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Protein Kinase A and Hedgehog Signaling in Drosophila Limb Development

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Summary

The Drosophila *hedgehog* (*hh*) gene encodes a secreted protein involved in organizing growth and patterning in many developmental processes. Hh appears to act by inducing the localized expression of at least two other signaling molecules, decapentaplegic (dpp) and wingless (wg), which then govern cell proliferation and patterning in surrounding tissue. Here, we demonstrate that cyclic AMP (cAMP)-dependent protein kinase A (PKA) is essential during limb development to prevent inappropriate *dpp* and *wg* expression. We also show that a constitutively active form of PKA can prevent inappropriate *dpp* and *wg* expression, but does not interfere with their normal induction by hh. We propose that the basal activity of PKA imposes a block on the transcription of *dpp* and *wg* and that hh exerts its organizing influence by alleviating this block.

Introduction

hedgehog (*hh*) encodes the founding member of a family of secreted proteins involved in organizing the development of body segments, limbs, eyes, and other organ systems in vertebrates and invertebrates (reviewed by Ingham, 1994). In Drosophila, *hh* is normally expressed in each limb primordium only by cells in the posterior compartment (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993). Conversely, all cells in the anterior compartment appear competent to respond to hh by secreting two other signaling molecules, decapentaplegic (dpp) and wingless (wg) (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994). As a consequence, hh secreted by posterior cells induces neighboring anterior cells to secrete dpp and wg, and these molecules, in turn, appear to organize cell proliferation and patterning in both compartments (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994). Similar models have been proposed to account for the organizing activity of hh in the larval segments and eye in Drosophila and in the vertebrate limb (Ingham et al., 1991; Heberlein et al., 1993; Ma et al., 1993; Laufer et al., 1994).

Although the available evidence in Drosophila argues that hh protein acts through its ability to induce *dpp* and *wg* expression, relatively little is known about the mechanism of hh signal transduction. For example, there is at present no compelling candidate for a hh receptor. Moreover, relatively few intracellular components have been identified that mediate *wg* or *dpp* transcription in response to hh signaling (reviewed by Hooper and Scott, 1992). However, one gene that plays a critical role is *patched* (*ptc*). *ptc* encodes a transmembrane protein that is expressed in all anterior cells in both the embryonic ectoderm and in the imaginal discs and that appears to block *wg* and *dpp* expression in cells that are not exposed to hh protein (Hooper and Scott, 1989; Nakano et al., 1989; Hidalgo and Ingham, 1990; Phillips et al., 1990; Ingham et al., 1991; Capdevila et al., 1994). Hence, hh signaling may induce *wg* and *dpp* expression by counteracting the action of ptc protein.

In the process of screening for new mutations affecting limb development, we have identified a recessive lethal mutation that causes anterior cells to behave as if they have received the hh signal, even when they are not near *hh*-expressing cells. Like anterior cells exposed to ectopic hh protein (Basler and Struhl, 1994), these mutant cells cause dramatic reorganizations of normal anterior pattern, including the formation of supernumerary double-anterior limbs. We show that this mutation is a loss-of-function allele of the previously identified *DC0* gene, which encodes the catalytic subunit of the Drosophila cyclic AMP (cAMP)-dependent protein kinase A (PKA) (Kalderon and Rubin, 1988; Lane and Kalderon, 1993). In addition, we demonstrate that the elimination of PKA activity in anterior cells causes them to express *dpp* and *wg*, and as a consequence, causes the reorganization of surrounding wild-type tissue. Finally, we show that expression of a constitutively active form of PKA is sufficient to suppress this inappropriate *wg* and *dpp* expression but does not interfere with the normal induction of these genes by hh, or their inappropriate expression in the absence of *ptc* gene activity. Based on these results, we propose that the basal activity of PKA imposes a block on *dpp* and *wg* transcription and that hh protein normally activates *dpp* and *wg* by counteracting this block.

Results

Similar Phenotypes Caused by Loss of PKA Activity and Ectopic *hh* Expression

The legs and wings of Drosophila are each subdivided into anterior and posterior compartments (Garcia-Bellido et al., 1973). As shown previously (Basler and Struhl, 1994), clones of anterior cells that express the hh protein ectopically can cause dramatic reorganizations of limb pattern (Figure 1B). In genetic screens for mutations that alter growth and patterning of the adult appendages, we obtained a recessive lethal mutation, *E95*, which causes a phenotype similar to that caused by ectopic *hh* expression (Figure 1A; see below).

The *E95* mutation maps to the chromosomal interval 30C, a region that includes the *DC0* gene, which encodes the catalytic subunit for cAMP-dependent PKA (Kalderon and Rubin, 1988; Lane and Kalderon, 1993). *E95* appears to be a loss-of-function allele of *DC0* by the following criteria: it does not complement a known *DC0* null allele ($DC0^{B3}$; Lane and Kalderon, 1993); $E95/DC0^{B3}$ zygotes that also

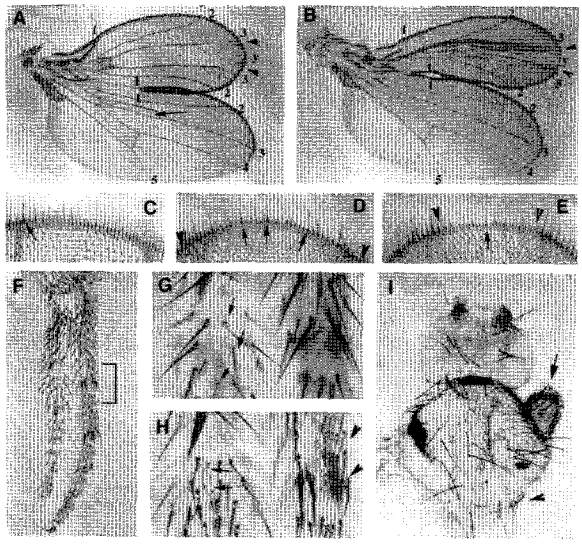

Figure 1. Organizing Influence of Anterior Compartment Clones Lacking PKA Activity

(A and B) Supernumerary double-anterior wings organized by a clone of *DC0*⁻ cells (A) or by a *tub>hh* clone that expresses *hh* constitutively under the control of the *tubulinα1* promoter (B). The anterior compartment of the normal wing (lower portion in each photograph) includes longitudinal veins 1–3, the posterior compartment includes longitudinal veins 4 and 5, and the compartment boundary runs proximodistally in a straight line just anterior to vein 4. Both clones form portions of the wing margin as well as a narrow stripe of cells on each surface of the wing that extends along the plane of symmetry of the supernumerary wing (arrowheads mark the boundaries of the clone along the wing margin; the *y* and *stc* markers are not visible in this photograph). The "normal" wing in (A) contains a second, small clone on the dorsal surface of the wing (arrow) that is associated with the formation of an ectopic vein by surrounding cells.

(C–E) Wing margin formed by wild-type cells close to the compartment boundary (C), or by cells exposed to ectopic hh protein (E) differentiate no, or very few, chemosensory bristles (arrows), in contrast with *DC0*⁻ cells (D), which do (arrowheads mark the clone boundaries).

(F–H) Supernumerary double-anterior hindleg (F; left) associated with anterior *DC0*⁻ clones positioned both dorsally (G) and ventrally (H) (arrows indicate mutant cells marked by *y*; arrowheads indicate posterior compartment structures [transverse row bristles] formed only by the normal limb; bracket in [F] indicates region enlarged in [G] and [H]).

(I) Two clones of *DC0*⁻ cells in the mesonotum associated with outgrowths of notal tissue (arrow) or the formation of extra scutellar bristles (arrowhead).

carry a *DC0*-rescuing transgene can develop into morphologically normal adults; the mutant phenotypes associated with the *E95* and *DC0*^{B3} mutations appear indistinguishable (data not shown). Hence, *E95* behaves genetically like a null allele of the *DC0* gene, and we refer to it subsequently as *DC0*⁻.

Clones of cells homozygous for the *DC0*⁻ mutation and marked by the mutations *yellow* (*y*) and *stubby chaete* (*stc*) were generated during the first and second instar using flp-mediated mitotic recombination (Golic, 1991; see Experimental Procedures). We describe the phenotypes associated with these clones in the wing and legs.

DC0⁻ clones in the wing fall into two classes (here and below; see Experimental Procedures for numbers of clones scored). Clones belonging to the first class appear to be completely normal and contribute to the posterior compartment or are restricted to the region between vein 3 and the compartment boundary in the anterior compartment (the region derived from cells that are normally exposed to hh protein). Clones belonging to the second class contribute to the remaining, more anterior portions of the wing and are invariably associated with abnormal patterns that resemble those caused by ectopic *hh* expression. Most strikingly, clones that contribute to both the dorsal and ventral surfaces of the wing appear to exert a long range influence on surrounding wild-type cells, including the formation of supernumerary double-anterior wings that display symmetric 123*y*321 vein patterns (Figure 1A; the numbers refer to longitudinal veins 1–3; "*y*" refers to the *DC0*⁻ mutant cells). As in the case of ectopic *hh*-expressing clones, *DC0*⁻ clones restricted to only the dorsal or ventral surface cause much less dramatic reorganizations of surrounding pattern, typically involving the formation of ectopic veins in close proximity to the *DC0*⁻ cells (Figure 1A, arrow). Finally, we note that *DC0*⁻ clones that contribute to the mesonotum (to which the wing is attached) cause outgrowths of surrounding tissue as well as the multiplication of particular macrochaetes (Figure 1I), providing further examples of anterior pattern duplication.

DC0⁻ clones differ from clones of ectopic *hh*-expressing cells in that *DC0*⁻ cells behave like anterior cells positioned a short distance from the compartment boundary (close to vein 3), whereas ectopic *hh*-expressing cells behave like anterior cells next to the boundary (close to vein 4). This difference is apparent in at least two ways. First, *DC0*⁻ cells that arise along the wing margin at a distance from the compartment boundary form chemosensory bristles interspersed with double-row bristles (Figure 1D), in contrast with similarly positioned ectopic *hh* clones that form these bristles only occasionally (Figure 1E). Second, the ectopic vein 3's associated with *DC0*⁻ clones arise immediately adjacent to the marked cells, whereas those associated with ectopic *hh*-expressing clones arise near, but not next to, these cells. Thus, the loss of PKA activity does not completely replicate the consequences of hh signaling at the normal compartment boundary or in response to ectopic *hh*-expressing cells. We note that anterior cells positioned along the compartment boundary express the *engrailed* (*en*) and/or *invected* (*inv*) genes late in larval life (Blair, 1992) and that this late *en–inv* gene activity appears to play a role in specifying the region of the wing lying between vein 3 and the compartment boundary (Hidalgo, 1994). Hence, anterior cells receiving hh signal may express *en* or *inv* late in larval life, which in turn blocks the differentiation of chemosensory bristles. In contrast, cells lacking PKA activity may be able to form chemosensory bristles because they do not express either *en* or *inv*.

The leg phenotypes associated with *DC0*⁻ clones are more complex but are consistent with the wing phenotypes. As in the wing, *DC0*⁻ clones in the posterior compartment appear phenotypically normal, whereas clones in the anterior compartment cause pattern duplications or the formation of supernumerary legs composed of symmetric anterior compartments (Figure 1F). Supernumerary legs with either complete or incomplete proximal–distal axes have been obtained and are usually associated with

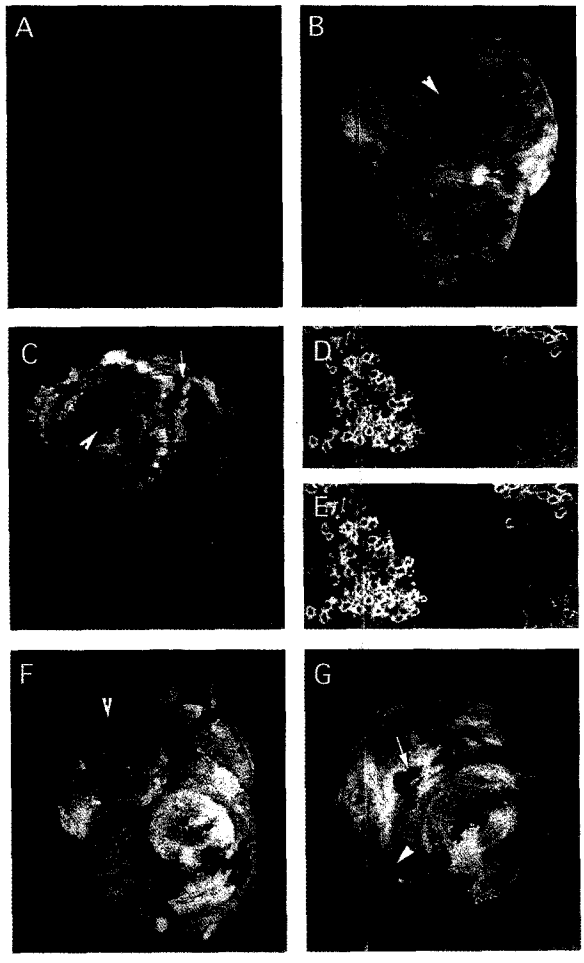

Figure 2. *dpp* and *wg*, but Not *hh*, Are Ectopically Expressed by Anterior Wing Cells Lacking PKA Activity

(A and B) Wing disc bearing clones of *DC0*$^-$ cells (e.g., arrowhead; clones are marked by the absence of green CD2 staining) in which *hh–lacZ* expression (red) is restricted to the posterior compartment.

(C) An anterior clone of *DC0*$^-$ cells (arrowhead) associated with ectopic *dpp–lacZ* expression (arrow marks a posterior clone that does not express *dpp–lacZ*).

(D and E) An anterior *DC0*$^-$ clone illustrating that only cells within such clones express the *dpp–lacZ* gene.

(F and G) Ectopic *dpp–lacZ* (F) and *wg–lacZ* (G) expression associated with anterior *DC0*$^-$ clones (arrowheads) positioned dorsally or ventrally, respectively, in the anterior compartment of the leg disc; arrow marks the absence of *wg–lacZ* expression in a dorsally positioned clone.

marked mutant cells both dorsally and ventrally (Figures 1G and IH). In both respects, *DC0*$^-$ mutant clones resemble clones of ectopic *hh*-expressing cells (Basler and Struhl, 1994).

Loss of PKA Activity Causes Ectopic Expression of *dpp* and *wg*, but Not *hh*

The similarity in phenotype caused by loss of PKA activity and ectopic *hh* expression could arise because cells lacking PKA activity inappropriately express *hh*. To test this possibility, we induced clones of *DC0*$^-$ cells in imaginal wing discs and assayed them for *hh* expression by in situ hybridization for *hh* transcripts, or by the expression of a *hh–lacZ* reporter gene (*hh*^{P30}; Lee et al., 1992). In all cases examined, *hh* expression was restricted to the posterior compartment (Figures 2A and 2B). Hence, the organizing activity associated with *DC0*$^-$ clones does not appear to be due to ectopic *hh* expression.

In the wing, hh normally induces only *dpp* expression, whereas in the legs, it induces *dpp* in dorsal cells and *wg* (as well as a low level of *dpp*) in ventral cells. We therefore tested whether *dpp* and *wg* are ectopically expressed in cells lacking PKA activity as assayed both by in situ hybridization and by the expression of *dpp–lacZ* and *wg–lacZ* reporter genes (*dpp–lacZ3.0*, Blackman et al., 1991; *wg–lacZ*, Struhl and Basler, 1993). We find that *dpp–lacZ* is ectopically expressed in *DC0*$^-$ clones located in the anterior but not in the posterior compartment of the wing disc (Figure 2C). Moreover, all *DC0*$^-$ cells within these clones appear to express *dpp–lacZ*, whereas none of the surrounding cells do so (Figures 2D and 2E). Similar results were also obtained in the leg discs, although in this case, high levels of ectopic *dpp–lacZ* expression were observed only in anterior *DC0*$^-$ cells positioned dorsally (Figure 2F), whereas ectopic expression of *wg–lacZ* was observed only in anterior *DC0*$^-$ clones positioned ventrally (Figure 2G). Thus, PKA is required in a cell-autonomous fashion to block inappropriate *dpp* and *wg* expression during wing and leg development.

Anterior Cells Lacking PKA Activity Reorganize Surrounding Tissue Because They Express *dpp* and *wg* Ectopically

To test whether the long range organizing activity of *DC0*$^-$ mutant cells is due to their expression of *dpp* or *wg*, we examined the phenotypes associated with *DC0*$^-$ clones that also lack the function of either *dpp* or *wg* or both. As described in Experimental Procedures, the null alleles *dpp*^{H61} (Posakony et al., 1990) and *wg*^{CX4} (Baker, 1987) were used to eliminate *dpp* and *wg* gene activity and are referred to subsequently as *dpp*$^-$ and *wg*$^-$.

Most *dpp*$^-$ *DC0*$^-$ clones in the wing, including those that arise anterior to the compartment boundary and contribute to both dorsal and ventral surfaces, do not appear to alter the normal shape or size of the appendage and can contribute to correctly patterned structures such as the wing margin (Figures 3A and 3B). Similarly, *dpp*$^-$ *DC0*$^-$ clones in the mesonotum do not appear to reorganize surrounding tissue (data not shown). Hence, we infer that singly mutant *DC0*$^-$ clones cause global reorganizations of wing and notum pattern because they express *dpp*.

We did observe one class of *dpp*$^-$ *DC0*$^-$ clones that exert a long range influence on the behavior of surrounding wild-type tissue. All of the clones in this class marked wing tissue in the vicinity of the compartment boundary (the domain of the wing in which *dpp* is normally induced in response to hh protein) and were associated with a marked reduction in wing size, as well as the loss of vein pattern in both the anterior and posterior compartments (Figure 3C). This phenotype is attributable simply to the loss of *dpp* gene activity, as it is observed in singly mutant *dpp*$^-$ clones (Posakony et al., 1990; data not shown).

We also note two phenotypes associated with *dpp*$^-$ *DC0*$^-$

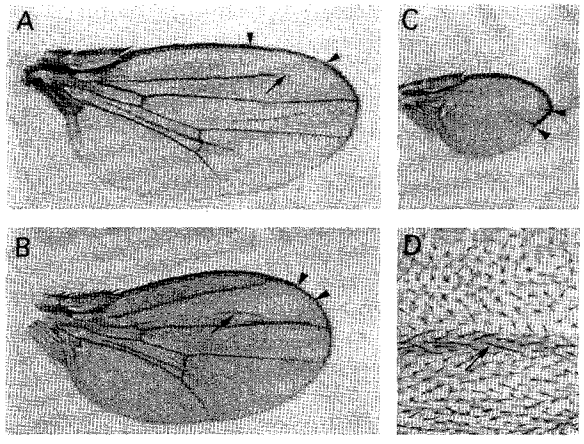

Figure 3. *dpp* Is Required for the Long-Range Organizing Influence Associated with Wing Clones Lacking PKA Activity

(A and B) *dpp*$^-$ *DC0*$^-$ clones that contribute to both the dorsal and ventral surfaces of the wing (arrowheads mark the boundaries of the clone along the wing margin; *y* and *stc* markers are not visible in these photographs). These clones do not reorganize wing pattern (compare with Figure 1A). The clones in (A) and (B) mark triple- and double-row bristles along the wing margin, respectively (not visible in this photograph). The clone in (A) fails to differentiate wing vein (arrow), and the clone in (B) is associated with an ectopic vein (arrow) formed by neighboring wild-type tissue (B and D).

(C) An anterior *dpp*$^-$ *DC0*$^-$ clone positioned in the vicinity of the compartment boundary that blocks proliferation and patterning in both compartments.

(D) Higher magnification of (B); note the presence of marked (smaller or tufted) hairs above the ectopic vein (arrow).

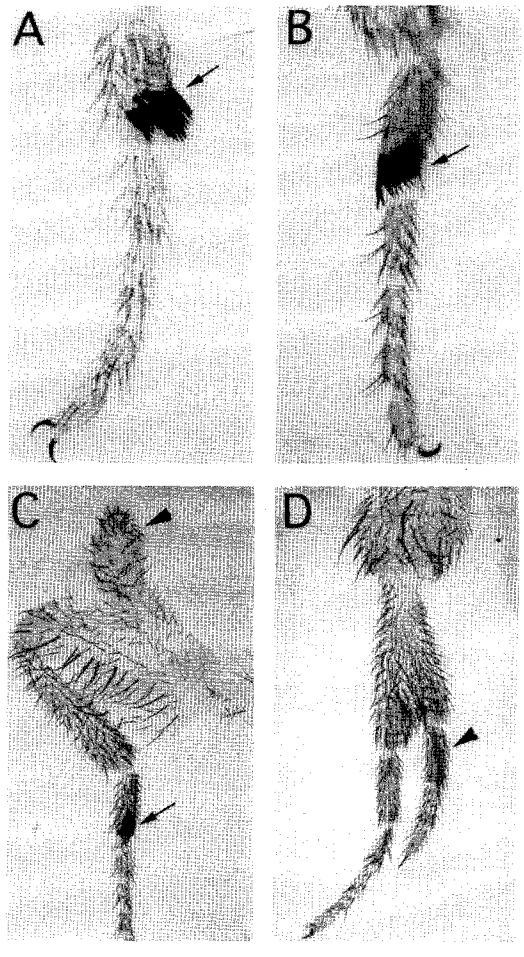

Figure 4. *dpp* and *wg* Are Required for the Long-Range Organizing Influence Associated with Leg Clones Lacking PKA Activity

(A and B) Forelegs bearing laterally positioned *dpp*$^-$ *DC0*$^-$ (A) and *dpp*$^-$ *wg*$^-$ *DC0*$^-$ (B) clones in the anterior compartment. These clones do not organize supernumerary limbs, but can induce neighboring cells to form extra ventrolateral structures such as sex comb teeth (arrows; compare with normal sex comb [arrow in C]).

(C and D) Forelegs bearing *wg*$^-$ *DC0*$^-$ clones causing a dorsolateral outgrowth (C; arrowhead) or associated with a supernumerary limb (D; arrowhead).

clones that appear to reflect short range, *dpp*-independent consequences of the loss of PKA activity. First, doubly mutant clones can induce their immediate neighbors to form ectopic veins (Figures 3B and 3D) and margin bristles (data not shown), a phenotype that is not associated with singly mutant *dpp*$^-$ clones (Posakony et al., 1990). Second, *dpp*$^-$ *DC0*$^-$ clones fail to differentiate wing veins in a cell-autonomous fashion (Figure 3A). The interpretation of this "no vein" phenotype is complicated by the fact that *dpp* has at least two distinct functions in wing development: the *disc* function required for the global control of cell proliferation and patterning and a genetically separable *shortvein* function required for wing vein differentiation (Segal and Gelbart, 1985; Posakony et al., 1990). Hence, the absence of vein differentiation in *dpp*$^-$ *DC0*$^-$ clones can be attributed simply to the complete absence of *dpp* gene function. However, we have also generated clones of *DC0*$^-$ cells that are homozygous for the *dpp*^{d12} mutation, which lacks the *disc* function but retains the *shortvein* function (Posakony et al., 1990). Such *dpp*^{d12} *DC0*$^-$ clones also fail to form veins in the anterior compartment and in all other respects appear indistinguishable from *dpp*$^-$ *DC0*$^-$ clones in this compartment (data not shown). Thus, the failure of *dpp*^{d12} *DC0*$^-$ cells to form anterior veins appears to reflect a second, *dpp*-independent consequence of the loss of PKA activity.

As in the wing, *dpp*$^-$ *DC0*$^-$ clones in the leg do not organize supernumerary double-anterior limbs (Figure 4A). This result is expected given that the formation of supernumerary legs in response to ectopic *hh* expression appears to depend on generating both ectopic *dpp* and *wg* expression (Basler and Struhl, 1994; Diaz-Benjumea et al., 1994; Campbell and Tomlinson, 1995). Similarly, we do not find supernumerary legs associated with *dpp*$^-$ *wg*$^-$ *DC0*$^-$ clones (Figure 4B). However, we were surprised to find that *wg*$^-$ *DC0*$^-$ clones can cause local outgrowths of leg tissue (Figure 4C) as well as the formation of supernumerary legs (Figure 4D). These supernumerary legs differ in several respects from the double-anterior limbs organized by singly mutant *DC0*$^-$ clones, suggesting that they are not equivalent. In particular, they appear to branch out ventrally rather than laterally from the normal leg and to form both anterior and posterior compartment patterns (data not shown). Moreover, they are relatively rare. Because we do not find these atypical limbs associated with

triply mutant *dpp⁻ wg⁻ DC0⁻* clones, we infer that their formation depends on ectopic *dpp* expression in *wg⁻ DC0⁻* cells. Hence, we suggest that ventrally situated *wg⁻ DC0⁻* clones can express elevated levels of *dpp* and thereby induce the formation of a supernumerary limb if they are positioned close to wild-type *wg*-expressing cells along the compartment boundary (see Discussion).

Finally, we find evidence that the pattern abnormalities associated with *DC0⁻* clones in the legs cannot be attributed solely to the ectopic expression of *dpp* and *wg*. In particular, triply mutant *dpp⁻ wg⁻ DC0⁻* clones that arise in the ventral portion of the leg can influence the behavior of their immediate neighbors, causing them to form extra ventrolateral structures such as sexcomb teeth (Figure 4B), in contrast with similarly positioned *dpp⁻ wg⁻* clones (data not shown).

These results show that clones of cells lacking PKA activity cause long range reorganizations of wing and leg pattern because they ectopically express *dpp* and *wg*. However, they also provide evidence for short range reorganizations of pattern that are *dpp* and *wg* independent.

Hh Does Not Induce *dpp* and *wg* Transcription by Down-Regulating cAMP-Dependent PKA Activity

In other systems, PKA is regulated by cAMP, which causes the dissociation of its component regulatory and catalytic subunits, thereby activating the kinase (reviewed by Taylor et al., 1990). To determine whether hh signal transduction involves a cAMP-dependent down-regulation of PKA, we asked whether a mutated form of the mouse catalytic subunit of PKA that is constitutively active because it cannot interact properly with the regulatory subunit (Orellana and McKnight, 1992) interferes with normal hh signaling. As both the regulatory and catalytic subunits of PKA are highly conserved, we reasoned that this mutant protein (referred to subsequently as PKA*) should behave constitutively when introduced into Drosophila.

Because constitutive expression of the *PKA** coding sequence might be lethal, we expressed it conditionally in two ways (Experimental Procedures). First, we have used the flp-out technique (Struhl and Basler, 1993) to create genetically marked clones of *Actin5C>PKA** cells that express the *PKA** coding sequence under the control of the constitutive *Actin5C* promoter. Second, we placed the *PKA** coding sequence under the direct control of the heat shock–inducible *hsp70* promoter.

We first examined whether constitutive PKA* activity can compensate for the loss of endogenous PKA activity without interfering with normal hh signaling. Clones of *DC0⁻* cells that also carry a single copy of the *Actin5C>PKA** transgene were generated in the wing disc (see Experimental Procedures). As shown in Figure 5A, these clones do not express the *dpp–lacZ* gene unless they are in the immediate vicinity of the compartment boundary, indicating that constitutive PKA* activity derived from the *Actin5C>PKA** transgene can block ectopic *dpp* expression without interfering with the normal induction of *dpp* by hh. Further evidence for rescue of the *DC0⁻* mutant phenotype was obtained by examining the phenotype of sibling clones in the adult mesonotum. Such *DC0⁻ Actin5C>PKA** clones formed normal notum patterns and did not appear to influence the pattern of surrounding tissue (data not shown).

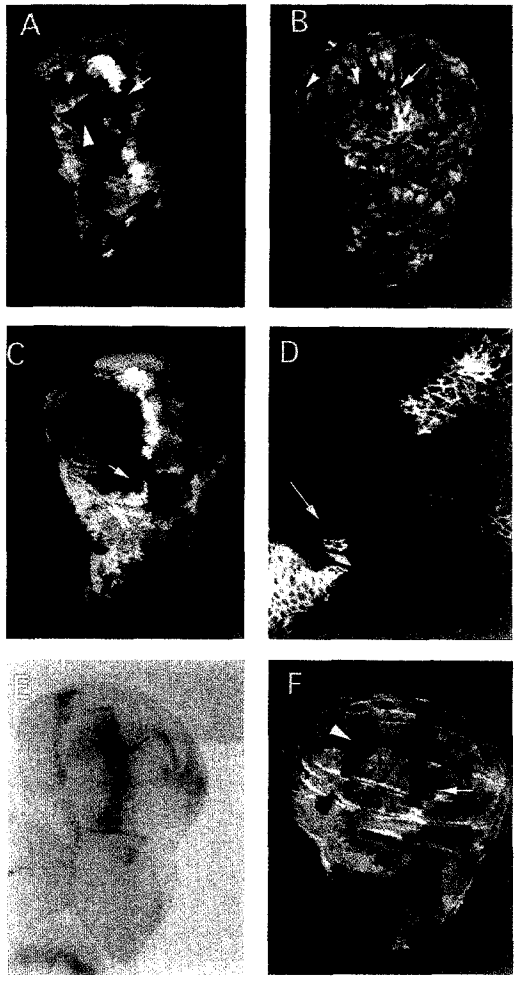

Figure 5. Constitutive PKA* Activity Compensates for Loss of Endogenous PKA Activity but Does Not Block Normal Hh Signaling or Compensate for the Loss of *ptc* Function

(A) Clones of anterior *Actin5C>PKA** *DC0⁻* cells (absence of green CD2 staining) positioned at a distance from the compartment boundary (e.g., arrowhead) do not express *dpp–lacZ* (red), whereas sibling anterior clones positioned along the compartment boundary do (arrow).

(B) A *hs–PKA** disc maintained at 25°C following the induction of many small *DC0⁻* clones (arrowheads) during the early third larval instar: most clones positioned at a distance from the compartment boundary (arrowhead) do not express *dpp–lacZ*, in contrast with clones along the compartment boundary (arrow), which do.

(C) Clones of anterior cells carrying three copies of the *Actin5C–PKA** gene positioned along the compartment boundary (arrow) express *dpp–lacZ*.

(D) High magnification of the clone indicated by the arrow in (C).

(E) Normal pattern of *dpp* transcripts along the compartment boundary (arrow) in a *hs–PKA** wing disc subjected to three 37°C heat shocks.

(F) Clones of anterior *Actin5C>PKA** *ptc⁻* cells (e.g., arrowhead) positioned at a distance from the compartment boundary (arrow) in the wing disc express *dpp–lacZ* (red).

Similar results were also obtained using the *hsp70–PKA** gene to provide constitutive PKA* activity. In this case, the basal level of *PKA** expression generated at 25°C from a single copy of the *hsp70–PKA** gene is suffi-

cient to suppress ectopic *dpp–lacZ* expression in most anterior compartment clones of *DC0*⁻ cells but does not interfere with normal *dpp–lacZ* expression along the compartment boundary (Figure 5B). Moreover, complete rescue is observed when larvae are shifted up to 33°C 1 day after the clones were induced (data not shown).

We next asked whether higher levels of constitutive PKA* activity might block hh signal transduction by examining *dpp–lacZ* expression in wing discs in which many cells carry three copies of the *Actin5C>PKA** gene. Such "three copy" cells express *dpp–lacZ* normally in the vicinity of the compartment boundary (Figures 5C and 5D). Moreover, the discs containing these clones are of normal size and morphology, providing further evidence that endogenous *dpp* expression is not affected. We also performed equivalent experiments using heat shock to drive expression of the *hsp70–PKA** transgene. In this case, larvae were subjected to a series of 37°C heat shocks during the mid– to late–third instar stage, and then assayed for *dpp* and *wg* expression by in situ hybridization (see Experimental Procedures). For both genes, we observe normal expression along the compartment boundary (Figure 5E).

Although we have not directly assayed PKA* activity under the conditions of these experiments, the level of PKA* activity in cells carrying three copies of the fused *Actin5C>PKA** gene is likely to be around three times higher than cells carrying only a single copy. Similarly, the level of PKA* activity driven by the *hsp70* promoter at 37°C should be 3-fold higher than that generated at 33°C, the temperature necessary to block ectopic *dpp–lacZ* expression completely in all *DC0*⁻ clones, and at least 10-fold higher than that generated at 25°C, which is sufficient to block ectopic *dpp–lacZ* expression in most *DC0*⁻ clones (O'Brien and Lis, 1991). Thus, levels of constitutive PKA* activity that are several fold higher than levels sufficient to block ectopic *dpp* and *wg* expression do not appear to interfere with the normal induction of these genes by hh. We infer from this result that hh does not induce *dpp* and *wg* expression simply by down-regulating PKA activity through a cAMP-dependent mechanism.

Constitutive PKA* Activity Does Not Compensate for the Loss of *ptc* Gene Function

As described in the Introduction, *ptc* encodes a transmembrane protein implicated in hh signal transduction that resembles PKA in that it is normally required to block inappropriate *dpp* and *wg* expression. We therefore asked whether constitutive PKA* activity could compensate for the loss of endogenous *ptc* activity.

First, we examined the phenotypes associated with clones of cells homozygous for the amorphic allele ptc^{S2} and find that in most respects they behave like clones of *DC0*⁻ mutant cells (Experimental Procedures; see also Phillips et al., 1990). In particular, they cause ectopic *dpp* and *wg* expression in the wing and leg discs as well as similar reorganizations of anterior wing, notum, and leg pattern in the adult (data not shown). We then tested whether constitutive PKA* expression derived either from a single copy of the *Actin5C>PKA** gene or from the *hsp70–PKA** gene in larvae maintained at 33°C can block ectopic expression of *dpp* in *ptc*⁻ clones in the wing disc (see Experimental Procedures). As shown in Figure 5F, neither treatment appears to block ectopic expression of *dpp* in these clones. Similarly, we observed no rescue of the notum phenotype of *ptc*⁻ cells that also carry a single copy of the fused *Actin5C>PKA** gene (data not shown). Thus, constitutive PKA* activity does not appear to bypass the requirement for *ptc* gene function.

Discussion

cAMP-dependent PKA plays a central role in mediating cellular and physiological responses to a large number of extracellular signals. PKA exists in two states: as an inactive tetramer of two regulatory (R) and two catalytic (C) subunits and as the dissociated R and catalytically active C monomers (reviewed by Taylor et al., 1990). The equilibrium between these states is controlled by the level of cAMP: binding of cAMP to the R subunits promotes dissociation of the tetramer, thereby promoting catalytic activity of the C subunits. PKA is believed to be the principal, if not sole, direct sensor for cAMP levels within most cells.

Here, we report evidence implicating PKA in the transduction of the extracellular signal hh, a major organizer of segment, limb, and neural patterning in animal development. However, our findings argue against the notion that PKA mediates hh signaling simply through its conventional role as a sensor for changes in intracellular cAMP levels. Instead, it appears that a basal level of PKA activity is both necessary and sufficient to impose a block on the transcription of two genes that encode downstream signaling molecules, *dpp* and *wg*. Hence, the hh signal appears to act by alleviating this block, leading to the secretion of dpp and wg proteins, which govern proliferation and patterning in surrounding cells.

PKA in Hh Signal Transduction

Our proposal that hh signaling counteracts a transcriptional block imposed by the basal activity of PKA is based on two findings that for simplicity we summarize for the case of the developing wing. First, only anterior cells that are exposed to hh protein normally transcribe *dpp*; however, in the absence of PKA activity, all anterior cells express *dpp*. Second, a constitutively active form of PKA (PKA*) can block ectopic *dpp* expression in the absence of endogenous PKA activity, but similar or several fold higher levels of PKA* activity do not alter the normal induction of *dpp* by hh. The first result establishes that PKA activity imposes a block on *dpp* transcription that is alleviated in response to hh signaling. The second result argues that hh does not induce *dpp* transcription by down-regulating PKA activity through a modulation of cAMP metabolism.

One simple hypothesis to account for the role of PKA in hh signal transduction is that the basal level of PKA activity is sufficient to phosphorylate and hence down-regulate a factor that would otherwise activate *dpp* transcription in anterior cells and that transduction of the hh signal counteracts the consequences of PKA activity (e.g.,

by stimulating a phosphatase that removes the phosphate applied by PKA, or by stimulating another kinase that adds a second phosphate that restores activity). This model accounts for our finding that loss of PKA activity in anterior cells causes *dpp* expression, whereas levels of constitutive PKA* activity that are sufficient to compensate for the loss of endogenous PKA activity do not interfere with the normal induction of *dpp* by hh. Several additional points about this model are worth noting.

First, because hh signal transduction and PKA activity are proposed to have opposite effects on the activity of a particular target protein through modifications of that protein, the two inputs may be competitive (e.g., if hh signaling acts to remove phosphates added by PKA). Hence, it is possible that extremely high levels of PKA activity (e.g., generated experimentally by massive overexpression of a constitutively active form of PKA) could prevent a cell from responding to hh, even though such levels might not occur under normal physiological conditions.

Second, our experiments do not address the possibility that hh signaling might directly down-regulate PKA activity through a non–cAMP-dependent mechanism. The existence of such a mechanism would allow hh signaling to block both endogenous PKA and exogenous PKA* activities and hence undermine our experimental evidence that hh signaling counteracts the downstream consequences of PKA activity. However, we are not aware of a precedent for the down-regulation of PKA by means other than the modulation of cAMP, and hence consider this possibility unlikely.

Third, the transcriptional block of *dpp* and *wg* imposed by PKA depends on developmental context. For example, in the wing disc, *wg* is expressed in a complex pattern in the anterior compartment (e.g., Kassis et al., 1992) despite the effective block of *dpp* transcription in many of the same cells. Conversely, in the leg disc, release from this block due to hh signaling or loss of PKA activity causes high levels of transcription of *wg*, but not *dpp*, in ventral anterior cells. We infer that these differences reflect the presence of other factors specific for regulating either *wg* or *dpp* expression that can override the transcriptional block imposed by PKA activity or that can independently repress transcription, irrespective of hh signaling. As we discuss below, wg itself may play such a role in down-regulating ventral *dpp* expression in the leg discs.

Finally, the role of PKA in hh signal transduction appears similar in several respects to that of the kinase *zeste-white3–shaggy* (*zw3–sgg*) in wg signal transduction, and hence, may provide insight into how the wg signal is transduced. *zw3–sgg*, like PKA, encodes a highly conserved intracellular kinase that mediates a broad range of cellular responses (Bourouis et al., 1990; Siegfried et al., 1990, 1992). Loss of *zw3–sgg* activity causes cells that are not exposed to wg protein to behave as if they have been exposed. Moreover, *zw3–sgg*-deficient cells express genes that would otherwise be repressed (Siegfried et al., 1992) and appear to exert a long range influence over the behavior of neighboring cells (Diaz-Benjumea and Cohen, 1994). Hence, the role of *zw3–sgg* in wg signal transduction appears to be analogous to that of PKA in hh signal transduction, raising the possibility that wg signaling acts by alleviating a transcriptional block imposed by the basal activity of the *zw3–sgg* kinase.

PKA, Ptc, and Possible Pathways of Hh Signal Transduction

The *ptc* gene encodes a transmembrane protein that, like PKA, blocks inappropriate *wg* and *dpp* expression in the absence of hh signaling (Hooper and Scott, 1989; Nakano et al., 1989; Hidalgo and Ingham, 1990; Phillips et al., 1990; Ingham et al., 1991; Capdevila et al., 1994; this paper). In principle, hh signal transduction could involve the modulation of ptc activity. Alternatively, ptc may play a similar role to PKA, imposing a block to transcription of *dpp* and *wg* that is alleviated by hh signaling. In Figure 6, we outline two possible pathways for the relationship between ptc and PKA in hh signal transduction. Both pathways are consistent with what we presently know about the functional relationships between hh, ptc, and PKA, including the failure of constitutive PKA* activity to compensate for the loss of *ptc* gene function. However, they differ in whether ptc is placed upstream (Figure 6A) or downstream (Figure 6B) of PKA and whether hh signal induction involves a modulation of ptc activity (Figure 6B).

Although our experiments do not distinguish whether PKA acts upstream or downstream from ptc, we nevertheless favor the view that PKA acts downstream because cells lacking PKA activity do not exhibit all the attributes of cells that receive the hh signal or that lack ptc activity. In particular, they behave like cells positioned a short distance from the compartment boundary rather than like cells positioned next to the boundary. These differences could be due to residual PKA activity, whether encoded by the mutant *DC0*^{E95} allele or by another *DC0*-like gene (Kalderon and Rubin, 1988). Alternatively, it may indicate that PKA blocks only a subset of the responses normally blocked by ptc and induced by hh. This second possibility can be readily accommodated in pathways in which ptc

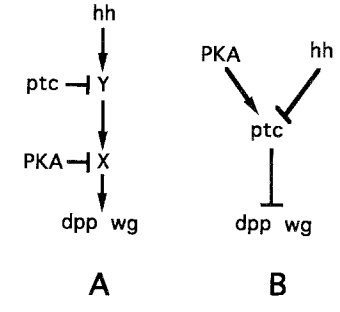

Figure 6. Possible Pathways for Hh Signal Transduction

(A) A pathway in which PKA and ptc impose blocks on *dpp* and *wg* transcription by modifying factors X and Y and hence repressing their intrinsic activities (solid arrows denotes up-regulation, and solid tees denotes down-regulation). We favor the view that hh signaling counteracts the consequences of PKA and ptc activity (e.g., as diagrammed) and that ptc acts upstream of PKA (see Discussion).

(B) Alternative pathway in which PKA acts upstream of ptc: an extreme case is shown in which ptc is itself the substrate that is regulated in opposite directions by modifications resulting from PKA and hh signal transduction.

acts upstream of PKA (Figure 6A) if we posit a second output of ptc that is not down-regulated by PKA. However, it is more difficult to incorporate in pathways in which PKA is placed upstream of ptc (Figure 6B).

Down-Regulation of *dpp* by Wg in the Developing Leg

In leg discs, loss of PKA activity causes high levels of ectopic *dpp* and *wg* expression in mutually exclusive subpopulations of dorsal and ventral leg cells and can cause the formation of supernumerary double-anterior limbs, provided that PKA activity is lacking in both dorsally and ventrally positioned cells. These results support the proposal that juxtapositions of *dpp*-expressing and *wg*-expressing cells organize growth and patterning along the proximodistal axis (Campbell et al., 1993; Basler and Struhl, 1994; Diaz-Benjumea et al., 1994; Campbell and Tomlinson, 1995). Hence, we expected that clones of cells that lack either *dpp* or *wg* activity as well as PKA activity would not be able to organize supernumerary legs. We were therefore surprised to find that clones of cells lacking both PKA and *wg* activity could organize supernumerary legs, although of different character than those normally associated with clones deficient for PKA.

One explanation of this result is suggested by the observation that both hh signaling as well as the loss of PKA activity cause ventral leg cells to express high levels of *wg* and low levels of *dpp* (Basler and Struhl, 1994; this paper), raising the possibility that the level of *dpp* expression may be down-regulated by wg. If such a regulatory relationship exists, clones of ventral leg cells that lack *wg* and PKA activity should express high levels of *dpp*. Moreover, such clones should trigger the formation of supernumerary limbs if they arise close to wild-type *wg*-expressing cells along the compartment boundary. Consistent with this interpretation, we find that triply mutant clones that lack *dpp*, *wg*, and PKA activity do not organize supernumerary legs. We therefore posit that *wg* activity induced by *hh* in the developing leg normally down-regulates *dpp* expression induced by hh in the same cells. We also suggest that this down-regulation may be essential to generate adjacent populations of dorsal *dpp* and ventral *wg*-expressing cells and hence to organize growth and patterning along the proximodistal axis.

Distinct Long and Short Range Outputs of Hh Signaling

When we eliminate *dpp* and/or *wg* gene function as well as PKA activity, we create cells that have, in effect, transduced the hh signal but that are unable to respond by secreting either or both dpp and wg protein. Hence, these experiments provide a test for the hypothesis that the long range influence of hh signaling is elicited through the subordinate organizing activities of dpp and wg (Basler and Struhl, 1994). In the developing wing, in which hh signaling induces only *dpp* expression, we observe that cells lacking both PKA and *dpp* activity do not cause global reorganizations of anterior pattern. Similarly, in the developing leg, we find that cells lacking *wg*, *dpp*, and PKA activity do not organize supernumerary limbs. Thus, we infer that most or all of the long range organizing activities of hh protein in the limbs are mediated by dpp and/or wg signaling.

However, we also observe that the loss of PKA activity can cause local reorganizations of pattern that appear to be independent of either *dpp* or *wg* activity, such as the autonomous failure to form wing veins, the induction of ectopic veins in adjacent wing tissue, and the recruitment of surrounding leg cells to form inappropriate ventrolateral structures such as sex comb teeth in the foreleg. Thus, our results provide evidence for distinct short range outputs of hh signaling that do not depend on either *wg* or *dpp*.

Experimental Procedures

Genes and Transgenes Employed

DC0^{E95}
An apparent null allele of the *DC0* gene (see Results) induced by EMS mutagenesis and identified in a screen for mutations that cause abnormal adult patterns in somatic clones generated by flp-mediated mitotic recombination (Golic, 1991).

stc (stubby chaete)
A recessive lethal mutation, mapping in the vicinity of *Adh*, obtained from M. Ashburner. Causes stubby bristles and replaces hairs by smaller hairs or tufts of hairs.

FRT39, w$^+$
A w^+ minigene flanked by flp-recombinase targets (FRTs) (Golic and Lindquist, 1989) inserted in the proximal 2L (Chou and Perrimon, 1992).

FRT42, neo^R
An FRT target site inserted in the proximal 2R (Xu and Rubin, 1993).

H700.1
A *tub>y$^+$>*hh transgene on chromosome 2 (Basler and Struhl, 1994).

A901.2, A901.3
*hsp70–PKA** transgenes (see below) inserted on chromosomes 2 and 3.

A902.2, A902.3
*Actin5C>CD2, y$^+$>PKA** transgenes (see below) inserted on chromosomes 2 and 3.

CD2L.1, y$^+$, CD2R.1, y$^+$
hsp70–CD2, y$^+$ transgenes (see below) inserted on the left and right arms of chromosome 2.

hsp70-flp.1, hsp70-flp.3
hsp70–flp transgenes (Struhl and Basler, 1993) inserted on chromosomes 1 and 3.

Dp(2;2) VT1,dpp+
A tandem duplication of the *dpp* region obtained from V. Twombly and W. Gelbart (personal communication).

See text and Lindsley and Zimm (1992) for other mutations and chromosomal aberrations.

Composition of Transgenes

hsp70-CD2, y$^+$
A modified form of the *hsp70–flp* transgene (Struhl and Basler, 1993) in which the *ry$^+$* gene is replaced by *y$^+$* and the *flp* coding sequence is replaced by the *CD2* coding sequence (Dunin-Borkowski and Brown, 1995; here and below, details available on request).

hsp70-PKA* (A901)
A modified form of the *hsp70–CD2, y$^+$* transgene that lacks the *y$^+$* gene and contains the coding sequence of the catalytic subunit of mouse PKA with the His(87)Gln and Trp(196)Arg mutations in place of the *flp* coding sequence (this mutated form of the catalytic subunit does not bind the regulatory subunit with high affinity and hence is constitutively active, irrespective of cAMP concentration; Orellana and McKnight, 1992). Animals carrying two copies of the *A901* gene are subviable, although survivors appear morphologically normal.

Actin5C>CD2, y$^+$>PKA* (A902)
A modified form of *A901* in which the *hsp70* promoter was replaced

by the *Actin5C* promoter (Struhl and Basler, 1993) and the promoter was separated from the *PKA** coding sequence by a *>y⁺>* flp-out cassette that contains the *CD2* coding sequence just downstream from the first FRT. Zygotes carrying the fused *Actin5C>PKA** gene do not survive to adulthood. However, clones of *Actin5C>PKA** cells form normally patterned legs and wings, except that mutant cells in the wing blade are associated with blisters. For both the *A901* and fused *A902* transgenes, we infer that viability is compromised because exogenous PKA* protein cannot be down-regulated by cAMP and hence interferes with PKA-mediated signaling events that are normally modulated by cAMP.

Generating Clones of Marked Cells

Clones of mutant cells were generated by *flp*-mediated mitotic recombination as described in Golic (1991), Chou and Perrimon (1992), and Xu and Rubin (1993) (see below for specific genotypes employed). The presence of *DC0⁻* clones in imaginal discs was confirmed using a new cell marker transgene, *hsp70–CD2*, (Dunin-Borkowski and Brown, 1995; see below) in trans to the mutant-bearing chromosome. For the induction of the *hsp70–CD2* gene, larvae were heat shocked at 37°C for 60 min and then processed for antibody staining following a 60 min recovery period at 25°C.

Clones of cells carrying one or more copies of the *Actin5C>PKA** transgene were obtained using the flp-out technique (Struhl and Basler, 1993) as follows. Virtually all cells of larvae carrying both the *Actin5C>CD2, y⁺>PKA** and *hsp70–flp* transgenes (see below) express the reporter protein CD2, but not PKA*, under the control of the *Actin5C* promoter. However, heat shock–induced expression of the flp recombinase can cause excision of the *>CD2, y⁺> flp*-out cassette to generate cells that now express PKA*, but not CD2; these cells can be readily identifed by the absence of CD2 protein expression. Clones of cells lacking PKA or *ptc* activity but carrying the *Actin5C–PKA** gene were generated by combining the flp-out and mitotic recombination techniques and recognized in the imaginal discs by the absence of CD2 expression and in the adults by the *y* and *stc* markers (see genotypes below).

Genotypes Used for Generating Clones by Flp-Mediated Mitotic Recombination and by the Flp-Out Technique

Clones in Adults

DC0⁻ Clones

yw; DC0^E95 stc FRT39, w⁺/Dp(1;2)sc¹⁹, y⁺ FRT39, w⁺; hsp70–flp.3/+.

wg⁻ DC0⁻ Clones

As above, except for the addition of *wg^CX4* in *cis* with *DC0^E95*.

dpp⁻ DC0⁻ Clones

yw; dpp^H61 DC0^E95 stc FRT39, w⁺/Dp(2;2)VT1, dpp⁺ Dp(1;2)sc¹⁹, y⁺ FRT39, w⁺; hsp70–flp.3/+.

dpp⁻ wg⁻ DC0⁻ Clones

As above, except for the addition of *wg^CX4* in *cis* with *DC0^E95*.

ptc⁻ Clones

y hsp70–flp/+ or *Y; FRT42 ptc^S2/FRT42, neo^R CD2R.1, y⁺.*

Clones in Discs

DC0⁻ Clones

y hsp70–flp.1/+ or *Y; DC0^E95 stc FRT39, w⁻/CD2L.1, y⁺ FRT39, w⁺* with (i) *dpp–lacZ3.0* or *wg–lacZ* present in *cis* with *DC0^E95* or (ii) *hh^P30/+*.

DC0⁻ Clones in *hsp70–PKA** Discs

Same as above except for the additional presence of one copy of transgene *A901.3*.

DC0⁻ Clones in Discs with *Actin5C>PKA** Flp-Out Clones

y hsp70–flp.1/+ or *Y; DC0^E95 stc FRT39, w⁺ dpp–lacZ3.0/CD2L.1, y⁺ FRT39, w⁺; A902.3/+.*

*Actin5C>PKA** Flp-Out Clones:

With One Copy of the A902 Transgene

y hsp70–flp.1/+ or *Y; dpp–lacZ3.0/+; A902.3/+.*

With Three Copies of the A902 Transgene

y hsp70–flp.1/+ or *Y; A902.2/dpp–lacZ3.0; A902.3/A902.3.*

ptc⁻ Clones

Same as *ptc^S2* clones in adults, except for the presence of *dpp^P10638* (a *dpp–lacZ* reporter gene containing a *lacZ* enhancer trap insertion in the *dpp* locus; R. Blackman, personal communication) in *cis* on the *ptcS2* chromosome.

ptc⁻ Clones in *hsp70–PKA** Discs or in Discs with *Actin5C>PKA** Clones

Same as above, except for the additional presence of the *A901.3* or *A902.3* transgene.

Heat Shock Treatments

Clones were induced by incubating tubes with first or second instar larvae in a water bath at 35°C or 37°C for 60 min. The same protocol was used to induce flp-out clones of the *A902* transgene, except that two to three heat shocks (with recovery periods of 3 hr at ~21°C) were applied when three *A902* transgenes were used, or when clones of *DC0⁻* cells were induced in the same animals. To assay the ability of *hs–PKA** expression to compensate for the loss of endogenous PKA activity, *DC0⁻* clones were induced during the second or early third instar by a single heat shock and then maintained subsequently at 25°C for 3 days, or for 1 day followed by a shift up to 33°C for 2 days. To test whether high levels of *hs–PKA** activity interfere with normal *dpp* and *wg* expression, mid– to late–third instar larvae were subjected to three 37°C heat shocks (each of 1 hr, with recovery periods of 2 hr at ~21°C), and fixed for in situ hybridization 1 hr after the last heat shock.

Quantitation

For clones of each genotype, 50 or more wings with abnormal pattern were analyzed. For *DC0^E95* clones, 100 wings of normal morphology were inspected under the compound microscope and 37 clones in the posterior compartment or close to the compartment boundary in the anterior compartment were observed. In the leg, 20 or more appendages with anterior compartment clones of each genotype were analyzed. For clones in the imaginal discs, at least 15 discs were examined for each genotype for each method of detection. Typically, several clones were observed in each disc (Figures 2 and 5).

Visualization of Protein and RNA Expression

Standard protocols for immunofluorescence, immunohistochemistry, and in situ hybridization were used and *lacZ* expression was monitored as described previously (Basler and Struhl, 1994; Sturtevant et al., 1993). CD2 protein expression was monitored using the monoclonal antibody OX34 (Serotec).

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