Development 121, 4161-4170 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995
DEV6187

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 *hedgehog*^{Mrt} 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 (Préat 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 *Eco*RI/*Spe*I fragment of a *ptc* cDNA (Schuske et al., 1994) containing the entire coding region of *ptc* was cloned in the sense orientation into the *Eco*RI/*Xba*I sites of pUAST (Brand and Perrimon, 1993). This vector, which contains the mini *white* gene, was injected into Df(1)*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* Gal4$^{559.1}$(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), *hh*^{Mrt}, *hh*^{MrtR1}, *hh*^{MrtR2} (Tabata and Kornberg, 1994), *cos-2* ^{V1}*ptc* ^{tuf}, *ci*^{Ce} and *ci*^{M62f} (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*; 71*ptc* 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 71*ptc* 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 NH_4OAc 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 2× EtOH, 1× MeOH, 1× 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 53ºC in 50% formamide, 5× 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 5× 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 (PBT). 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 PBT ON at 4°C, followed by PT washes and 1:500 goat anti-rat biotin in PBT. 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 *PKA*^{B3} *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 71*ptc*.

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 (Figs 2, 5A).

The 71*ptc* wing phenotype is as follows (Fig. 2): In a wild-type wing, longitudinal veins L3 and L4 run parallel and close to the A/P border along the entire proximal/distal axis of the wing blade. In the proximal third of the wing, the anterior cross

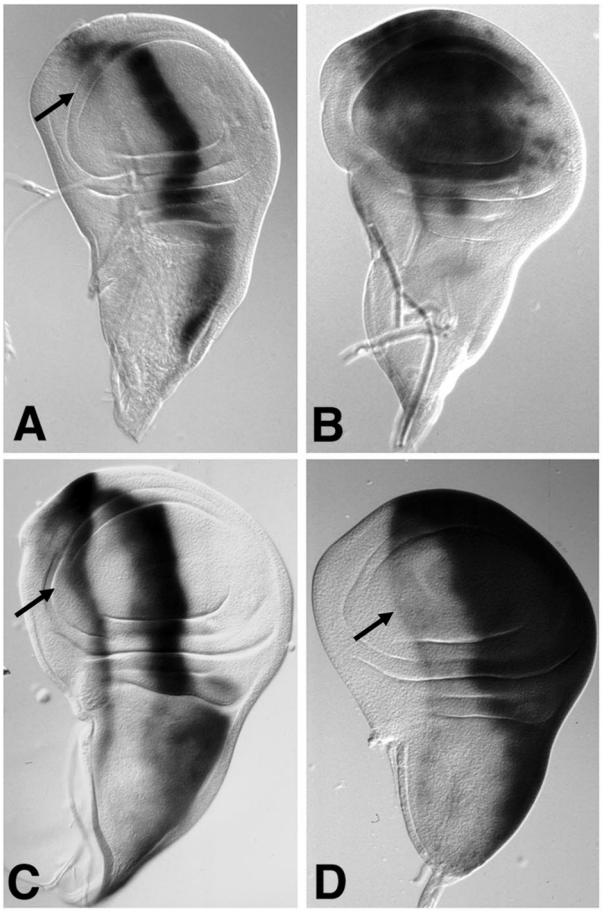

Fig. 1. Wing disc expression patterns of enhancer *lacZ* or Gal4-driven UAS *lacZ* lines. X-gal staining in wing imaginal discs is shown for *ptc* enhancer trap (A), 71B Gal4 (B), *ptc* Gal4 (C) and *en* Gal4 (D). The arrows indicate peripodial staining which lies above the anterior region of the imaginal tissue layer.

vein (ACV) intersects the A/P border to connect the two veins. 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.

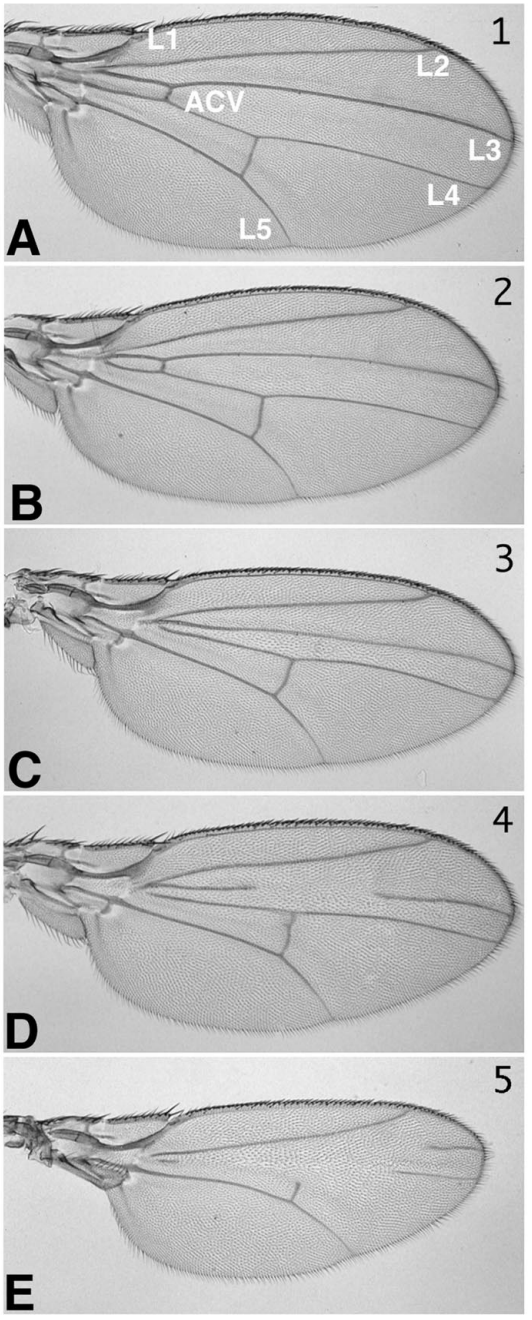

Fig. 2. Phenotypic series of wing patterning defects caused by 71*ptc*. UAS *ptc* expression in the 71B Gal4 pattern causes alterations in the L3 and L4 veins at the A/P border. (A) A wild-type wing is denoted as class 1 and the 5 longitudinal veins (L1-L5) and anterior cross vein (ACV) are indicated. This completely penetrant 71*ptc* phenotype is scored from mild (class 2) to severe (class 5). (B,C) Class 2 and 3 phenotypes show loss of the ACV or fusion of L3 and L4 at the proximal end. (D,E) Class 4 and 5 phenotypes result in deletion of L3 and L4, alteration of L2 and L5, and reduction in wing blade size. The phenotype rating is indicated in the upper right-hand corner of each panel.

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 ^{tuf}/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*ptc* 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. *neuralized* (*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*ptc* 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*ptc* 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*ptc*. A dominant *hh* allele, *Moonrat* (*hh^{Mrt}*), 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^{Mrt}* background, the 71*ptc* 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^{Mrt}* gain of function since two X-ray revertants, *hh^{MrtR1}* and *hh^{MrtR2}*, which have lost the wing overgrowth (Tabata and Kornberg, 1994), fail to suppress the 71*ptc* 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*ptc* phenotype is greatly enhanced in the backgrounds of two *ci* alleles, *ci^{M62f}* and *ci^{Ce}* (Fig. 4C-F; Table 2). Wing veins L3 and L4 are extensively deleted when 71*ptc* is combined with *ci^{M62f}*, a deficiency that removes the entire *ci* locus (Fig. 4D). *ci^{Ce}*, a dominant allele of *ci* which produces a truncated *ci* protein (Slusarski et al., 1995), has a wing phenotype similar to that of 71*ptc*; a fusion of L3 and L4 and loss of the ACV (Fig. 4E). The 71*ptc* chromosome appears to enhance the *ci^{Ce}* 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* overex-

Table 1. Expressivity of 71*ptc* wing phenotype

	Phenotype class (%)						
	1	2	3	4	5	N	°C
71*ptc* / +	0	35	37	28	<1	115	24
71*ptc* / 71*ptc*	0	0	14	31	56	36	
71*ptc* / +	0	0	1	4	95	179	29
71*ptc* / 71*ptc*			lethal				

Wings from 71*ptc* 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*ptc* homozygotes had wings had severe vein alterations at 24°C and died as pupae at 29°C.

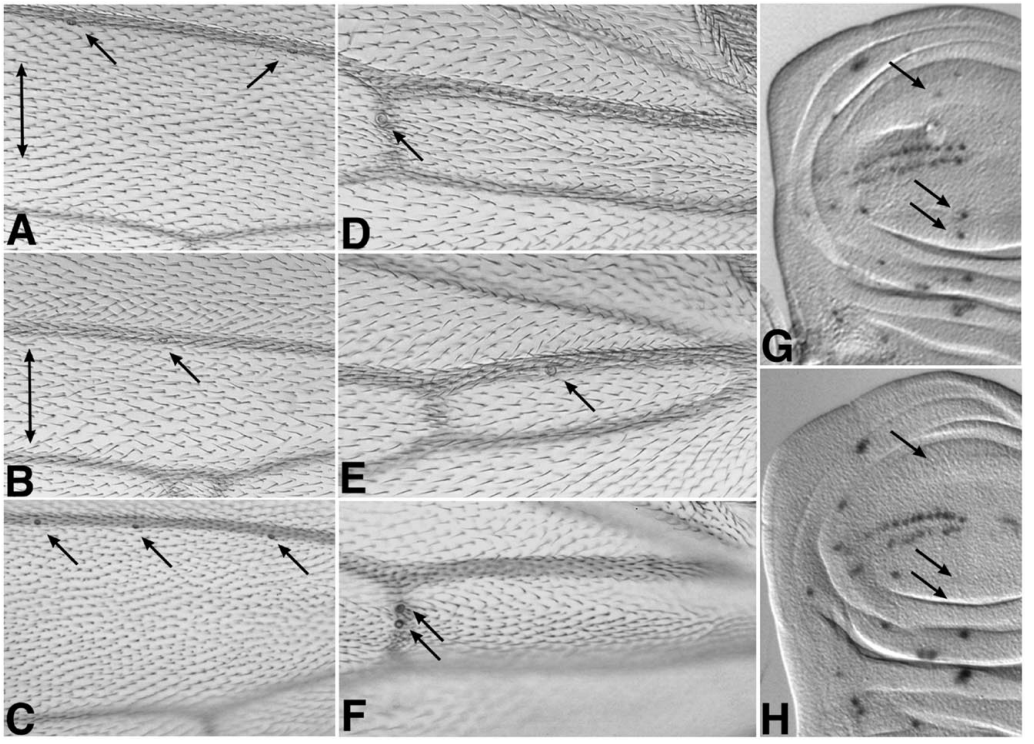

Fig. 3. *ptc* inhibits the formation of the campaniform sensory neurons (CSN) in wings and their precursors, the sensory mother cells, in imaginal discs. CSNs (arrows) along the L3 (A-C) and ACV (D-F) regions of the wing blade are shown for wild type (A,D), 71*ptc* (B,E), and *ptc^{tuf}* (C,F). The double-headed arrow (A,B) indicates the reduced distance between L3 and L4 in 71*ptc* wings. *neu lacZ* staining of wing imaginal discs show the presence (wild type; G) or absence (71*ptc*; H) of the L3 sensory mother cells (arrows).

pression in the anterior compartment or by ectopic *ptc* expression in the posterior. To examine these two possibilities, *ptc* Gal4 (Gal4$^{559.1}$; 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*ptc* expression in the *ptc* Gal4 pattern results in a mild 71*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*ptc* lines crossed to *ptc* Gal4, two show loss of the anterior crossvein (Fig. 5B and data not shown) while a third line (UAS*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 at 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

ptc presumably mediates its effects on wing disc patterning at least in part by modulating the activities of *hh* target genes. Since *ptc* and *dpp* are activated locally near *hh*-expressing regions (Basler and Struhl, 1994; Capdevila et al., 1994; Tabata and Kornberg, 1994), we determined whether *ptc* overexpression alters *dpp* or *ptc* transcription levels. In 71*ptc* wing discs, the A/P stripe of *ptc lacZ* staining is reduced in the wing pouch where *ptc* is highly expressed, while levels in the notum where no extra *ptc* is produced are comparable to wild type (Fig. 6A,E). This result is consistent with previous experiments showing that *ptc* negatively autoregulates in the wing disc (Capdevila et al., 1994). *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 *ci* is required to activate *hh* target genes and is downstream of *ptc* (Forbes et al., 1993). *ptc* might inhibit transcription of these genes by regulating *ci* RNA or protein levels. *ci* RNA is homogeneously distributed throughout the anterior compartment of both wild-type (Eaton and Kornberg, 1990; Orenic et al., 1990) and 71*ptc* imaginal discs (Fig. 6C,G). In contrast, 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 Ci staining suggests that the Ci level may be modulated by *ptc*.

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

	Phenotype class (%)					
	1	2	3	4	5	N
71*ptc* / +	0	6	20	50	23	64
71*ptc* / *hh^{Mrt}*	0	24	68	8	0	66
71*ptc* / +	0	2	6	36	57	340
71*ptc* / *hh^{MrtR1}*	0	1	12	35	53	430
71*ptc* ; +	0	8	68	24	<1	199
71*ptc* ; *ci^{M62f}*	0	1	4	27	67	346
+ ; *ci^{Ce}*	8	73	19	0	0	292
71*ptc* ; +	0	25	58	15	1	259
71*ptc* ; *ci^{Ce}*	0	<1	13	18	68	261

The 71*ptc* wing phenotype is suppressed from class 4 to class 3 in a *hh^{Mrt}* background but is unaffected in a *hh^{MrtR1}* background. Loss of one copy of the *ci* locus (*ci^{M62f}*) enhances the severity of the 71*ptc* phenotype from class 3 to class 5. The combination of 71*ptc* and *ci^{Ce}* chromosomes causes much more severe vein alterations than either 71*ptc* or *ci^{Ce}* alone.

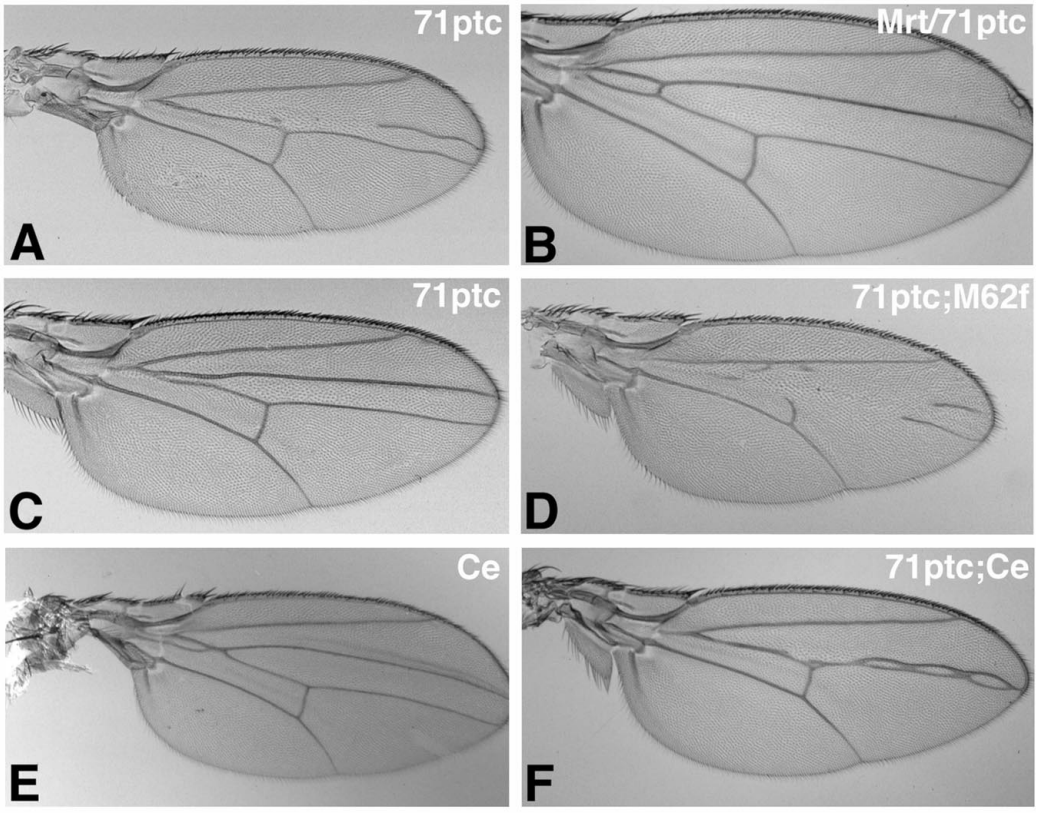

Fig. 4. *hh^{Mrt}* suppresses and *ci* alleles enhance the 71*ptc* wing phenotype. Wings of sibling flies from 71*ptc* × *hh^{Mrt}* (A,B), 71*ptc* × *ci^{M62f}* (C,D) and 71*ptc* × *ci^{Ce}* crosses are shown. In a *hh^{Mrt}* background, where *hh* is ectopicly expressed in the anterior, the severe 71*ptc* phenotype (A) is suppressed (B). In contrast, loss of a copy of the *ci* locus enhances a mild 71*ptc* phenotype (C) to a more severe defect (D). The wing vein fusions of a second *ci* allele, *ci^{Ce}*, (E) are enhanced in combination with 71*ptc* (F). See also Table 2.

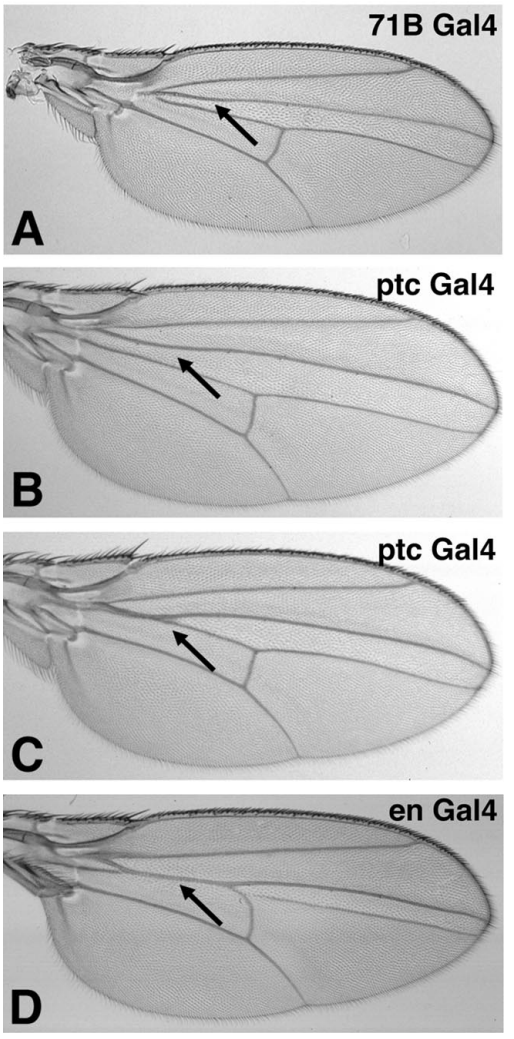

Fig. 5. Ptc overexpression in the *ptc* or *en* pattern causes wing phenotypes similar to that of 71*ptc*. UAS *ptc* expression in the 71B Gal4 (A), *ptc* Gal4 (B,C) and *en* Gal4 (D) patterns results in related phenotypes at the A/P border, exemplified by loss of the ACV and/or L3 and L4 fusions (arrows).

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 71*ptc* discs (Fig. 6H). In contrast, the Ci level is unaffected in the notum, where *ptc* is not overexpressed. Furthermore, in hh^{Mrt} 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 hh^{MrtR1} 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 71*ptc* 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 *lac Z* 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 71*ptc* wing phenotype is suppressed in a hh^{Mrt} background where Hh is ectopically expressed. Furthermore, the tran-

scription of *hh* target genes, such as *ptc* and *dpp*, is repressed. The most severe 71*ptc* 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 71*ptc* 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.,

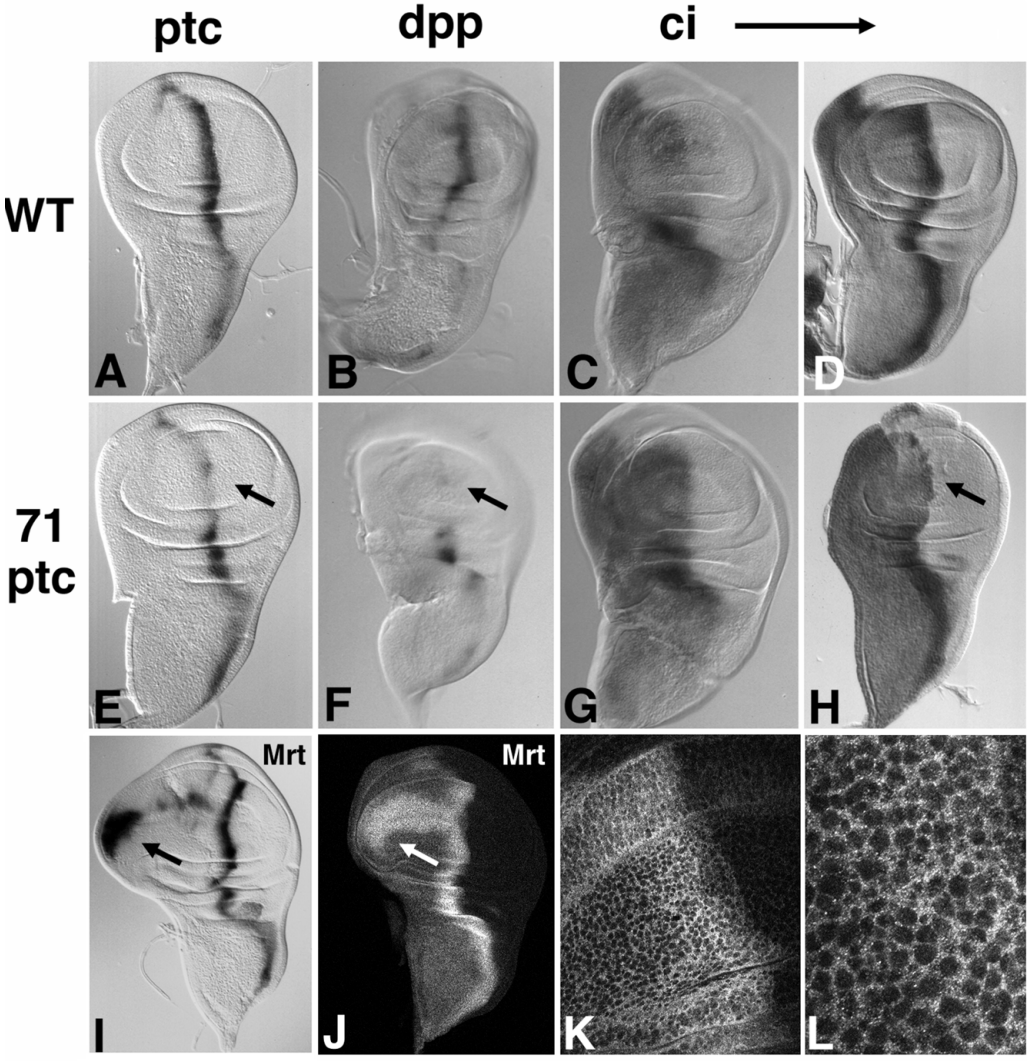

Fig. 6. Ptc activity modulates the transcriptional or protein levels of several genes in wing imaginal discs. *ptc lacZ* (A,E,I), *dpp* RNA (B,F), *ci* RNA (C,G) and *ci* protein (D,H,J-L) expression was examined in wing discs from wild type (A-D,K,L), 71*ptc* (E-H), or hh^{Mrt} (I,J). *ptc* overexpression reduces the A/P stripe of *ptc* (E) and *dpp* (F) transcription and *ci* protein (H) in the wing pouch of 71*ptc* 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).

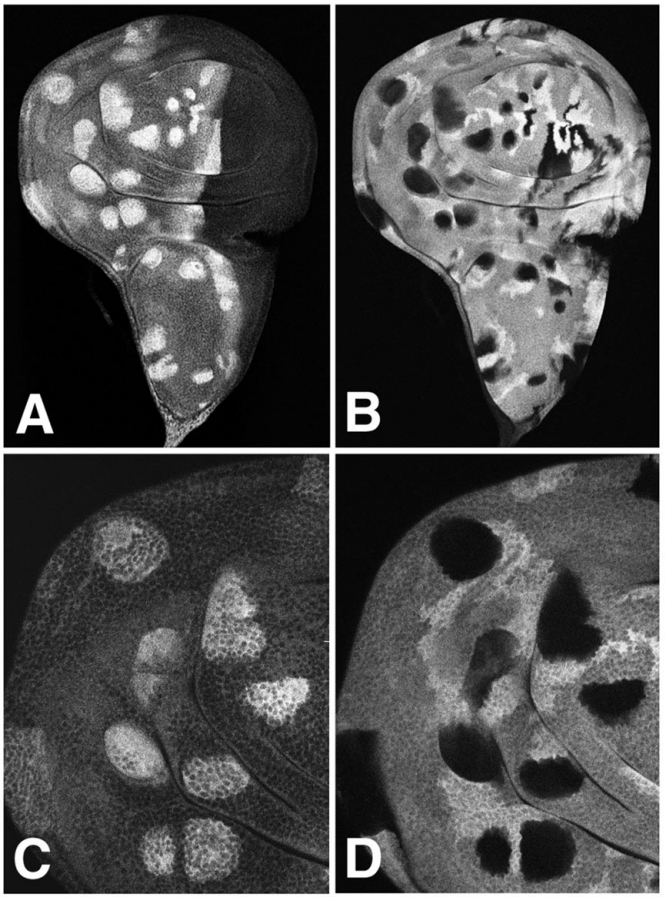

Fig. 7. PKA regulates Ci levels in the wing disc anterior. Wing imaginal discs which contain clones mutant for PKA, were doubly stained with antibodies to Ci (A,C) and β-gal (B,D). Clones that lack PKA (marked by the absence of β-gal staining) have high levels of Ci only in the anterior compartment (A). Accompanying the clones lacking PKA are clones with two doses of *lacZ*, which are visible as more intensely stained cells (B,D). Higher magnification (C) suggests that the subcellular distribution of Ci remains cytoplasmic. Dorsal is up and anterior to the left in all panels.

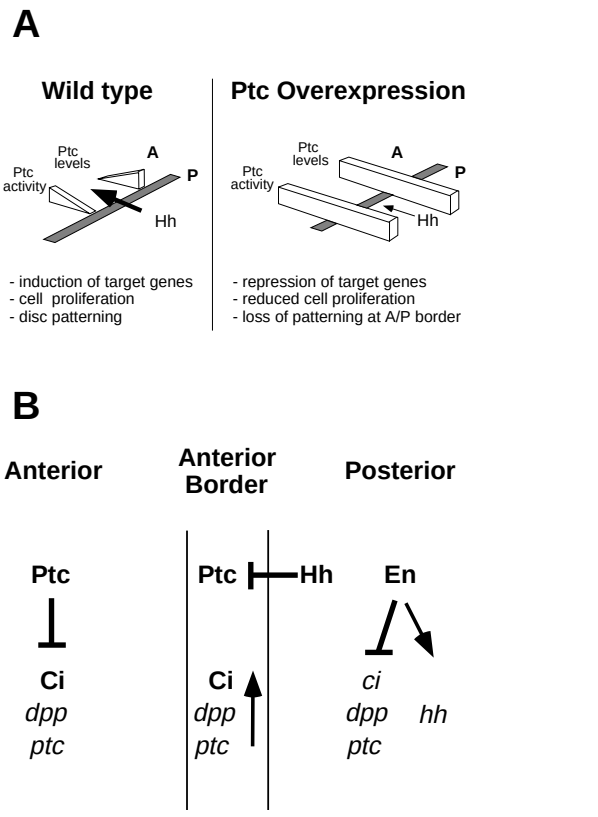

Fig. 8. Models of Ptc function and relations to other genes. (A) A model of Ptc function in patterning wild type and *ptc* overexpression wing discs. In the wild-type wing disc, Hh blocks Ptc activity locally at the A/P border to activate expression of target genes such as *ptc* and *dpp*. When *ptc* is sufficiently overexpressed, the Hh signal is reduced, which results in the loss of target gene expression and subsequent patterning of both compartments at the A/P boundary. (B) Regulatory relationships among genes required for A/P wing patterning. Gene transcription is shown in italics while regulation at the protein level is shown in bold. In the posterior, the transcription factor *engrailed* (En) represses *ci*, *ptc* and *dpp* transcription while activating *hh* expression. Production of Hh blocks Ptc activity, to permit accumulation of Ci protein and *ptc* and *dpp* transcription at the compartment border, which is normally prevented by Ptc in the remainder of the anterior. PKA has been omitted from the diagram since its expression pattern in the wing disc is not determined. Its effect, like that of Ptc, is negative, but it is not possible to say whether it acts in the same pathway as Ptc or a parallel one.

1994). 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^{Mrt} 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^{Ce} 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^{M62f} 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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(Accepted 22 August 1995)