 is not known, *dpp* is involved in generating the proximodistal axis in discs (Spencer et al., 1982). The distal extreme in discs is located where the band of *dpp*-expressing cells along the A–P border transect the band of cells that express *wingless*. The causal relationship between the secreted signaling proteins *dpp* and *wingless* encode is indicated by the duplicated appendages that have been observed after ectopic induction of *wingless* (Campbell et al., 1993). Presumably, these duplicated appendages arise because *dpp-* and *wingless*-expressing cells find a new region of close association and there create a new proximodistal axis. We propose that similar interactions lead to the *Mrt* phenotype. For reasons that are not understood, *hedgehog* is expressed ectopically along the wing margin in the anterior compartment in *Mrt* wing discs. The immediate consequence, presumably, is to inhibit *patched* in this region of the anterior compartment. This ultimately leads to expression of the genes that *patched* normally represses, including *dpp*, *LF06*, P1531 (Figure 6), and *aristaless* (data not shown). Since in these mutant discs *dpp* and *wingless* intersect abnormally along the anterior wing margin (*wingless* is normally expressed along the wing margin), an extended ectopic proximodistal axis is created that causes overgrowth. The proposed role of *hedgehog* in generating the proximodistal axis is consistent with the loss of distal structures in legs and antennae associated with clones of *hedgehog* mutant clones (Mohler, 1988).

We consider two issues related to this model. Studies of regeneration in insects led to rules of pattern regulation along the proximodistal axis. These rules are formally described by a model in which positional information is specified in terms of polar coordinates, of which one component is a value corresponding to position on a circle, and the second is a value for position on a radiating diagonal (Bryant et al., 1981). This model is complicated by the observation that positional values were nonrandomly distributed within discs, and that regeneration along the proximodistal axis required certain specific regions (Karlsson, 1980; Schubiger and Schubiger, 1978). A boundary model was then formulated in which compartment borders were proposed to act as organizing centers, with the most distal structure formed around the intersection of A–P and D–V compartment borders (Meinhardt, 1983). This boundary model is consistent with the proposal that the A–P compartment border delineates the domain of *dpp* expression, and that the distal extreme is localized in the region in which cells espressing *wingless* and *dpp* cells intersect.

Experiments comparing the regenerative capacities of different fragments of wing imaginal discs revealed that proximal fragments that consist entirely of either anterior or posterior compartment cells could not regenerate distally. In contrast, many fragments containing tissue from both compartments could regenerate distal structures (Karlsson, 1980). More recently, a systematic screen of enhancer trap lines identified several lines with altered expression in regenerating discs (Brook et al., 1993). Several of the lines express *lacZ* along the A–P border in normal discs and express *lacZ* in regenerating discs only when the anterior and posterior compartments are juxtaposed by wound healing. One of these lines is an insertion at the *dpp* locus, and its expression is restricted to cells on one side of the wound heal. We suggest that when fragments from anterior and posterior compartments are juxtaposed, *hedgehog* inhibition of *patched* at the region of juxtaposition leads to *dpp* induction, to new positional values, and to regeneration of distal structures.

Finally, we consider the distance over which the dominant and nonautonomous effects of HH protein extend. It would seem that, in embryos, patched activity more than a single cell distant from the stripe of *hedgehog*-expressing cells is immune to HH-dependent inhibition. In discs, this distance appears to be greater. However, we note that in the wing blade, clones of *patched* mutant cells differ radically in phenotype depending upon their location within the anterior compartment. Clones close to the A–P compartment border developed normally; centrally-located clones developed abnormal patterns; and clones near the anterior wing margin died (Phillips et al., 1990). Thus, the apparent requirement for *patched* function was graded, and we wonder whether this graded requirement might be a consequence of a gradient of HH protein. We did not observe a HH protein gradient, so for this conjecture to be valid the concentration of HH protein in the gradient must have been below the level that our HH-Ab was capable of detecting, and the response of *patched* to the gradient must be nonlinear.

Experimental Procedures

Preparation of Anti-Hedgehog Antiserum and Western Blot Analysis

A plasmid expressing HH protein sequences C-terminal to the putative transmembrane domain (residues 90–471) was constructed by cloning a EcoNI–HpaI 1.7 kb fragment from chh46 (Tabata et al., 1992) into pET15b (Novagene). The recombinant protein was induced in *Escherichia coli* BL21 (DE3) with isopropyl-β-D-thiogalactopryanoside and was recovered as an inclusion body. The protein was purified through SDS-PAGE and electro-eluted from the gel. Rabbits were immunized with the purified protein by standard methods. Antiserum (HH-Ab) was affinity-purified by adsorption to bacterially produced HH protein that had been immobilized on nitrocellulose filters. Protein samples from embryos, Schneider cells, and imaginal discs were resolved on 10% SDS gels and were electroblotted onto nitrocellulose filters. The filters were incubated with HH-Ab, washed, and incubated with biotin-conjugated donkey anti-rabbit antibody. After further washing, streptavidin-conjugated horseradish peroxidase was added. The filters were developed using chemiluminescence reagents (Amersham).

Schneider Cell Transformation

Schneider line 2 cells were transfected with a plasmid (Act-hh-neo) in which the *hedgehog* cDNA is constitutively expressed under the control of an actin promoter fragment. Act-hh-neo was constructed by

placing the *hedgehog* cDNA into pUChsneo-act (Thummel et al., 1988). Permanent cell lines were established by long-term selection in the presence of geneticin (G418).

Immunocytochemistry and Immunofluorescence

HH-Ab was preadsorbed for 2 hr at a dilution of 1:1500 with 0.2 volume of fixed wild-type embryos. Preadsorbed antiserum was then incubated with 0.1 volume of embryos at 4°C overnight. Secondary antibody (donkey anti-rabbit antibody conjugated to biotin) was added and incubated for 2 hr, followed by a 1 hr incubation with preformed AB complex (Vectorstain). The DAB staining kit (Vectorstain) was used to visualize the reaction.

Immunofluorescence double-labeling of embryos and imaginal discs was performed with the following antibodies, essentially as described above. For a primary, mouse anti-engrailed (4D9, [Patel et al., 1989]), rabbit anti-wingless (van den Heuvel et al., 1989), and mouse (Promega) and rabbit (Cappel) anti-β-galactosidase antibodies were used. For a secondary, donkey anti-mouse conjugated to biotin, donkey anti-mouse conjugated to Cy5, and donkey anti-rabbit conjugated to fluorescein isothiocyanate were used. For the enhancing the signal, streptavidin conjugated to fluorescein isothiocynate or Cy5 were used in the proper combinations. Images were taken on a Laser Scanning Confocal Microscope (Bio-Rad MRC600).

Monensin Treatment of Embryos

Wild-type embryos were treated with monensin essentially as described by González et al. (1991). Embryos aged 4–6.5 hr post fertilization were treated with 50% bleach to remove the egg shell chorion and followed by a 1:1 mixture of octane and insect tissue culture medium (GIBCO BRL) to render them permeable. The incubation with monensin (70 nM) was for 1 hr and was followed by the standard protocol for fixation and processing for light microscopy.

Ubiquitous Expression of Hedgehog Protein

A full-length *hedgehog* cDNA (the 2.2 kb DraIII-HpaI fragment of chh46) was subcloned into the pCaSpeR-hs vector, so that the hsp70 promoter is upstream of the cDNA. Flies were transformed by the standard methods for P element-mediated transformation (Spradling, 1982). Homologous fly lines carrying a P element insert on the X chromosome were used for heat shock treatments as follows. Embryos collected 2.5–3.5 hr after egg laying were subjected to three cycles of heat treatment at 37°C. Each heat shock was for 30 min and the embryos were allowed to recover at 25°C for 30 min between each heat treatment. After the third heat treatment, the embryos were either allowed to develop at 25°C for 2.5 hr before fixation and processing for antibody staining or in situ hybridization, or they were allowed to develop for 36 hr at 25°C prior to cuticle preparation.

For the analysis of the adult wing phenotype, third instar larvae were subjected to the heat treatments of 30 min at 37°C followed by 30 min at 25°C as above, except that 10 cycles of heat treatment per day were performed for 3 days.

Reverting the *Moonrat* Mutation

Mrt males were irradiated with X-rays (3000 rad) and were mated to *Dr/TM3 Sb* females. Of 8620 F1 flies, two putative revertants with wild-type wings were obtained. Both revertants were embryonic lethal and produced embryonic cuticle with a strong *hedgehog* phenotype; neither complemented the embryonic lethality or cuticle phenotype hh^{IJ}. Southern blot analysis revealed the DNA in the *hedgehog* region in both revertants to be altered (data not shown).

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