**patched overexpression alters wing disc size and pattern: transcriptional and post-transcriptional effects on hedgehog targets**

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**SUMMARY**

The membrane protein, Patched, plays a critical role in patterning embryonic and imaginal tissues in Drosophila. patched constitutively inactivates the transcription of target genes such as wingless, decapentaplegic, and patched itself. The secreted protein, Hedgehog, induces transcription of target genes by opposing the Patched signaling pathway. Using the Gal4 UAS system we have overexpressed patched in wing imaginal discs and found that high Patched levels, expressed in either normal or ectopic patterns, result in loss of wing vein patterning in both compartments centering at the anterior/posterior border. In addition, patched inhibits the formation of the mechanosensory neurons, the campaniform sensilla, in the wing blade. The patched wing vein phenotype is modulated by mutations in hedgehog and cubitus interruptus (ci). Patched overexpression inhibits transcription of patched and decapentaplegic and post-transcriptionally decreases the amount of Ci protein at the anterior/posterior boundary. In hedgehogMr wing discs, which express ectopic hedgehog, Ci levels are correspondingly elevated, suggesting that hedgehog relieves patched repression of Ci accumulation. Protein kinase A also regulates Ci; protein kinase A mutant clones in the anterior compartment have increased levels of Ci protein. Thus patched influences wing disc patterning by decreasing Ci protein levels and inactivating hedgehog target genes in the anterior compartment.

Key words: patched, hedgehog, cubitus interruptus, segment polarity, imaginal disc development, signaling, anterior/posterior patterning, campaniform sensilla, protein kinase A

**INTRODUCTION**

Imaginal discs are precursors to adult tissues in Drosophila and are patterned during late embryogenesis and early larval development. The anterior/posterior (A/P) axis of the wing disc is patterned by cell-cell signaling between two adjacent compartments of cells (Garcia-Bellido et al., 1973). The segment polarity genes hedgehog (hh) and patched (ptc) are critical in patterning the A/P axis. Hh, a novel secreted protein, is expressed only in the posterior compartment (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993; Taylor et al., 1993; Tabata and Kornberg, 1994) while Ptc, a multiple transmembrane protein (Hooper and Scott, 1989; Nakano et al., 1989), is expressed at low levels in the anterior compartment except at the A/P border, where it is present at high levels (Phillips et al., 1990; Capdevila et al., 1994).

Hh is proposed to act locally at the compartment border to induce the expression of border-specific genes in anterior cells (Basler and Struhl, 1994; Capdevila et al., 1994; Kojima et al., 1994; Tabata and Kornberg, 1994) such as decapentaplegic (dpp), whose product is a member of the TGF-β family of signaling proteins (Padgett et al., 1987) and ptc itself. Expression of hh in the anterior compartment of the wing disc results in ectopic expression of dpp, ptc and other border-specific genes as well as mirror-image duplications of anterior wing structures (Basler and Struhl, 1994; Kojima et al., 1994; Tabata and Kornberg, 1994).

Ptc opposes the Hh signal in embryonic and imaginal tissues to inactivate transcription of target genes. ptc overexpression in the embryonic ectoderm blocks the hh-dependent transcription of wingless and ptc (Schuske et al., 1994) while in the wing disc, dpp and ptc transcription are inhibited (Capdevila et al., 1994). Several reduced-function alleles of ptc show mirror-image duplications of the wing anterior (Phillips et al., 1990; Capdevila et al., 1994) similar to that of ectopic hh expression (Basler and Struhl, 1994; Tabata and Kornberg, 1994). Furthermore, dpp and ptc transcription are induced in ptc mutant clones in the anterior compartment of the wing disc (Capdevila et al., 1994; Li et al., 1995).

Additional genes have been identified that interact with ptc and hh to pattern tissues in Drosophila. fused (fu), which encodes a putative serine/threonine kinase (Prétat et al., 1990; Therond et al., 1993), and cubitus interruptus (ci), which encodes a zinc-finger-containing protein homologous to the vertebrate family of GLI transcription factors (Orenic et al., 1990), are required to maintain hh-dependent transcription in...
the embryonic ectoderm (Forbes et al., 1993). Adult viable alleles of ci and fu have phenotypes in which wing veins are fused or altered (Fausto-Sterling, 1978; Slusarski et al., 1995). Recently, protein kinase A (PKA) has been shown to influence wing patterning in a manner similar to ptc by inactivating the transcription of hh target genes (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995).

To better understand the role of ptc in hh signaling and in wing development, we ectopically expressed ptc in different patterns in the wing disc. Prior work has shown that heat-shock expression of ptc can rescue the wing defects and ectopic ptc and dpp transcription in weak ptc alleles (Capdevila et al., 1994). We show here that ptc overexpression in normal or novel patterns in the wing disc causes alterations in the vein and campaniform sensilla patterns at the A/P border. ptc overexpression antagonizes hh signaling by inactivating endogenous ptc and dpp transcription. We also show that ptc post- transcriptionally prevents the accumulation of Ci protein at the compartment boundary, the first demonstration of ptc regulation of a protein target. Like ptc, PKA negatively regulates Ci levels.

MATERIALS AND METHODS

UAS ptc construction and germline transformation

A 4 kb EcoRI/SpeI fragment of a ptc cDNA (Schuske et al., 1994) containing the entire coding region of ptc was cloned in the sense orientation into the EcoRI/XbaI sites of pUASt (Brand and Perrimon, 1993). This vector, which contains the mini white gene, was injected into Df(1R)white embryos (Spradling and Rubin, 1982) and four independent transgenic lines were recovered: UAS ptc B1 (chromosome III), UAS ptc B2 (chromosome III), UAS ptc B3 (chromosome II) and UAS ptc C (chromosome I).

Fly stocks

Gal4 and UAS lines used were E16E en Gal4 (gift of N. Perrimon), ptc Gal4559 (Hinz et al., 1994), 71B Gal4 and UAS lacZ (Brand and Perrimon, 1993). Enhancer trap lines used were A101 neu (Bellen et al., 1989) and AT90 ptc (provided by C. Goodman). Segment polarity gene alleles used were Df(2R) P14TE (Hooper and Scott, 1989), hhMtk1, hhMtk2, hhMtk3 (Tabata and Kornberg, 1994), cos-2vl ptc, c191 and cP83 (Lindsley and Zimm, 1992). Fly crosses were incubated at 29°C to maximize expressivity of the imaginal disc and wing phenotypes.

Mounting of wings

Wings were mounted as previously described (Basler and Struhl, 1994). yw flies were used for wild-type controls.

β-galactosidase, in situ and antibody staining

Third instar larvae were dissected in phosphate-buffered saline, leaving the wing imaginal discs attached to the cuticle. After staining, wing discs were dissected and mounted either in 80% glycerol in PBS (antibody and β-gal) or Canada Balsam (in situ).

β-gal staining was as described (Brand and Perrimon, 1993) except no Triton X-100 was used. lacZ; TM6B and lacZ; 71ptc larvae were distinguished by the Tb marker on the balancer chromosome TM6B.

For in situ hybridization, a dpp probe was made as described (Mathies et al., 1994) and for ci, a 0.8 kb fragment of the ci cDNA, DC1 (provided by T. Kornberg), was labeled with digoxigenin dNTPs and Klenow polymerase. yw and homozygous 71ptc discs were fixed in 4% paraformaldehyde on ice for 20 minutes and changed to fresh fix with 0.1% deoxycholate and 0.1% Triton X-100 for 15 minutes at RT. Fix was replaced with 0.5 ml 0.3 M NH4OAc and dehydrated by dropwise addition of 0.5 ml EtOH. After several EtOH washes, discs were extracted in 50% EtOH/50% xylene for 10 minutes, washed 2x EtOH, 1x MeOH, 1x 50% MeOH/50% post fix (PBS+0.1% Tween 20 (PTw)+5% formaldehyde) and then postfixed for 20 minutes. Following several PTw washes, discs were prehybridized at least 2 hours at 53°C in 50% formamide, 5X SSC, 0.1 mg/ml salmon sperm DNA, 0.1 mg/ml yeast tRNA, 50 ug/ml heparin, and 0.1% Tween 20. Discs were hybridized overnight at 53°C, followed by 5x PTw washes at 53°C and 1:2000 anti-DIG antibody for 1 hour at room temperature. After PTw washes, alkaline phosphatase substrate was added.

For antibody staining, discs were fixed in 4% paraformaldehyde for 15 minutes, washed in PBS+0.5% Triton X-100 (PT) and blocked several hours in PT+5% BSA (PTB). A rat anti-Ci monoclonal antibody (1:4 dilution, generous gift of R. Holmgren) or rabbit anti-β-gal polyclonal antibody (1:1000 dilution) was used in PTB ON at 4°C, followed by PT washes and 1:500 goat anti-rat biotin in PTB. After PT washes, discs were incubated with Vectastain for 30 minutes, washed and HRP substrate was added.

Induction of PKA clones

HS Flp: arm lacZ Frt 40A virgins were mated with PKA3 Frt 40A/Cyo males (Pan and Rubin, 1995) and eggs were collected at 24 hour intervals at room temperature. Embryo collections were heat shocked 1 and 2 days later for 30 minutes at 38°C. Wandering third instar larvae were dissected and their imaginal discs stained with β-gal and Ci antibodies.

RESULTS

Gal4-driven ptc expression

To create adult phenotypes, the UAS ptc B1 line was crossed to each of more than a hundred enhancer trap Gal4 lines (Brand and Perrimon, 1993; Lin and Goodman, 1994). Sixteen crosses resulted in detectable phenotypes including pupal lethality, loss or alteration of leg structures and wing defects (data not shown). We focused on the wing phenotype created by the line 71B Gal4 (Brand and Perrimon, 1993) to further understand the role of ptc in wing patterning. This phenotype is fully penetrant and reproducible with three independent UAS ptc lines. To enable easy genetic manipulation, a stable line was made by recombining the 71B Gal4 and UAS ptc B1 transgenes onto a single third chromosome. This line and its resultant phenotype is named 71ptc.

In the wing disc, endogenous ptc RNA is present at low levels throughout the anterior compartment except near the A/P boundary, where a stripe of high ptc expression occurs (Phillips et al., 1990; Fig. 1A). In contrast, the 71B Gal4 expression pattern is restricted to the wing pouch, with weaker staining detected at the presumptive margin and the anterior and posterior edges of the imaginal disc (Brand and Perrimon, 1993; Fig. 1B). We expected ptc overexpression in the 71B pattern to alter development in the more anterior and posterior regions of the wing blade. However the major effect of ptc misexpression is along the A/P border, the site of highest normal ptc expression. When Gal4, and therefore ptc, activity is raised to higher levels by raising the temperature, the effects extend into both anterior and posterior compartments.
ptc overexpression in wing discs

The mildest phenotype of $71^{ptc}$ is a fusion of L3 and L4 at the proximal end of the wing and/or the absence of the ACV. In more severe cases, L3 and L4 fuse more distally or become progressively deleted while the posterior cross vein has altered or ectopic venation. In the extreme phenotype, L3 and L4 are almost entirely missing and L2 and L5 are partially deleted. In this phenotypic series, the wing morphology is maintained, but the overall size progressively decreases, indicating that increased $ptc$ function leads to an inhibition of growth.

The expressivity of the $71^{ptc}$ phenotype is modulated by both temperature and dosage (Table 1). To score changes in expressivity, a scale ranging from 1 (wild type) to 5 (most severe) is used (Fig. 2). At room temperature (24°C), 72% of the wings from $71^{ptc}$ heterozygotes have an intermediate phenotype (class 2-3) while, at 29°C, 95% of the wings have the strongest phenotype (class 5). Introducing a second copy of the $71^{ptc}$ chromosome causes a stronger phenotype at 24°C and homozygotes raised at 29°C die as pharate adults.

**Effects of $ptc$ function on sensilla patterning**

In addition to wing vein alterations, $ptc$ overexpression inhibits the formation of the campaniform sensilla on the wing blade.

$ptc$ effects on the wing vein and sensilla phenotypes are independent and separable since sensilla can form in the absence of the underlying vein and veins can form in the absence of sensilla. The sensory organs appear in stereotypical locations.
in the wing with four sensilla positioned along L3 (L3-v, 1, 2 and 3), one along the ACV and two at the anterior proximal wing margin (Huang et al., 1991; Fig. 3A,D). In ptc<sup>116</sup>/Df(2R) P14TE flies, a reduced-function heteroallelic combination of ptc, extra sensilla are present along L3 and in the ACV (Fig. 3C,F). In contrast, 71<sup>ptc</sup> wings have many of these sensilla missing or in new locations (Fig. 3B,E).

ptc affects the initiation of sensilla formation, since the precursors to the sensilla, the sensory mother cells (SMC), are missing. neutralized (neu) expression is a marker for early entry into SMC formation in both the future notum and wing. In the wing pouch of mid 3rd instar larvae, neu lacZ marks the positions consistent with the L3-v, L3-2 and ACV precursors along the A/P boundary (Cubas et al., 1991; Huang et al., 1991; Fig. 3G arrows). In many 71<sup>ptc</sup> wing discs, neu lacZ staining is absent from these specific positions in the wing pouch, indicating that the precursors to the campaniform sensilla do not form (Fig. 3H).

71<sup>ptc</sup> interacts genetically with hh and ci

hh and ptc act in opposition, so added hh expression would be expected to suppress the effects of 71<sup>ptc</sup>. A dominant hh allele, Moonrat (hh<sup>Mrt</sup>), has normal hh expression in the posterior compartment of the wing disc, but causes ectopic hh product to accumulate in the anterior. This causes overgrowth and ectopic venation in the anterior distal portions of the wing (Tabata and Kornberg, 1994; Felsenfeld and Kennison, 1995).

In a hh<sup>Mrt</sup> background, the 71<sup>ptc</sup> phenotype is suppressed from severe wing vein deletions (73% class 4-5) to mild vein fusions (92% class 2-3; Fig. 4A,B, Table 2). Furthermore, the wing blade is restored to its normal size. This suppression is specific to hh<sup>Mrt</sup> gain of function since two X-ray revertants, hh<sup>MrtR1</sup> and hh<sup>MrtR2</sup>, which have lost the hh product (Tabata and Kornberg, 1994), fail to suppress the 71<sup>ptc</sup> phenotype (Table 2 and data not shown).

ptc is proposed to negatively regulate ci activity since, in the embryo, ci is epistatic to ptc and is required to maintain wg and ptc transcription (Forbes et al., 1993). Increased ptc expression in the wing disc should have greater ability to repress ci function. Indeed, the 71<sup>ptc</sup> phenotype is greatly enhanced in the backgrounds of two ci alleles, ci<sup>M62f</sup> and ci<sup>Ce</sup> (Fig. 4C-F; Table 2). Wing veins L3 and L4 are extensively deleted when 71<sup>ptc</sup> is combined with ci<sup>M62f</sup>, a deficiency that removes the entire ci locus (Fig. 4D). ci<sup>Ce</sup>, a dominant allele of ci which produces a truncated ci protein (Slusarski et al., 1995), has a wing phenotype similar to that of 71<sup>ptc</sup>; a fusion of L3 and L4 and loss of the ACV (Fig. 4E). The 71<sup>ptc</sup> chromosome appears to enhance the ci<sup>Ce</sup> phenotype since the wing vein fusions extend along the entire A/P border of the wing (Fig. 4F). This enhancement by two independent ci alleles is consistent with ptc counteracting ci activity, since removal of a single dose of ci enables ptc to exert a stronger effect.

Similar wing defects are created by either ptc overexpression or misexpression

71B Gal4 expression is detected in much of the wing pouch and the wing phenotype could be caused either by ptc overexp-

<table>
<thead>
<tr>
<th>Phenotype class (%)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>N</th>
<th>9°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>71&lt;sup&gt;ptc&lt;/sup&gt; / +</td>
<td>0</td>
<td>35</td>
<td>37</td>
<td>28</td>
<td>&lt;1</td>
<td>115</td>
<td>24</td>
</tr>
<tr>
<td>71&lt;sup&gt;ptc&lt;/sup&gt; / 71&lt;sup&gt;ptc&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>31</td>
<td>56</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>71&lt;sup&gt;ptc&lt;/sup&gt; / +</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>95</td>
<td>179</td>
<td>29</td>
</tr>
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</table>

Wings from 71<sup>ptc</sup> heterozygotes and homozygotes raised at 24°C or 29°C were mounted and their vein phenotypes were ranked. At 24°C, heterozygotes had moderate vein alterations (class 2-4) while at 29°C, almost all wings had a severe phenotype (class 5). 71<sup>ptc</sup> homozygotes had wings with severe vein alterations at 24°C and died as pupae at 29°C.

Fig. 3. ptc inhibits the formation of the campaniform sensory neurons (CSN) in wings and their precursors, the sensory mother cells, in imaginal discs. CSNs (arrows) along the L3 (A-C) and ACV (D-F) regions of the wing blade are shown for wild type (A,D), 71<sup>ptc</sup> (B,E), and ptc<sup>116</sup>/Df(2R) (C,F). The double-headed arrow (A,B) indicates the reduced distance between L3 and L4 in 71<sup>ptc</sup> wings. neu lacZ staining of wing imaginal discs show the presence (wild type; G) or absence (71<sup>ptc</sup>; H) of the L3 sensory mother cells (arrows).
expression in the anterior compartment or by ectopic ptc expression in the posterior. To examine these two possibilities, ptc Gal4 (Gal4 \textsuperscript{59}; Hinz et al., 1994; Speicher et al., 1994) and en Gal4 enhancer trap lines (E16E) were used to overexpress ptc. β-gal staining demonstrates that both Gal4 lines reproduce the endogenous ptc and en expression pattern in wing discs (Kornberg et al., 1985; Phillips et al., 1990; Fig. 1C,D). UAS\textit{ptc} expression in the ptc Gal4 pattern results in a mild 71\textit{ptc} wing phenotype (class 2; Fig. 5A-C). This lessened effect may be caused in part by lower Gal4 production by the ptc Gal4 line or by ptc negative regulation of ptc Gal4 transcription. Of the three UAS\textit{ptc} lines crossed to ptc Gal4, two show loss of the anterior crossvein (Fig. 5B and data not shown) while a third line (UAS\textit{ptc} B2) shows partial lethality with escapers having wing vein fusions at the A/P border (Fig. 5C). Similarly, ectopic expression of ptc solely in the posterior by en Gal4 also results in defects in both compartments, specifically fusion of L3 and L4. Thus either a hypermorphic or neomorphic alteration in ptc expression creates a similar phenotype – loss of pattern in both compartments of the A/P border. It appears that both the amounts and the location of ptc expression are important determinants in patterning the wing disc.

**Overexpressed ptc inactivates hh target genes at the transcriptional and posttranscriptional level**

\textit{ptc} presumably mediates its effects on wing disc patterning at least in part by modulating the activities of \textit{hh} target genes. Since \textit{ptc} and \textit{dpp} are activated locally near \textit{hh}-expressing regions (Basler and Struhl, 1994; Capdevila et al., 1994; Tabata and Kornberg, 1994), we determined whether \textit{ptc} overexpression alters \textit{dpp} or \textit{ptc} transcription levels. In 71\textit{ptc} wing discs, the A/P stripe of \textit{ptc} lacZ staining is reduced in the wing pouch where \textit{ptc} is highly expressed, while levels in the notum where no extra \textit{ptc} is produced are comparable to wild type (Fig. 6A,E). This result is consistent with previous experiments showing that \textit{ptc} negatively autoregulates in the wing disc (Capdevila et al., 1994). \textit{dpp} transcription is also reduced in the wing pouch as compared to the notum (Fig. 6B,F).

In the embryo, genetic studies have shown that \textit{ci} is required to activate \textit{hh} target genes and is downstream of \textit{ptc} (Forbes et al., 1993). \textit{ptc} might inhibit transcription of these genes by regulating \textit{ci} RNA or protein levels. \textit{ci} RNA is homogeneously distributed throughout the anterior compartment of both wild-type (Eaton and Kornberg, 1990; Orenic et al., 1990) and 71\textit{ptc} imaginal discs (Fig. 6C,G). In contrast, \textit{Ci} protein is present at low levels in the anterior, except at the A/P border where it is expressed as an intense stripe (Slusarski et al., 1995; Fig. 6D). This stripe of increased \textit{Ci} staining suggests that the \textit{Ci} level may be modulated by \textit{ptc}.

**Table 2. Expressivity of 71\textit{ptc} wing phenotype in \textit{ci} and \textit{hh}\textsuperscript{Mrt} backgrounds**

<table>
<thead>
<tr>
<th>Phenotype class (%)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td>71\textit{ptc} / +</td>
<td>0</td>
<td>6</td>
<td>20</td>
<td>50</td>
<td>23</td>
</tr>
<tr>
<td>71\textit{ptc} / \textit{hh}\textsuperscript{Mrt}</td>
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<td>24</td>
<td>68</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>71\textit{ptc} / +</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>36</td>
<td>57</td>
</tr>
<tr>
<td>71\textit{ptc} / \textit{hh}\textsuperscript{MrtR1}</td>
<td>0</td>
<td>1</td>
<td>12</td>
<td>35</td>
<td>53</td>
</tr>
<tr>
<td>71\textit{ptc} : +</td>
<td>0</td>
<td>8</td>
<td>68</td>
<td>24</td>
<td>&lt;1</td>
</tr>
<tr>
<td>71\textit{ptc} : \textit{ci}\textsuperscript{M62f}</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>27</td>
<td>67</td>
</tr>
<tr>
<td>71\textit{ptc} : \textit{ci}\textsuperscript{Ce}</td>
<td>0</td>
<td>25</td>
<td>58</td>
<td>15</td>
<td>1</td>
</tr>
<tr>
<td>71\textit{ptc} : \textit{ci}\textsuperscript{Ce}</td>
<td>0</td>
<td>&lt;1</td>
<td>13</td>
<td>18</td>
<td>68</td>
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The 71\textit{ptc} wing phenotype is suppressed from class 4 to class 3 in a \textit{hh}\textsuperscript{Mrt} background but is unaffected in a \textit{hh}\textsuperscript{MrtR1} background. Loss of one copy of the \textit{ci} locus (\textit{ci}\textsuperscript{M62f}) enhances the severity of the 71\textit{ptc} phenotype from class 3 to class 5. The combination of 71\textit{ptc} and \textit{ci}\textsuperscript{Ce} chromosomes causes much more severe vein alterations than either 71\textit{ptc} or \textit{ci}\textsuperscript{Ce} alone.

**Fig. 4. \textit{hh}\textsuperscript{Mrt} suppresses and \textit{ci} alleles enhance the 71\textit{ptc} wing phenotype.** Wings of sibling flies from 71\textit{ptc} × \textit{hh}\textsuperscript{Mrt} (A,B), 71\textit{ptc} × \textit{ci}\textsuperscript{M62f} (C,D) and 71\textit{ptc} × \textit{ci}\textsuperscript{Ce} crosses are shown. In a \textit{hh}\textsuperscript{Mrt} background, where \textit{hh} is ectopically expressed in the anterior, the severe 71\textit{ptc} phenotype (A) is suppressed (B). In contrast, loss of a copy of the \textit{ci} locus enhances a mild 71\textit{ptc} phenotype (C) to a more severe defect (D). The wing vein fusions of a second \textit{ci} allele, \textit{ci}\textsuperscript{Ce}, (E) are enhanced in combination with 71\textit{ptc} (F). See also Table 2.
Fig. 5. Ptc overexpression in the ptc or en pattern causes wing phenotypes similar to that of 71ptc. UAS ptc expression in the 71B Gal4 (A), ptc Gal4 (B,C) and en Gal4 (D) patterns results in related phenotypes at the A/P border, exemplified by loss of the ACV and/or intense Ci staining is detected (Fig. 6J). In a Kornberg, 1994; Felsenfeld and Kennison, 1995; Fig. 6I), more anterior margin induces target gene expression (Tabata and Kornberg, 1994; Ingham et al., 1991; Capdevila et al., 1994). The phenotype is enhanced by increasing 71ptc dosage indicating that the severity is due directly to the amount of Ptc produced. The proximal regions of the A/P boundary of the wing pouch are most sensitive to Ptc overproduction where Gal4-driven lacZ staining is highest (Fig. 1B). As the phenotypic series progresses, the defects propagate distally along and away from the A/P border (Fig. 2).

Consistent with this hypothesis, the A/P stripe of Ci, like that of ptc and dpp, is almost entirely absent in the wing pouch of 71ptc discs (Fig. 6H). In contrast, the Ci level is unaffected in the notum, where ptc is not overexpressed. Furthermore, in hhMrt wing discs where ectopic Hh expression along the anterior margin induces target gene expression (Tabata and Kornberg, 1994; Felsenfeld and Kennison, 1995; Fig. 6I), more intense Ci staining is detected (Fig. 6J). In a hhMrt51 X-ray revertant, Ci levels are as in wild type, with no intense Ci staining along the anterior margin (data not shown). Confocal images of wild-type wing discs show that within detection limits, Ci protein is located in the cytoplasm, not in the nucleus (Fig. 6K,L, and R. Holmgren, personal communication). The cellular location of Ci does not appear to be altered by the Hh signal since the subcellular Ci distribution is the same throughout the anterior; only the amount of Ci increases at the A/P border and no protein is detected above background in the nucleus.

Protein kinase A regulates Ci levels
Recent studies have shown that PKA, like ptc, inactivates the transcription of hh target genes, in the wing imaginal disc. Mutant clones of PKA in the anterior compartment autonomously express ptc and dpp while, in the surrounding wild-type cells, transcription of these genes is repressed (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995). Since ptc and PKA regulate hh target genes similarly, we determined whether loss of PKA function, like hh inhibition of ptc activity, would cause Ci protein levels to increase. PKA clones were induced in first and second instar larvae and wing discs were doubly stained with β-gal and Ci antibodies. Mutant clones of PKA, identified by the absence of β-gal staining, autonomously express high levels of Ci only in the anterior compartment (Fig. 7A,B). The high expression of Ci in anterior clones is unchanged irrespective of the distance away from the A/P border, suggesting that this effect is independent of the Hh signal. The level of Ci within the clones is generally higher than the level within the Hh-induced stripe at the AP boundary. The subcellular distribution of Ci also appears unchanged within the clones (Fig. 7C). Since ci RNA levels are unaffected in the clones (data not shown) PKA, like ptc, modulates Ci protein levels.

DISCUSSION
Antagonistic functions of Ptc and Hh
Our experiments demonstrate that wing development requires a proper balance between ptc and hh function. Earlier work has shown that increased hh expression causes anterior wing overgrowth (Basler and Struhl, 1994; Kojima et al., 1994; Tabata and Kornberg, 1994; Felsenfeld and Kennison, 1995) and anterior limb overgrowth in chick limb buds (Riddle et al., 1993; Chang et al., 1994). In contrast, ptc overexpression causes reduced wing growth, altered or deleted vein pattern, and changes in sensilla formation at the A/P boundary. The phenotype is enhanced by increasing 71ptc dosage indicating that the severity is due directly to the amount of Ptc produced. The proximal regions of the A/P boundary of the wing pouch are most sensitive to Ptc overproduction where Gal4-driven lacZ staining is highest (Fig. 1B). As the phenotypic series progresses, the defects propagate distally along and away from the A/P border (Fig. 2).

Hh has been proposed to signal unidirectionally from the posterior compartment to adjacent cells in the anterior. This local Hh signal induces target genes, such as dpp, whose product, the signaling molecule, acts bidirectionally to pattern both compartments (Posakony et al., 1990; Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994; Ingham and Fietz, 1995). dpp expression is restricted to a stripe on the anterior side of the compartment border because en represses dpp transcription in the posterior compartment (Sanicola et al., 1995). Ptc activity, unless locally inhibited by Hh, prevents transcription in the anterior (Basler and Struhl, 1994; Tabata and Kornberg, 1994).

Our results fit nicely with models for the antagonistic relation between Hh and Ptc (Ingham et al., 1991; Capdevila et al., 1994 Fig. 8). The 71ptc wing phenotype is suppressed in a hhMrt background where Hh is ectopically expressed. Furthermore, the tran-
scription of hh target genes, such as ptc and dpp, is repressed. The most severe 71ptc wing phenotypes are similar to those of reduced-function hh and dpp wings, where pattern in both the anterior and posterior is greatly diminished and venation is absent or fused (Posakony et al., 1991; Basler and Struhl, 1994). Hence, elevated Ptc levels in the wing disc dampen the signaling ability of Hh. While Hh has been proposed to bind Ptc in a ligand-receptor interaction (Ingham et al., 1991), Hh action could oppose Ptc effects either at the membrane or at other points during the transduction of the Ptc signal from membrane to nucleus.

In addition to providing positional information for patterning the A/P axis, ptc appears to have a second role in regulating neurogenesis in the wing disc. ptc overexpression reduces the number of campaniform sensilla in the wing blade, while reduced ptc function causes ectopic sensilla to form. Two lines of evidence make it unlikely that this effect is due to the alteration of positional information during early disc development. First, in 71ptc wings veins can form normally in the absence of CSNs indicating that the correct positional information is present, but the CSNs do not differentiate. Second, ptc can prevent the formation of the twin campaniform sensilla at the anterior margin far away from the vein defects at the A/P border. Furthermore, previous studies have shown that in ptc mutant embryos defects and supernumerary neurons arise in the central and peripheral nervous system (Patel et al., 1989; Schmucker et al., 1994).

It is difficult to create dominant phenotypes by ubiquitous ptc expression. Modest heat-shock promoter-driven misexpression of ptc causes almost no dominant effects in embryonic (Ingham et al., 1991; Sampedro and Guerrero, 1991) and imaginal (Capdevila et al., 1994) tissues, but can partially rescue defects caused by loss of ptc function. Only with persistent heat shocks of HS ptc flies can an embryonic cuticular defect be generated; such embryos have a lawn of denticles similar to a late loss of wg phenotype (Schuske et al., 1994). It is likely that the difficulty in obtaining a phenotype results from rapid degradation of Ptc. In the embryo, Ptc protein distribution follows the RNA pattern closely. At stage 11, Ptc turns over rapidly in anterior parts of segments where RNA and protein disappear (Capdevila et al., 1994). In the wing disc, much of the A/P stripe of Ptc is gone within an hour after hh is inactivated (Capdevila et al., 1994). Our success in making dominant ptc phenotypes is probably due to the stability and continuous activation by the Gal4 transcription factor.

**The defects caused by ptc overexpression occur where ptc expression is normally at its highest levels**

At first glance, it is counterintuitive that the defects caused by ptc overexpression occur preferentially where Ptc is normally at its highest levels. Endogenous ptc RNA and protein are expressed at low levels throughout the anterior compartment except at the compartment boundary where a stripe of high expression is detected (Phillips et al., 1990; Capdevila et al.,

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**Fig. 6.** Ptc activity modulates the transcriptional or protein levels of several genes in wing imaginal discs. ptc lacZ (A,E,I), dpp RNA (B,F), ci RNA (C,G) and ci protein (D,H,J,L) expression was examined in wing discs from wild type (A-D,K,L), 71ptc (E-H), or hh Mrt (I,J). ptc overexpression reduces the A/P stripe of ptc (E) and dpp (F) transcription and ci protein (H) in the wing pouch of 71ptc discs while ci RNA levels are unaffected (G). In hh Mrt discs, ptc lacZ (I) and Ci (J) levels are elevated along the anterior margin of the wing pouch (arrows) corresponding to where hh is ectopically expressed. Ci localizes to the cytoplasm of cells in the anterior compartment (K) and at the A/P border (L, higher magnification).
However, because \( ptc \) negatively regulates its own transcription, areas of high \( Ptc \) activity have low amounts of \( Ptc \) expression, while in areas where \( Ptc \) function is inactivated (e.g. the A/P border), its transcription and protein levels are much higher (Fig. 7). Hence boosting the levels of \( Ptc \) at the compartment border should enable \( Ptc \) to overcome \( Hh \) negative regulation and become active. Indeed, \( Ptc \) activity in \( 71\) \( ptc \) wing discs is reflected by the inactivation of \( ptc \) and \( dpp \) transcription at the A/P border (Fig. 6E,F). Overexpression of \( ptc \) in either normal \( ptc \) Gal4 or ectopic patterns \( 71B \) Gal4 and \( en \) Gal4) results in a similar wing phenotype (Fig. 5). This suggests that regulation of \( Ptc \) levels and spatial distribution are both important for proper patterning. It is surprising that UAS\( ptc \) expression by \( en \) Gal4 results in defects at the A/P border since \( en \)-expressing cells do not transcribe \( dpp \) (Sanicola et al., 1995). \( Ptc \) may either be inactivating \( dpp \) expression nonautonomously in adjacent anterior cells or interfering with a \( dpp \)-independent pathway in the posterior cells. High \( Ptc \) levels in the posterior may restrict the range of \( Hh \) movement and limit its ability to signal to neighboring anterior cells. In the embryo, \( hh \) protein diffuses further in \( ptc \)-mutants than in wild type (Taylor et al., 1993), suggesting that \( Ptc \) can indeed confine \( Hh \) movement.

**Possible Ptc regulation of \( Hh \) signaling by modulation of \( Ci \) protein**

\( Ptc \) may inactivate the transcription of \( hh \) target genes by preventing the accumulation of \( Ci \) protein. The lowering of \( Ci \) levels closely follows the increase in \( Ptc \) activity. Near \( Hh \)-expressing cells, whether at the A/P border in wild-type discs or further into the anterior compartment in \( hh^{Min} \) discs, \( Ptc \) is presumably functionally inactivated and the \( Ci \) level is elevated (Fig. 6D,J). Conversely, in \( 71\) \( ptc \) wing discs, where \( Ptc \) overexpression has overcome inhibition by \( Hh \), \( Ci \) levels are reduced to a basal state (Fig. 6H). \( Ptc \) may reduce \( Ci \) levels by
enhancing protein degradation or preventing Ci translation. In ci<sup>Ex</sup> wing discs, Ci accumulates at high levels throughout the anterior (Slusarski et al., 1995) suggesting that Ci negatively regulates its own translation, possibly by binding its mRNA.

PKA and ptc appear to modulate hh target genes similarly. Previous studies have shown the induction of dpp and ptc expression in PKA mutant clones in wing discs (Jiang and Struhl, 1995; Lepage et al., 1995; Li et al., 1995; Pan and Rubin, 1995). In this study we have found that PKA, like ptc, also prevents the accumulation of Ci. Throughout the anterior compartment, clones of cells that lack PKA activity have increased amounts of Ci protein, which are even higher than the Hh-induced Ci levels at the A/P border. The effect of PKA is cell autonomous since the regions of high Ci protein are coincident with the cells lacking PKA, suggesting that PKA does not control a diffusible signal that induces high level Ci. Since ptc and PKA appear to behave indistinguishably, we are not able to determine whether these two genes act in the same or in parallel pathways.

Ptc and PKA activity appear to regulate the abundance of Ci, not its distribution, since the cellular localization of Ci is unchanged whether near or far from Hh-expressing cells or in PKA mutant clones (Figs 6L, 7C). Ci has been proposed to bind directly to hh target genes to activate transcription (Forbes et al., 1993), yet its apparent absence from the nucleus in PKA clones or in the presence of the Hh signal makes this scenario less tenable. However, we cannot rule out the possibility that low levels of Ci are present in the nucleus.

In fused mutant imaginal discs, Ci protein accumulates at high levels throughout the anterior (Slusarski et al., 1995). This suggests that reduced fu function, like that of reduced ptc activity, allows Ci levels to elevate. However, experiments in the embryo show that fu and ptc have opposing functions; fu is required to maintain hh-dependent gene expression while ptc inactivates this transcription (Forbes et al., 1993). Hence, one would expect Ci levels to fall in fu wing discs, not accumulate. We currently cannot resolve this paradox. It may be that fu acts in opposite ways to maintain hh target gene expression and prevent Ci accumulation.

We thank Drs D. Lin and C. Goodman for providing their Gal4 enhancer trap lines, Dr R. Holmgren for ci antibody, ci<sup>M62f</sup> stock and communication of unpublished data, Drs D. Pan and G. Rubin for the PKA stocks, L. Mathies for the dpp probe and Kaye Suyama for confocal microscopy of wing discs. We are also grateful to L. Goodrich and Drs G. Hime, K. Cadigan and R. Nusse for helpful discussions and critical reading of the manuscript. R. L. J. was supported by the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Fellowship, DRG 1218. This research was supported by the Howard Hughes Medical Institute.

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Nature 341, 508-513.


(Accepted 22 August 1995)