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The *Drosophila* segment polarity gene *patched* interacts with *decapentaplegic* in wing development

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The *decapentaplegic (dpp)* gene of *Drosophila melanogaster* encodes a polypeptide of the transforming growth factor-β family of secreted factors. It is required for the proper development of both embryonic and adult structures, and may act as a morphogen in the embryo. In wing imaginal discs, *dpp* is expressed and required in a stripe of cells near the anterior-posterior compartment boundary. Here we show that viable mutations in the segment polarity genes *patched (ptc)* and *costal-2 (cos2)* cause specific alterations in *dpp* expression within the anterior compartment of the wing imaginal disc. The interaction between *ptc* and *dpp* is particularly interesting; both genes are expressed with similar patterns at the anterior-posterior compartment boundary of the disc, and mis-expressed in a similar way in segment polarity mutant backgrounds like *ptc* and *cos2*. This mis-expression of *dpp* could be correlated with some of the features of the adult mutant phenotypes. We propose that *ptc* controls *dpp* expression in the imaginal discs, and that the restricted expression of *dpp* near the anterior-posterior compartment boundary is essential to maintain the wild-type morphology of the wing disc.

Key words: decapentaplegic/Drosophila segment polarity genes/*patched*/transforming growth factor-β/wing development

Introduction

Although little is known about the mechanisms that underlie pattern formation in *Drosophila* imaginal discs, several classes of developmental genes are known to play a role in this process. The segment polarity genes constitute one of such classes. These genes define the correct pattern within each embryonic segment (Nüsslein-Volhard and Wieschaus, 1980; reviewed in Ingham, 1991; Klingensmith and Perrimon, 1991; Ingham and Martínez-Arias, 1992; Peifer and Bejsovec, 1992), and they are also needed for the correct formation of adult structures (reviewed in Whittle, 1990; Wilkins and Gubb, 1991). Most of these structures are derived from groups of cells set aside during embryonic development, constituting the imaginal discs (Bate and Martínez-Arias, 1991; Cohen *et al.*, 1991). From early in development, most of the imaginal discs are subdivided into two groups of cells that would form the anterior and posterior compartments (García-Bellido *et al.*, 1973, 1976; Morata and Lawrence, 1977). The involvement of the segment polarity genes in patterning the imaginal discs is demonstrated by the phenotypes of viable alleles and by the study of mitotic recombinant clones of lethal alleles. For instance, in en^1, a viable allele of the segment polarity gene *engrailed (en)*, there is a transformation of part of the posterior compartment of several imaginal discs into the anterior compartment (García Bellido and Santamaría, 1972). Viable mutants of the segment polarity gene *wingless (wg)* cause a severe loss of adult structures and/or duplications in the entire wing disc (Sharma and Chopra, 1976; Morata and Lawrence, 1977; Baker, 1988a,b; Couso *et al.*, 1993). Although mutations in the segment polarity genes affect the derivatives of several imaginal discs, in this work we are mostly concerned with the development of the wing disc.

Mutations in other genes of this class alter the morphology of the anterior compartment of the wing disc, leaving the posterior apparently unaltered. Thus, viable mutations in the *patched (ptc)* gene result in local overgrowth within the anterior compartment of the wing, loss of specific structures, and alterations in venation and in the pattern of sensory organs (Phillips *et al.*, 1990). The *ptc* gene encodes a transmembrane protein (Hooper and Scott, 1989; Nakano *et al.*, 1989) and is transcribed throughout the anterior compartment with a stronger expression near the anterior-posterior compartment boundary of the wing imaginal disc (Phillips *et al.*, 1990). This boundary could play an important role in the establishment of positional information over the whole surface of the disc, in a similar way to that proposed for the parasegmental border in the embryo (Martínez-Arias and Lawrence, 1985; reviewed in Ingham and Martínez-Arias, 1992). Mutations in the *costal-2 (cos2)* gene give rise to duplications of the anterior part of the wing blade (Whittle, 1976; Simpson and Grau, 1987). Embryos mutant for lethal alleles of *cos2* derived from a mutant female germ line display a cuticular phenotype similar to that of *ptc* mutant embryos (Grau and Simpson, 1987).

Many other genes, not included within the segment polarity class, also participate in the formation of imaginal structures. The *decapentaplegic (dpp)* gene is a clear example of this. This gene is needed for the development of derivatives of most or all imaginal discs (Spencer *et al.*, 1982). In the wing disc, for example, absence of *dpp* produces flies with smaller wings, absent in extreme cases. This is due to massive apoptotic cell death in mid-third instar wing imaginal discs, resulting in smaller discs (Bryant, 1988). Clonal analysis of *dpp* mutations revealed that *dpp* expression is required solely in the cells just anterior to the anterior-posterior compartment boundary of the wing disc (Posakony *et al.*, 1991). Consistent with this, *dpp* is transcribed precisely in this subset of cells (Masucci *et al.*, 1990; Posakony *et al.*, 1991; Raftery *et al.*, 1991). *dpp* encodes a secreted factor of the transforming growth factor-β (TGF-β) family (Padgett *et al.*, 1987) with a probable role in cell-cell communication. In agreement with this, *dpp*

presents non-autonomous behaviour in mosaics. In the case of wing development, *dpp* expression along the anterior–posterior compartment boundary has been suggested to provide a signal for the establishment of proximal–distal positional information (Gelbart, 1989). In addition, *dpp* has recently been proposed in the embryo to act as a classical morphogen in the correct specification of dorsal structures (Ferguson and Anderson, 1992; Wharton *et al.*, 1993).

The expression of *ptc* and *dpp* genes in the wing disc bears a certain similitude: *ptc* presents a maximal and clear accumulation of expression in a region that roughly coincides with that of *dpp*. This similar pattern of transcription suggests that a relationship between *ptc* and *dpp*, or a response of both of them to a third factor, might be present in imaginal discs. We have explored this possibility by analyzing the expression of *ptc* and *dpp* in different combinations of *ptc* alleles, as well as in the $cos2^{v1}$ $Cos1^{2}$ mutant combination. The expression of *dpp* was also studied in clones of cells lacking *ptc* function, as well as in cases where *ptc* is overexpressed in the whole disc under heat shock control. Our results indicate that *ptc* is required to prevent *dpp* transcription throughout the anterior compartment, thus restricting *dpp* expression to the cells near the anterior–posterior compartment boundary. *dpp* mis-expression may be responsible for the adult phenotypes of some segment polarity genes like *ptc*.

Results

Localization of ptc product in the wing imaginal discs

In third instar wing imaginal discs, ptc protein is expressed throughout the anterior compartment, with a stripe of maximal intensity near the anterior–posterior compartment boundary. The width of this stripe increases during the third instar and in mature discs it is 8–10 cell diameters wide. There is a stronger staining in the five or six cell diameters closer to the posterior compartment all along the ptc stripe (Figure 1b). The cells of the stripe belonging to the presumptive wing margin give a weaker ptc signal (Figure 1b). In the rest of the compartment, there is a uniform basal level of ptc expression. This pattern coincides with that previously described for the RNA expression by *in situ* hybridization (Phillips *et al.*, 1990).

In order to localize more precisely the ptc stripe with respect to the compartment boundary, a double staining for ptc and a posterior compartment marker was performed. For this purpose, a strain expressing the bacterial *β-galactosidase* (*β-gal*) gene under the control of the *en* promoter was used (*en–lacZ;* Hama *et al.*, 1990). To detect the ptc antigen, the discs were stained with an anti-ptc monoclonal antibody (Capdevila *et al.*, submitted), and to detect β-gal activity X-Gal staining was used (Figure 1a). Although expression of en protein has generally been taken to define the anterior–posterior compartment boundary, it has recently been shown (Blair, 1992) that from mid-third instar onwards, en protein expression extends several cell diameters more anterior than the lineage restriction boundary determined by clonal analysis. This is also true for *en–lacZ* expression, although the anterior expression of the construct is faint (Blair, 1992). We have observed that cells expressing the ptc protein are anterior to the β-gal-expressing cells in third instar wing discs from the *en–lacZ* stock (Figure 1a and b), but a few cells in the stripe near the anterior–posterior compartment boundary co-express both ptc and β-gal antigens (see arrows in Figure 1b). This observation agrees with that of Phillips *et al.* (1990).

Wing imaginal discs were also doubly stained to compare *ptc* and *dpp* expression (Figure 1c–f). For this purpose, a strain expressing a *dpp–lacZ* construct, which mimics the expression of the *dpp* RNA, was used (Blackman *et al.*, 1991). This expression is observed in a band of cells abutting the anterior–posterior compartment boundary. In Figure 1e and f, it is shown that the patterns of expression of ptc and *dpp–lacZ* partially overlap, although the pattern evolves during the third instar. The posterior edge of the ptc stripe runs straight, crossing the wing pouch, whilst the posterior edge of β-gal expression is more anterior, some cells being stained for ptc, but not for β-gal (Figure 1e and f). This is more clearly seen in the distal regions of the wing pouch, whilst in the proximal regions the posterior edge of *dpp–lacZ* coincides with that of ptc in early third instar discs (Figure 1f). In older discs, fewer cells in the posterior edge of the ptc stripe co-express ptc and the *dpp–lacZ* construct (Figure 1e).

Expression of ptc in the compartment boundary is dependent on hh activity

It has previously been shown that the segment polarity gene *hedgehog (hh)* is expressed in posterior regions of embryonic segments and in the posterior compartments of the imaginal discs. It encodes a putative transmembrane protein (Lee *et al.*, 1992; Mohler and Vani, 1992; Tabata *et al.*, 1992; Tashiro *et al.*, 1993), including a putative site for proteolytic cleavage (Lee *et al.*, 1992). In clones, *hh* behaves non-autonomously, also affecting anterior structures (Mohler, 1988), which is consistent with a possible role as a signal molecule. *ptc* represses *ptc* and *wg* transcription in the embryo, except in the cells near the parasegmental boundary. It has been proposed that there is an interaction between *hh* and *ptc* that could block the repressive effect of *ptc* over *ptc* and *wg* (Ingham *et al.*, 1991). In accordance, the phenotype of *ptc; hh* double mutants in the embryo is like that of *ptc* alone (Hidalgo, 1991; Ingham *et al.*, 1991; Ingham and Hidalgo, 1993).

Using a temperature-sensitive allele of *hh* (hh^{9K}; Jürgens *et al.*, 1984; Mohler, 1988), we observe that the transient inactivation of the hh protein in third instar wing imaginal discs results in a dramatic reduction of the level of ptc protein in its stripe of strong expression near the anterior–posterior compartment boundary (Figure 2). After a pulse of 45 min at the restrictive temperature (25°C), the ptc protein, which is strongly accumulated in the cells of the stripe at the permissive temperature of 17°C (Figure 2A), begins to disappear and the remaining protein is located mostly in large intracellular deposits (Figure 2B). These deposits are present throughout the entire width of the stripe, and no changes are evident in the rest of the anterior compartment. Longer pulses at the restrictive temperature (20–24 h) result in the complete disappearance of the stripe of strong expression of ptc, although apparently wild-type levels of ptc staining remain in the whole anterior compartment (Figure 2C).

By contrast, after 24 h at the restrictive temperature the level of *dpp* expression in hh^{9K} wing discs is not different from that of the wild-type, as seen by *in situ* hybridization using a *dpp* probe (data not shown). When keeping the larvae at the restrictive temperature during the second and third instars, there is only a slight decrease in the width of the *dpp* stripe. Thus, normal hh activity is required in third instar

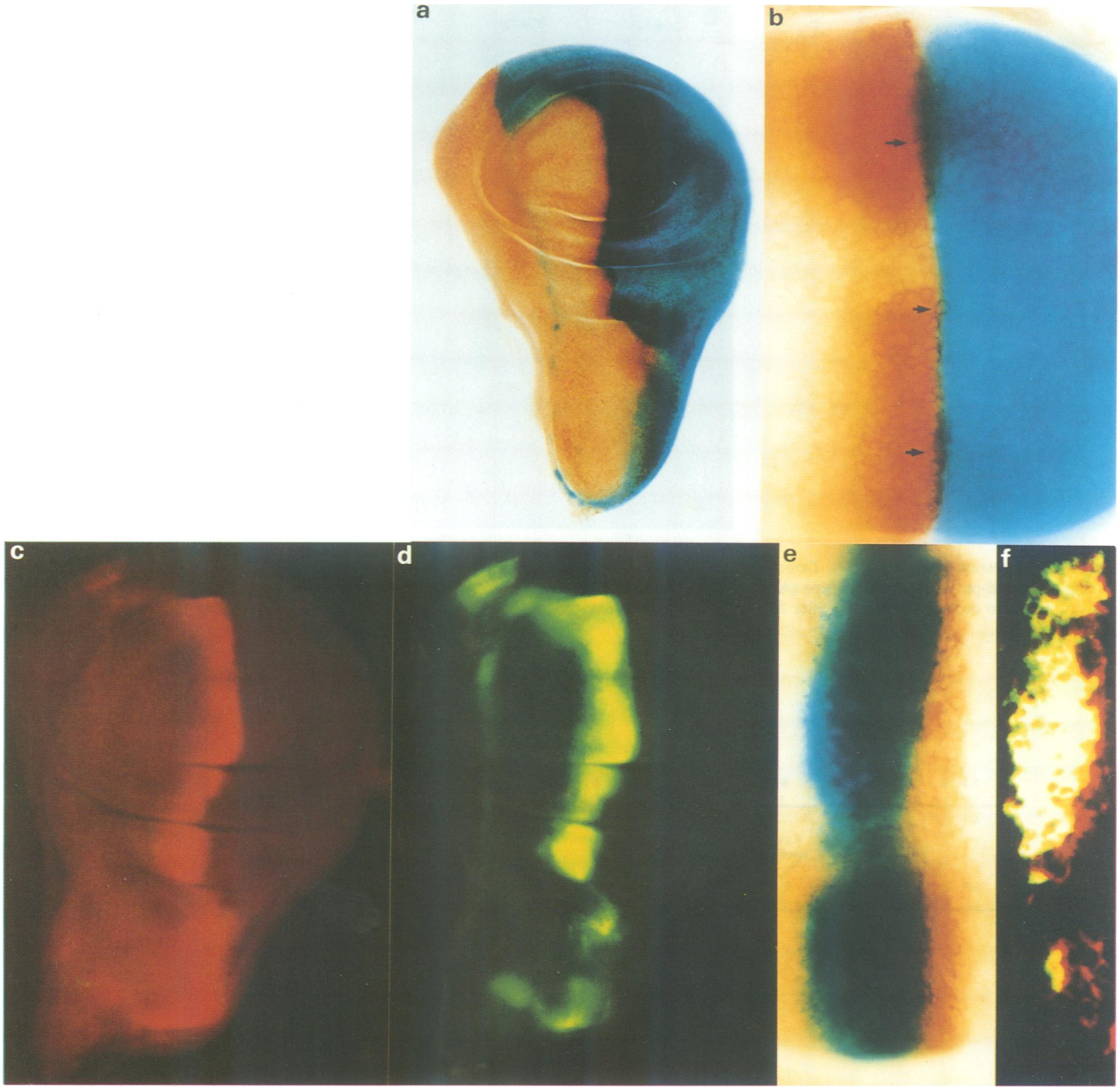

Fig. 1. ptc expression in the third instar wing imaginal disc relative to *en* and *dpp*. (**a**) Whole-mount double staining with anti-ptc (brown) and X-Gal (blue) in an *en−lacZ* disc. (**b**) Detail of the wing pouch of a similar disc, where some cells co-express ptc and β-galactosidase (arrows). (**c** and **d**) Whole-mount double immunofluorescence staining with anti-ptc (red, c) and anti-β-gal (green, d) in the same *dpp−lacZ* disc. (**e**) Detail of the wing pouch of a *dpp−lacZ* third instar imaginal disc doubly stained with anti-ptc monoclonal antibody (brown) and X-Gal (blue). The stripe of maximal intensity of ptc staining runs straight across the wing pouch, whilst *dpp−lacZ* expression is more anterior. (**f**) Detail of the wing pouch of a *dpp−lacZ* disc stained as in (c) and (d), observed under a confocal microscope. ptc staining is in red, β-gal in green and the extensive region of overlapping is seen in yellow. In this and all the subsequent figures, discs are oriented anterior to the left and ventral side up.

wing discs for the expression of the wild-type stripe of ptc near the compartment border, but not for *dpp* maintenance.

Molecular characterization of ptc mutants

We have characterized at the molecular level the two hypomorphic alleles of *ptc* used in this work. They were first studied by examining the restriction polymorphisms in the *ptc* genomic DNA region of these alleles. These polymorphisms were compared with the progenitor chromosome (in the case of ptc^{G20}), and with Oregon-R (in the case of tuf^{1}, since it is a spontaneous mutation for which the original chromosome is unknown). For tuf^{1} we found an insertion of ~7 kb in the 5′ regulatory region (Figure 3A). The insertion is located between −50 and −100 bp upstream of the transcription initiation site (Hooper and Scott, 1989; Nakano *et al.*, 1989). Since the progenitor chromosome of tuf^{1} is not available, we cannot be sure that the observed change in the promoter region is responsible for the phenotype. For ptc^{G20}, we found a deletion <100 bp in the 5′ region of the coding sequence (Figure 3B, left). To analyze this deletion accurately, a fragment corresponding to the first exon was amplified by polymerase chain reaction (PCR), cloned and sequenced. The lesion was found to be a deletion of 66 bp that eliminates 22 amino acids of the coding region (Figure 3B, right). At the 3′ end of this deletion five new bases appear. The deletion does not alter

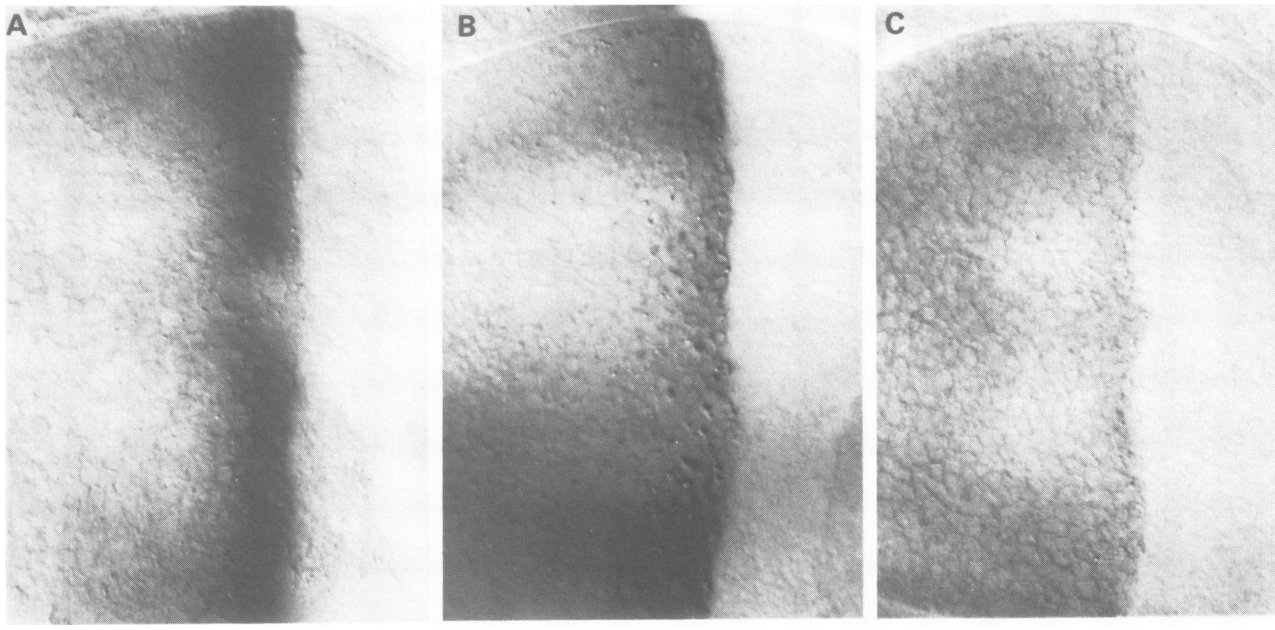

Fig. 2. ptc expression in hh^{9K} mutants. Detail of the ptc expression in the wing pouch of third instar wing imaginal discs detected by peroxidase staining using anti-ptc antibody. Wild-type (**A**). In hh^{9K}, after 45 min at the restrictive temperature, the level of ptc staining in its stripe of maximal intensity begins to decay (**B**). In hh^{9K}, after 24 h at the restrictive temperature, homogeneous levels of ptc protein are seen throughout the anterior compartment (**C**).

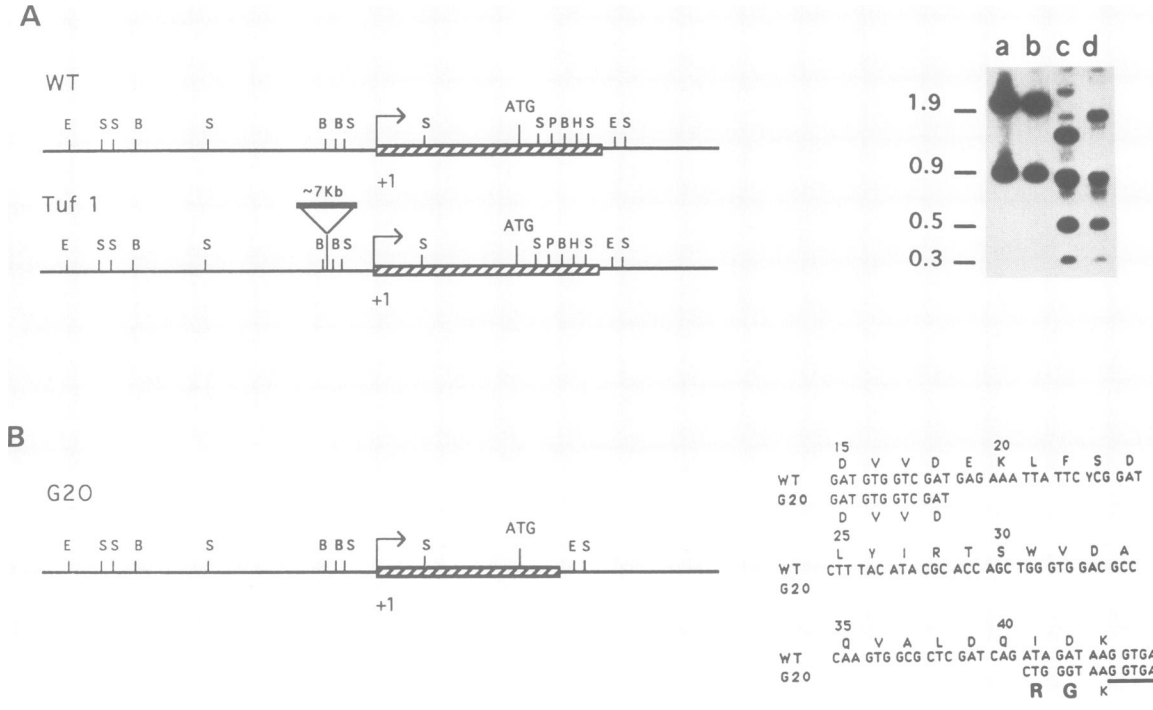

Fig. 3. Molecular characterization of tuf^1 and ptc^{G20} mutations. (**A**) Molecular analysis of tuf^1 mutation. Left: restriction map of the 5′ region of the *ptc* genomic DNA in wild-type and in tuf^1 chromosomes. tuf^1 presents a 7 kb insertion upstream of the transcription initiation site (indicated by the arrow). Right: Southern blot hybridized with an *Eco*RI DNA fragment used as a probe. Lanes a and b: genomic DNA from wild-type (a) and tuf^1 mutant (b), digested with *Bgl*I, where no differences in the restriction pattern are observed. Lanes c and d: genomic DNA from wild-type (c) and tuf^1 mutant (d), digested with *Sau*3aI; a restriction polymorphism in the 1.3 kb band is observed. An insertion is located between the two *Bgl*I sites (−50 and −100 bp from the transcription initiation site). Further digestions with other restriction enzymes (not shown) indicated that the size of the insertion was ~7 kb. *Bgl*I (B), *Eco*RI (E), *Hae*II (H), *Pvu*II (P), *Sau*3aI (S). Not all these restriction sites are indicated in the map. (**B**) Sequence alteration of the ptc^{G20} protein. Left: restriction map of the genomic region of ptc^{G20} mutation. It shows a small deletion just after the ATG (translation initiation site) of the *ptc* coding sequence, where several enzyme restriction sites are lost. Right: amino acid sequences of wild-type ptc and of ptc^{G20} proteins in the first exon, where the restriction polymorphism was found. A deletion of 71 bp and an insertion of five new base pairs were located at the end of the first exon. These changes do not alter either the reading frame or the splicing donor site (underlined). The mutation originates the loss of 22 amino acids close to the 5′ amino terminus of the ptc protein and the insertion of two new amino acids (bold letters). No further alterations in other exons have been found. The distances are only approximate and 1 cm in the figure roughly corresponds to 500 bp.

the open reading frame, introducing two new amino acids just before the last amino acids of exon 1.

Thus, the two lesions are different at the molecular level. In the case of tuf^1, the mutation affects a regulatory region, presumably reducing the level of transcription and producing low levels of wild-type ptc protein. This is confirmed by the fact that tuf^1/tuf^1 and tuf^1/ptc^{IIW} discs show lower levels of ptc protein than the wild-type discs (see below). In the ptc^{G20} allele, the sequence in the amino terminal part of the protein between amino acids 19 and 43 is changed, giving rise to an abnormal protein, presumably with incomplete ptc activity.

Expression of ptc and dpp is altered in a similar way in some viable segment polarity mutants

The patterns of expression of both *ptc* and *dpp* genes were studied in third instar wing imaginal discs in the heteroallelic combinations of two viable (tuf^1 and ptc^{G20}) and two embryonic lethal (ptc^{IN} and ptc^{IIW}) alleles of *ptc* (Figure 4). ptc^{IN} has a strong embryonic mutant phenotype, but produces protein, as detected by an anti-ptc antibody (data not shown). We consider ptc^{IIW} as a null allele, since it has a strong ptc phenotype in embryos and does not produce ptc protein. The mutant combination $cos2^{v1}Cos1^2/+$, that shows an adult phenotype in the wing similar in some characteristics to that of tuf^1 (Whittle, 1976; Simpson and Grau, 1987) (Figure 4l), was also studied. All these mutant combinations show severe overgrowth of the anterior compartment of the wing disc (Figure 4b, c and d; compare with the wild-type in a) and the adult wings (Figure 4j, k and l; compare with the wild-type in i), leaving the posterior compartment apparently unaffected. We have tried to correlate the extreme phenotypes of discs and adult cuticle displayed by these mutants with alterations in the *ptc* and *dpp* expression patterns.

Flies from the mutant combinations tuf^1/ptc^{IN} and tuf^1/ptc^{IIW} show a variable phenotype, including overgrowth of the anterior compartment of the wing, loss of costal structures, duplications or plexations of veins 1 and 2, and increase of the distance between veins 3 and 4 (Phillips *et al.*, 1990). Figure 4j shows an example of a tuf^1/ptc^{IIW} phenotype, although stronger phenotypes (including overgrowth similar to that described for *cos2 Cos1* mutants, Figure 4l), are also observed. There is also an increase in the number of scutellar bristles and several other defects. The expression of ptc protein in the third instar wing discs of both mutant combinations is altered: in the case of tuf^1/ptc^{IN}, the stripe of maximal ptc staining expands anteriorly several cell diameters, showing a level of ptc protein higher than the wild-type one. In the case of the tuf^1/ptc^{IIW} combination, the pattern is the same, but the protein level is very low (Figure 4b; compare with the wild type in a). This is because the ptc^{IIW} allele does not produce ptc protein and the tuf^1 mutation reduces the level of transcription of the gene (see above). In both mutant combinations, the resultant low level of *ptc* activity gives rise to ectopic *dpp* expression in the anterior edge of the presumptive wing pouch (Figure 4f). This region roughly corresponds to the presumptive costal region (Bryant, 1975). The stripe of *dpp* expression near the anterior−posterior compartment boundary also expands anteriorly (Figure 4f). We also note that the anterior expansion of both ptc and *dpp* stripes seems to correlate well with the observed increased distance between veins 3 and 4 (Figure 4j).

The combination ptc^{G20}/ptc^{IIW} is pupal lethal, but ptc^{G20}/ptc^{IN} is semi-viable, allowing the study of the adult phenotype. The wings of ptc^{G20}/ptc^{IN} flies show overgrowth of the anterior compartment and vein defects, including partial or complete loss of vein 2 and plexation of veins 1 and 3 (Phillips *et al.*, 1990; Figure 4k). Extra dorso-central and post-alar bristles appear in the notum (arrows in Figure 5b). There are also morphological alterations in other structures of these flies, like the legs. It has been previously shown that *ptc* activity is required to repress its own transcription in the developing embryo (Hidalgo and Ingham, 1990; Ingham *et al.*, 1991; Sampedro and Guerrero, 1991). In *ptc* mutant embryos, there are high levels of ectopic expression of *ptc*. We have seen that a similar situation is observed in the *ptc* mutant wing discs. In wing imaginal discs of both ptc^{G20}/ptc^{IIW} and ptc^{G20}/ptc^{IN} combinations, the stripe of maximal intensity of ptc staining is normal, but the expression in the rest of the anterior compartment is dramatically increased with respect to the wild-type, showing a high level of ptc mutant protein (Figure 4c, compare with the wild-type in 4a). The stripe of *dpp* expression in these genotypes is apparently normal, but there is heterogeneous ectopic expression throughout the anterior compartment, with higher levels in the anterior edge of the disc (Figure 4g). Again, the reduced levels of *ptc* activity result in the ectopic expression of the *dpp* gene. Although we have concentrated on studying the wing imaginal disc, we have observed similar changes in expression in other discs, like the leg discs.

In $cos2^{v1}Cos1^2/+$ imaginal discs, abnormal high levels of wild-type ptc protein are detected in the presumptive anterior edge of the wing disc, which shows variable overgrowth (Figure 4d, compare with the levels of wild-type ptc protein in the same region in 4a), and ectopic *dpp* expression appears in this same zone (Figure 4h). These regions of ptc and *dpp* ectopic expression will originate the structures affected in the adult wing (Figure 4l). These results suggest that the *cos2* segment polarity gene is involved in the mechanism of transcriptional control of both *ptc* and *dpp* genes. It is interesting to note that the cells of the anterior edge of the wing disc seem to be specially sensitive to the lack of *ptc* and *cos2*, since in both mutant backgrounds *dpp* is de-repressed in these cells.

We conclude that in the mutant backgrounds studied, the expression of *ptc* and *dpp* is altered in a similar way with slight differences, and that the sites of ectopic expression of *dpp* correlate well with the regions of the disc which will give rise to the structures affected in the adult.

Reduced dpp activity suppresses the ptc phenotype

The relationship between the regions where *dpp* is expressed ectopically and those where a phenotypic alteration is observed lead us to suspect that *dpp* mis-expression may be responsible for the phenotypes. To test this, we made stocks mutant for both *ptc* and *dpp* alleles. We have used the *dpp* alleles dpp^{d5} and dpp^{d12}. They both have alterations in the *disk* regulatory region of the *dpp* gene (St Johnston *et al.*, 1990), and this results in a reduction of the level of transcription of the gene (Masucci *et al.*, 1990). dpp^{d5} is recessive viable, and the homozygotes have reduced wings. The corresponding wing imaginal discs show reduced levels of *dpp* RNA (Masucci *et al.*, 1990). dpp^{d12} is recessive early pupal lethal, with homozygous larvae having greatly reduced imaginal discs (St Johnston *et al.*, 1990). The dpp^{d12}/dpp^{d5} combination is viable and flies have reduced

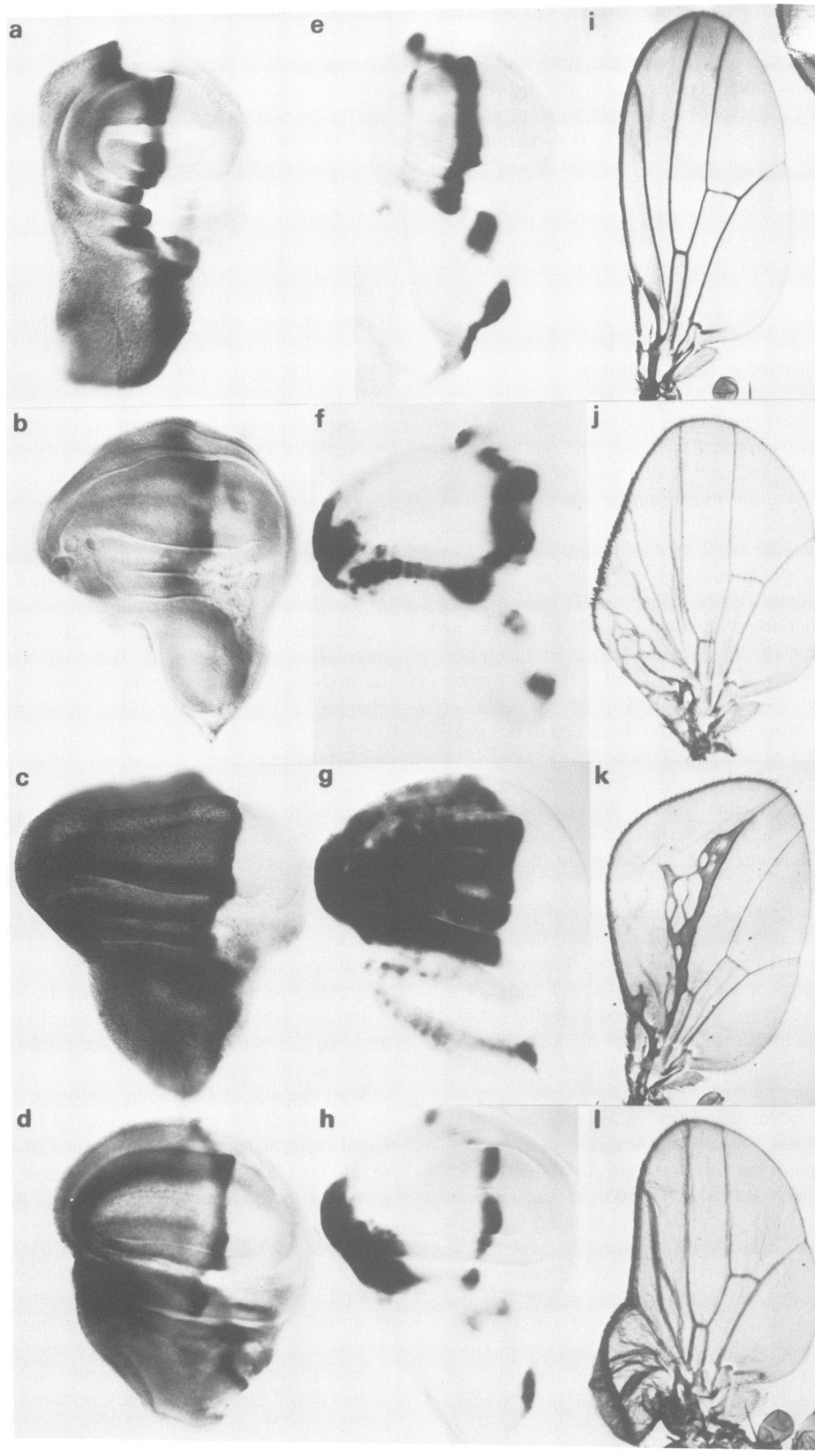

Fig. 4. Phenotypes and ectopic expression of ptc and *dpp* in *ptc* and *cos2 Cos1* mutants. (**a**, **e** and **i**) Wild-type wing phenotype (i) and ptc (a) and *dpp* (e) expression in third instar *dpp−lacZ* wing imaginal discs. (**b**, **f** and **j**) wing phenotype (j) and ptc (b) and *dpp* (f) expression in third instar wing imaginal discs of the combination $tuf^1/ptc^{IIW}dpp-lacZ$. (**c**, **g** and **k**) ptc^{G20}/ptc^{IN} wing phenotype (k) and ptc (c) and *dpp* (g) expression in third instar wing imaginal discs of the pupal lethal combination ptc^{G20}/ptc^{IIW} *dpp−lacZ*. (**d**, **h** and **l**) wing phenotype (l) and ptc (d) and *dpp* (h) expression in third instar wing imaginal discs of the combination $cos2^{v1}Cos1^2/dpp-lacZ$. To detect ptc antigen, peroxidase staining with anti-ptc monoclonal antibody was used, and for *dpp−lacZ* expression, wild-type and mutant imaginal discs carrying the *dpp−lacZ* construct were stained with X-Gal.

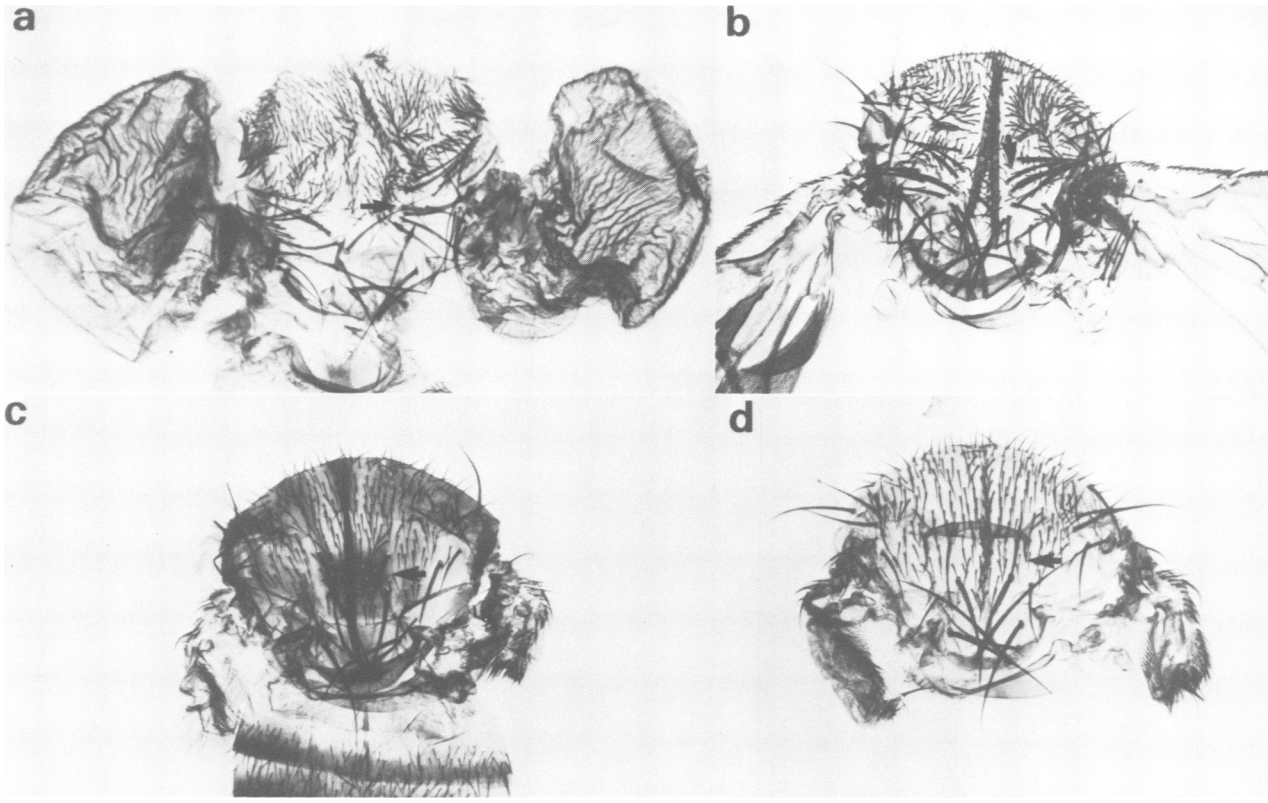

Fig. 5. Phenotypes of *ptc* mutants and *ptc dpp* double mutants. Wings and notum of a ptc^{G20}/ptc^{IIW} pharate adult (**a**) and a dpp^{d12} ptc^{G20}/dpp^{d5} ptc^{IIW} fly (**c**). Notum of a ptc^{G20}/ptc^{IN} fly (**b**), and the corresponding wing is shown in Figure 4k. Wings and notum of a dpp^{d12} ptc^{G20}/dpp^{d5} ptc^{IN} double mutant (**d**). The extra dorso-central bristles observed in single *ptc* mutants (arrows in a and b) are rescued in some cases to the wild-type number in the *ptc dpp* double mutants (arrows in c and d). This rescue is variable. It is also observed that the wings of the double *ptc dpp* mutants are greatly reduced, similarly to those of the dpp^{d12}/dpp^{d5} mutants.

wings. While flies of the genotype ptc^{G20}/ptc^{IIW} die in pupa, some flies of the genotype dpp^{d12} ptc^{G20}/dpp^{d5} ptc^{IIW} eclose and show a partial rescue of the *ptc* phenotype as compared with ptc^{G20}/ptc^{IIW} pharate adults (Figure 5a and c). For example, in ptc^{G20}/ptc^{IIW} pharate adults there is a great increase in the number of bristles in the notum (arrow in Figure 5a), legs and antennae, and in the number of teeth in the male sex comb. This number is substantially decreased in the same structures of dpp^{d12} ptc^{G20}/dpp^{d5} ptc^{IIW} flies (arrow in Figure 5c). In addition, a general reduction of the overgrowth characteristic of ptc^{G20}/ptc^{IIW} pharate adult legs is also observed. A partial phenotypic rescue is also observed in dpp^{d12} ptc^{G20}/dpp^{d5} ptc^{IN} adults (Figure 5d) in comparison with ptc^{G20}/ptc^{IN} flies (Figure 5b). As in the previous case, this is reflected in a reduction of overgrowth in legs and a decrease in the number of bristles of the thorax (arrow in Figure 5d), legs and antennae. In these double-mutant combinations, the wing phenotype is identical to that of the single dpp^{d12}/dpp^{d5} mutant. We conclude that the overgrowth observed in *ptc* mutants and some other features of the *ptc* phenotype can be alleviated by reducing *dpp* activity in the wing disc.

Effects of HSptc on dpp and ptc expression

The unrestricted expression of *ptc* under the control of a heat-shock promoter (HS*ptc*) partially rescues the ptc embryonic phenotype (Ingham *et al.*, 1991; Sampedro and Guerrero, 1991). We have investigated the effects of the overexpression of *ptc* on heteroallelic combinations of *ptc* alleles displaying adult mutant phenotypes. The basal, uninduced, activity of the heat-shock construct at 25°C partially rescues the sublethality of ptc^{G20}/ptc^{G20} and ptc^{G20}/ptc^{IN} flies, allowing a higher proportion of them to eclose. Defects in veins and in sensory organs of these ptc^{G20}/ptc^{IN}; HS*ptc* flies can be rescued by a series of heat-shock pulses starting from the first larval instar (see Materials and methods). Some flies emerge with wild-type notum and wings. Phenotypic rescue by the heat-shock treatment is also observed in the pupal lethal combination ptc^{G20}/ptc^{IIW}; HS*ptc*. The same treatment applied to tuf^{1}/ptc^{IN}; HS*ptc* flies corrects the wing phenotype and partially other defects. The same procedure applied to the ptc^{+}; HS*ptc* siblings or to flies which do not carry the HS*ptc* construct has no phenotypic effect.

To analyze the molecular basis of the phenotypic rescue, the effect of the heat-shock pulses on *ptc* expression was studied in wing imaginal discs from larvae carrying the HS*ptc* construct. A single pulse of 1 h results in the complete disappearance of the endogenous *ptc* transcription, as seen by *in situ* hybridization using a probe specific for the endogenous gene (not shown). The transcription begins to recover 4 h after the pulse. This repression of the endogenous *ptc* gene is never observed when heat-shocked larvae do not contain the HS*ptc* construct [the specificity of the effect of the HS*ptc* is also discussed in Sampedro and Guerrero (1991)]. In ptc^{G20}/ptc^{IN} and ptc^{G20}/ptc^{IIW} imaginal discs, there is a dramatic increase in the level of ptc mutant protein throughout the anterior compartment (Figure 4c), since the autorepressive mechanism of *ptc* fails to occur. A single

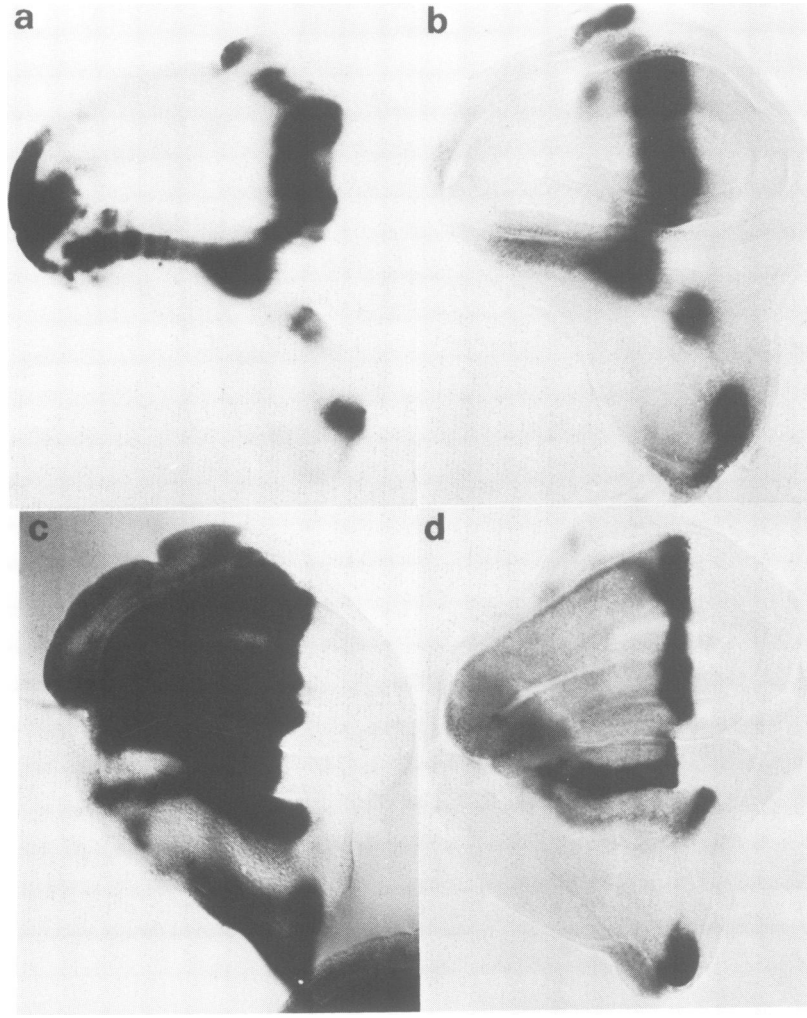

Fig. 6. Effect of HS*ptc* over *dpp* expression. (**a** and **b**) $tuf^1/ptc^{IIW}dpp-lacZ$; HS*ptc* and (**c** and **d**) $ptc^{G20}/ptc^{IIW}dpp-lacZ$; HS*ptc* third instar wing imaginal discs. *dpp−lacZ* expression (as seen by X-Gal staining) without heat treatment (a and c) and after four heat-shock cycles (b and d) as described in Materials and methods.

HS*ptc* pulse (see Materials and methods) is able to repress the abnormal expression of *ptc* in third instar wing imaginal discs from these combinations. We have used the staining in the posterior compartment of the wing disc as an internal control to monitor the decay of the exogenous ptc protein. When the exogenous protein starts to decay (4 h after the heat shock), endogenous *ptc* expression in its stripe of expression near the anterior−posterior compartment boundary begins to be distinguished. Six hours after the pulse, when the exogenous ptc protein has disappeared (as seen by the absence of staining in the posterior compartment), the wild-type pattern is observed in the whole disc but, soon after this, the mutant pattern reappears. This transient recovery of the wild-type pattern is probably the reason why continued heat-shock pulses are required to rescue the adult mutant phenotypes. In tuf^1/ptc^{IIW}; HS*ptc* imaginal discs, where only the tuf^1 allele produces ptc protein and at a low level (Figure 4b), the heat-shock treatment gives a slight rescue of the anterior expansion of the ptc stripe.

We have also looked at *dpp* expression in these ptc^-; HS*ptc* discs. We have observed by *in situ* hybridization that two heat-shock pulses are enough to recover the wild-type pattern of *dpp* expression. When *dpp−lacZ* is used as a reporter of the ectopic *dpp* expression (Figure 6), more pulses are needed to recover the wild-type pattern, due to the high stability of the β-galactosidase protein. Thus, four heat pulses suppress almost completely the ectopic expression of *dpp−lacZ* in the mutant combination ptc^{G20} $dpp-lacZ/ptc^{IIW}$; HS*ptc*, as detected by X-Gal staining (Figure 6d). In tuf^1/ptc^{IIW} *dpp−lacZ*; HS*ptc* discs, the ectopic expression of *dpp* in the anterior edge of the wing pouch is repressed after four heat pulses, but there is a variability in the width of the *dpp* stripe near the compartment boundary (Figure 6b; compare with the wild-type in 4e). We have never seen repression of the wild-type expression of the *dpp−lacZ* construct or of *dpp* transcription (as seen by *in situ* hybridization) due to the overexpression of *ptc*. The lack of effect of HS*ptc* on wild-type *dpp* expression explains the lack of effect of HS*ptc* on ptc^+; HS*ptc* flies, although HS*ptc* is able to correct the adult ptc mutant phenotypes.

dpp expression in ptc mutant clones

Since hypomorphic *ptc* mutations cause de-repression of *dpp*, we studied the effect of the absence of *ptc* function on the *dpp* expression by inducing mitotic recombination *ptc* mutant clones in the wing disc. We analyzed $Minute^+$ clones

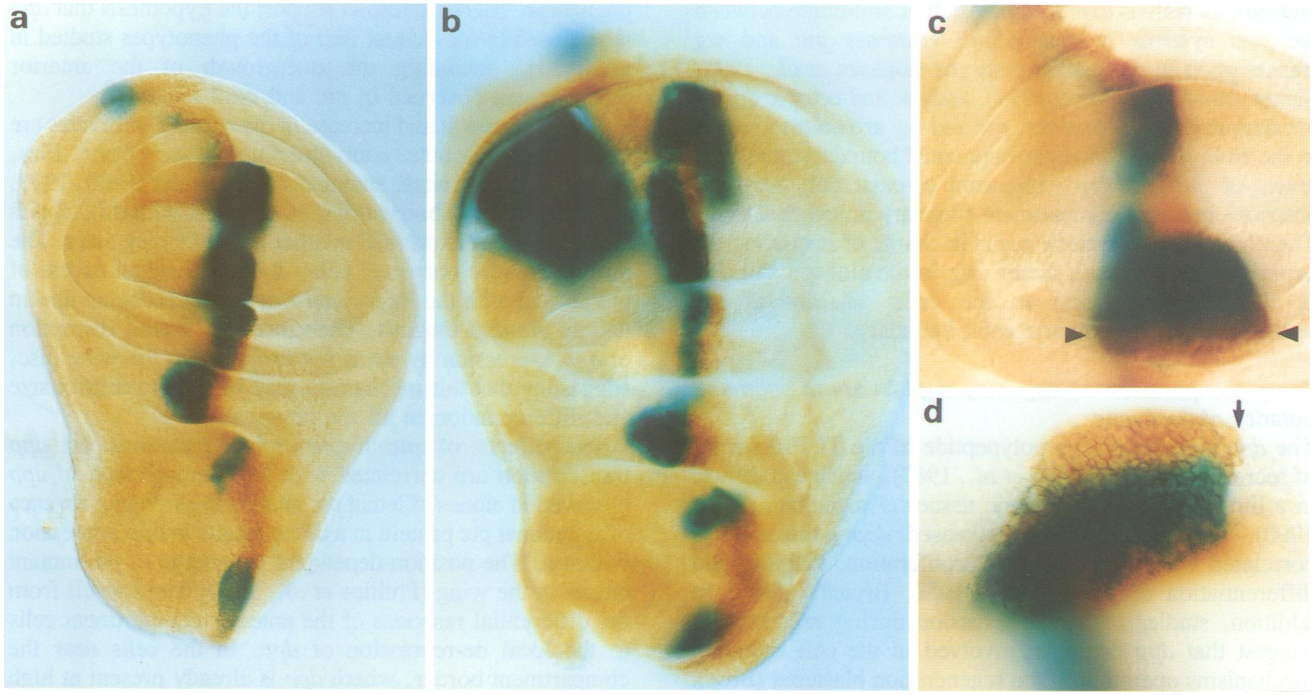

Fig. 7. Induction of *dpp* expression in *ptc* mutant clones. Double staining for ptc and *dpp−lacZ* expression of ptc^{IIW}*dpp−lacZ*/$Df(2R)M^{33a}$ third instar wing imaginal discs from larvae non-irradiated (**a**) and irradiated at the second instar (**b**) (see Materials and methods). ptc^{IIW} *dpp−lacZ* clones are identified by the lack of ptc protein, and *dpp−lacZ* expression is revealed by X-Gal staining. (**c** and **d**) Two examples of ptc^{6P} *dpp−lacZ* clones, identified in this case by the overexpression of non-functional ptc protein of the ptc^{6P} allele in the disc. Arrowheads in (c) show the anterior and posterior limits of a clone, as seen by the strong ptc staining. The arrow in (d) indicates the anterior−posterior compartment boundary of the wing disc. It is observed that not all the cells of the clone are expressing *dpp−lacZ*.

(Morata and Ripoll, 1975) homozygous for the null allele ptc^{IIW} and for *dpp−lacZ* in a *Minute* background. The clones were induced in second instar larvae, and wing imaginal discs dissected from wandering third instar larvae were doubly stained with anti-ptc antibody and X-Gal. As shown in Figure 7b, ptc^{IIW} *dpp−lacZ* clones (recognized by the lack of ptc protein in stained discs) also ectopically express β-gal (compare with the wild-type expression of *dpp−lacZ* in Figure 7a). The lack of ptc staining that identifies the ptc^{IIW} clone is only clearly visualized when the clones interrupt the stripe of strong expression of ptc near the anterior−posterior compartment boundary. To analyze the limits of the *ptc* mutant clones in different areas of the disc, we also obtained ptc^{6P} *dpp−lacZ* clones. ptc^{6P} is another allele of *ptc* that does produce protein, although it behaves as a null allele (Phillips *et al.*, 1990; our unpublished observations). Taking advantage of the fact that functional ptc protein is required to repress its own transcription in imaginal discs (this work), it is possible to identify ptc^{6P} *dpp−lacZ* clones in the anterior compartment by the overexpression of the ptc mutant protein (Figure 7c and d). In these clones, most of the cells also ectopically express the *dpp−lacZ* construct. We never observed de-repression of *dpp−lacZ* in the posterior compartment. As a further control, we irradiated ptc^{+} *dpp−lacZ* imaginal discs using the same experimental conditions and did not detect ectopic *dpp−lacZ* expression. We conclude that the lack of *ptc* function is able to de-repress the *dpp* gene. Sometimes, slight morphological alterations appear in the region affected by the clone. Since $Minute^{+}$ clones do not significantly alter the structure of a *Minute* disc (Brower *et al.*, 1981), we believe that the morphological alterations observed are due to the lack of *ptc*. We observe, as previously described (Brower *et al.*, 1981), that anterior $Minute^{+}$ clones growing in a *Minute* background are able to 'push' the anterior−posterior compartment border, and invade regions normally occupied by the posterior compartment.

In some cases, *dpp−lacZ* is not de-repressed in the whole *ptc* mutant clone, probably due to the differential responsiveness of the cells of the anterior compartment to the lack of *ptc*. This is in accordance with the fact that, in the extreme combination ptc^{G20}/ptc^{IIW}, where ptc mutant protein is homogeneously overexpressed in the anterior compartment, *dpp−lacZ* displays a heterogeneous pattern of de-repression (Figure 4c and g).

Discussion

ptc controls dpp expression in imaginal discs

In this paper, we suggest that *ptc* and *dpp* genes share a transcriptional control in imaginal discs dependent on *ptc* activity. This statement is based on several independent lines of evidence. First, in *ptc* and *cos2* mutant backgrounds both *ptc* and *dpp* expression show similar alterations. Second, *ptc* mutant clones in the anterior compartment induce ectopic expression of *dpp* inside the clone. Finally, heat-shock pulses applied to tuf^{ts}/ptc^{-}; HS*ptc* or ptc^{G20}/ptc^{-}; HS*ptc* mutant larvae repress the abnormal expression of both *ptc* and *dpp* genes.

These genetic interactions between *ptc* and *dpp* are very similar to those which occur within the embryonic segment between *ptc* and *wg*, where *ptc* is able to repress both *ptc* and *wg* genes in the anterior region of the segment (Martínez-Arias *et al.*, 1988; Hidalgo and Ingham, 1990; Ingham *et al.*, 1991; Sampedro and Guerrero, 1991). In wild-type imaginal discs, *ptc* represses its own transcription, as in the

embryo, as well as that of *dpp*. Another similitude between the two systems is that HS*ptc* represses *ptc* and *wg* transcription in *ptc* mutant embryos (Ingham *et al.*, 1991; Sampedro and Guerrero, 1991), and *ptc* and ectopic *dpp* in *ptc* mutant discs. Moreover, *ptc* and *wg* are co-transcribed in the embryo just at the parasegmental boundary, and high levels of *ptc* and *dpp* expression co-exist only near the anterior–posterior compartment boundary in imaginal discs. Therefore, in both systems *ptc* is in charge of restricting the expression of a growth factor (wg in the embryo and dpp in the imaginal discs) to the cells adjacent to the parasegmental or compartmental boundary.

Ectopic expression of dpp may explain some segment polarity phenotypes

The *dpp* gene encodes a polypeptide of the TGF-β family of secreted factors (Padgett *et al.*, 1987), which can diffuse in a limited way in embryonic tissues (Panganiban *et al.*, 1990). In imaginal discs, decreased *dpp* expression is correlated with defects in cell proliferation, viability and differentiation (Spencer *et al.*, 1982; Bryant, 1988). In addition, studies of gene expression during regeneration suggest that *dpp* could be involved in the cell signaling mechanisms operating in the regeneration blastema (Brook *et al.*, 1993). All this is in agreement with the fact that some members of the TGF-β family are known to affect cell proliferation and differentiation (reviewed in Massagué, 1990; Sporn and Roberts, 1992).

Our results indicate that the inappropriate expression of *dpp* is correlated with morphological defects in imaginal discs. We propose that some of the features of the adult segment polarity phenotypes studied in this work are due to the mis-expression of *dpp* in the anterior compartment of the wing disc. However, we cannot exclude the possibility that both the ectopic expression of *dpp* and the reduction of *ptc* activity are needed to obtain a phenotypic effect.

The two hypomorphic alleles of *ptc* used in this work, although they are different at the molecular level, have similar behaviour with respect to the de-repression of *dpp*. The tuf^1 mutation is due to an insertion in the promoter that probably diminishes the level of transcription of the *ptc* gene and may also disturb other regulatory regions. In tuf^1/ptc^- mutant wing discs, there is an anterior expansion of the normal *dpp* stripe, as well as ectopic expression of the *dpp* gene in the wing hinge, the anterior edge of the disc and the presumptive costal region. These flies present overgrowth of the anterior compartment of the wing, defects in the costa and a greater separation between veins 3 and 4, i.e. in regions with abnormal *dpp* expression. On the other hand, the ptc^{G20} mutation produces an altered ptc protein which presumably displays incomplete activity. In ptc^{G20}/ptc^- mutant wing discs, *dpp* is de-repressed throughout most of the anterior compartment. These flies (pharate adults in some combinations) present alterations on the wing and an increase in the number of sensory elements in the notum. The lethality of the ptc^{G20}/ptc^{IIW} combination is reduced if the flies are also mutant for viable *dpp* alleles, and the notal phenotype is partially rescued. Since these *dpp* alleles have reduced levels of *dpp* expression (Masucci *et al.*, 1990), the result suggests that a reduction of *dpp* expression could account for the phenotypic rescue of the *ptc* mutants. Moreover, an excess of wild-type ptc protein provided by the HS*ptc* abolishes the ectopic *dpp* expression and rescues the ptc phenotype. All these results support the hypothesis that *dpp* is responsible for at least part of the phenotypes studied in this work, including the overgrowth of the anterior compartment observed in *ptc* and *cos2* mutants.

The overgrowth and increase in the number of bristles are also observed in other adult structures of ptc^{G20}/ptc^- flies, such as legs, sternites, antennae and genitalia (Held, 1993; our unpublished observations). Consistent with this, *dpp* is also ectopically expressed in other discs, like leg discs. The defects are also corrected either by providing an excess of ptc protein with the HS*ptc* construct or by reducing *dpp* in double *ptc dpp* mutants. Therefore, the spatial restriction of *dpp* expression by *ptc* is not exclusive to the wing disc, suggesting that this mechanism may be used to control size and differentiation in all the imaginal discs.

The effects of *ptc* hypomorphic mutations on *dpp* transcription are correlated with the de-repression of *dpp* observed in clones of lethal *ptc* alleles. We find that absence of functional ptc protein in a clone results in *dpp* expression within it. The position-dependent phenotype of *ptc* mutant clones in the wing (Phillips *et al.*, 1990) could result from the differential response of the anterior compartment cells to the local de-repression of *dpp*. In the cells near the compartment border, where *dpp* is already present at high levels in the wild-type, a *ptc* mutant clone in the cuticle would have no effect, as it happens (Phillips *et al.*, 1990). In the rest of the anterior compartment, clones induced in the costal region, i.e. further in the anterior–posterior axis from the *dpp* transcribing region, do not appear, while clones between this region and the anterior–posterior compartment boundary survive, causing diverse phenotypes (Phillips *et al.*, 1990). This may suggest that different regions of the wing disc respond differently to the ectopic *dpp* expression and that this response may be related to the distance in the disc to the *dpp* site of expression near the anterior–posterior compartment boundary. Although *dpp* transcription could be the local source of this differential competence of the anterior cells, it does not imply that dpp protein diffusion during wing disc development is providing this difference (see Wharton *et al.*, 1993). Another factor could act, mediating the response of the cells to *dpp*.

This relationship between *dpp* ectopic expression and phenotypic effect may not be limited to the *ptc* alleles. Heterozygotes for the double-mutant combination $cos2^{v1}Cos1^2$ present a dominant phenotype in wings, consisting of duplication of part of its anterior region (Whittle, 1976; Grau and Simpson, 1987; Simpson and Grau, 1987). Wing discs of this combination show ectopic expression of wild-type ptc protein and *dpp* in the presumptive costal region, and in the anterior edge of the disc, the regions affected in the adult wing. Abnormal *dpp* expression in the wing disc is also observed in mutations for other segment polarity genes, and this is also correlated with adult phenotypes (Capdevila *et al.*, in preparation).

Interactions across the anterior–posterior boundary

The wing disc is divided from early in development into an anterior and a posterior compartment (García-Bellido *et al.*, 1973, 1976; Morata and Lawrence, 1977). This boundary does not coincide with any obvious morphological landmark in the wing, lying in the region between veins 3 and 4, but in some regions of the prospective wing blade a boundary of morphologically aligned cells is observed which coincides

with the anterior–posterior cell lineage restriction (Blair, 1992). The main place of expression of *dpp* in the wing disc is in a stripe near the anterior–posterior compartment boundary (Masucci *et al.*, 1990) and high levels of *ptc* are present in this region (Phillips *et al.*, 1990; this work). The region near this boundary, therefore, represents a zone of developmental restriction and particular gene expression.

The compartment border of the discs corresponds in the embryo to the parasegmental border, defined by the expression of the *en* and *wg* genes (Martínez-Arias and Lawrence, 1985; Baker, 1987; DiNardo *et al.*, 1988; van den Heuvel *et al.*, 1989). The expression of these genes in the embryo is interdependent, and other segment polarity genes like *ptc* are required in this regulatory loop (reviewed in Ingham and Martínez-Arias, 1992). Although *ptc* is involved in the repression of *wg* (and *ptc*) transcription in the embryo, both genes are co-expressed at the parasegmental border. It has been proposed that this co-expression is due to a signal from the *en*-expressing cells, mediated by the *hh* gene, that prevents this repression by *ptc*. As a result, the cells near the parasegment border maintain the wild-type transcription of both *ptc* and *wg* genes (Ingham *et al.*, 1991; Ingham and Hidalgo, 1993). This hypothesis is supported by two facts: first, *hh* is expressed in the cells of the posterior region in the embryonic segment and in posterior compartments in imaginal discs (Lee *et al.*, 1992; Mohler and Vani, 1992; Tabata *et al.*, 1992; Tashiro *et al.*, 1993), and it can be secreted (Lee *et al.*, 1992); second, in the absence of *ptc*, *hh* is dispensable for *wg* maintenance (Ingham *et al.*, 1991; Ingham and Hidalgo, 1993). We have previously shown the similitude between the relationship of *ptc* and *wg* in the embryo and that of *ptc* and *dpp* in imaginal discs. This extends to the effect of *hh* on *ptc* expression, since in *hh* mutant embryos the expression of *ptc* and *wg* genes decays (Hidalgo and Ingham, 1990), and our results demonstrate that the transient inactivation of hh protein results in loss of the high levels of ptc protein in the cells near the compartment border of the wing disc. However, contrary to what happens to *wg* in the embryo, absence of hh function only slightly affects *dpp* transcription, and this small effect is observed much later than that on ptc. Besides, while HS*ptc* represses *wg* in the embryo (Sampedro and Guerrero, 1991), it does not affect wild-type *dpp* expression in the disc. HS*ptc*, however, is able to suppress the ectopic *dpp* expression observed in *ptc* viable alleles, indicating that only the cells expressing *dpp* near the compartment boundary are insensitive (or much less sensitive) to an excess of wild-type ptc protein. It is possible, however, that *hh* is required for the correct *dpp* expression in the initial stages of disc development, becoming independent of *hh* and *ptc* later on. The extensive 3′ *cis*-regulatory sequences of the *dpp* gene (St Johnston *et al.*, 1990) suggest that a complex set of regulatory interactions could mediate the correct allocation of *dpp* expression. Autocatalysis, as shown to occur in the visceral mesoderm (Hursh *et al.*, 1993), could be another mechanism involved in the maintenance of *dpp* expression near the compartment boundary.

In this work, we demonstrate that changes in the level of active ptc product alter the profile of *dpp* transcription in wing imaginal discs. The release of the transcriptional control of *dpp* in *pte* mutants produces significant alterations in the pattern and morphology of the adult cuticle. This fact points to dpp as a key signal mediating the correct morphology of the discs, but further experiments are required to know whether *dpp* expression in the discs has an instructive role in pattern formation.

Materials and methods

Fly stocks

Wild-type embryos were from Oregon R strain (Lindsley and Zimm, 1992). Mutant stocks are the following: ptc^{G20} (kindly provided by J.R.S.Whittle; Phillips *et al.*, 1990), tuf^{1} (Sturtevant, 1948; Phillips *et al.*, 1990), ptc^{IN} (Hooper and Scott, 1989; Tearle and Nüsslein-Volhard, 1987), ptc^{IIW} and ptc^{6P} (Tearle and Nüsslein-Volhard, 1987), hh^{9K} (Jürgens *et al.*, 1984; Lindsley and Zimm, 1992), $cos2^{v1}Cos1^{2}$ (kindly provided by P.Simpson; Grau and Simpson, 1987; Lindsley and Zimm, 1992). dpp^{d5} and dpp^{d12} were obtained from M.Hoffmann. The *en–lacZ* stock (*ryxho25*, Hama *et al.*, 1990) and the *dpp–lacZ* stock (BS3.0, Blackman *et al.*, 1991) were kindly provided by T.Kornberg and W.Gelbart, respectively. The HS*ptc* fly stock was described in Sampedro and Guerrero (1991).

Whole-mount antibody staining of imaginal discs

Imaginal discs from wandering third instar larvae were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature and washed in PBS. For diaminobenzidine (DAB) staining, PBS containing 0.2% bovine serum albumin (BSA), 0.1% saponin and 5% goat serum was used for blocking and for antibody incubations. Tissue was blocked for 1 h and incubated overnight at 4°C in a 1/200 dilution of anti-ptc antiserum, washed and incubated in a 1/300 dilution of biotinylated anti-mouse (Amersham) for 1 h at room temperature. Discs were then washed in PBT (PBS containing 0.1% Tween 20) and incubated for 30 min in Vector AB elite solution in PBT. After several washes in PBT, the reaction was developed in 0.5 mg/ml DAB (Sigma) in PBS containing 0.06% H_2O_2. Discs were mounted under coverslips in epon–araldite (Fluka) after dehydration.

For double immunofluorescence staining, PBS containing 0.3% Triton X-100 and 5% goat serum was used for blocking and for antibody incubations. After fixation, discs were blocked for 1 h and incubated overnight at 4°C in a mix of two primary antibodies: anti-ptc antiserum diluted 1/40 and anti-β-gal (Amersham) diluted 1/1000 in incubation buffer. For detection of ptc and β-gal antigens in *en–lacZ* discs, tissue was incubated after several washes in Texas-Red anti-rabbit (Amersham, 1/50), followed by biotinylated anti-mouse (1/300) and Streptavidin–fluorescein isothiocyanate (FITC) (Janssen, 1/1000). For detection of ptc and β-gal antigens in *dpp–lacZ* discs, Texas Red anti-mouse (Amersham, 1/50) was followed by biotinylated anti-rabbit (Amersham, 1/300) and Streptavidin–FITC. After several washes in PBS, discs were mounted under coverslips in 4% *n*-propylgallate in 80% glycerol–PBS.

Imaginal discs were observed and photographed under a Zeiss Axiophot microscope and a Zeiss Laser Scan Microscope.

X-Gal staining

Imaginal discs were first fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, fixed again in 0.5% glutaraldehyde (Fluka) in PBS on ice for 2 min and washed in PBS. The reaction was developed in 5 mM $K_4[Fe^{II}(CN)_6]$, 5 mM $K_3[Fe^{III}(CN)_6]$, 1 mM $MgCl_2$ and 0.2% X-Gal in PBS containing 0.3% Triton X-100. Discs were mounted and observed as described for DAB staining.

Whole-mount RNA in situ hybridization

Digoxigenin-labeled *dpp* probe was prepared for hybridization as follows. A purified *Eco*RI–*Hin*dIII fragment of *dpp* cDNA clone (Padgett *et al.*, 1987) was labeled using the Genius kit (Boehringer Manheim) following the manufacturer's instructions. RNA *in situ* hybridization was performed following Tautz and Pfeiffle (1989) with minor modifications according to Masucci *et al.* (1991). Imaginal discs were mounted in araldite after dehydration.

Molecular characterization of ptc mutants

The restriction polymorphisms of the *ptc* mutants were studied by Southern blot analysis using different restriction enzymes. The DNA was extracted by standard methods (Sambrook *et al.*, 1989) from *cn* ptc^{G20} *bw sp/Cy0* and tuf^{1} mutant flies and from Oregon-R or *cn bw sp* (original chromosome for ptc^{G20}) flies. Different genomic fragments of the *ptc* wild-type DNA were used as probes (Nakano *et al.*, 1989). The mutant fragment of the first exon of *ptc* was cloned and sequenced as follows: the oligonucleotides 5′ GCAAAATGCAGCCAAACA 3′ (18mer) and 5′ GGATCCTC-

CGAATACGA 3′ (17mer), were used and a fragment of 426 bp of the coding region of the exon 1 was amplified in the $ptc^{G20}/Cy0$ DNA using PCR (Mullis and Faloona, 1987). The fragment was subcloned in the *Eco*RI−*Eco*RV site of BlueScript vector, and the sequence was obtained by the dideoxy method (Sanger *et al.*, 1977).

Heat-shock treatment

Larvae aged 24−48 h from the crosses ptc^{G20}/CyO; *HSptc/HSptc* × ptc^{IIW} *dpp−lacZ/SM5* and tuf^{1}/CyO; *HSptc/HSptc* × ptc^{IIW} *dpp−lacZ/SM5* were subjected to repetitive heat-shock cycles of 1 h at 37°C and 4 h at 25°C within a programmed incubator. Imaginal discs from wandering third instar larvae treated following this procedure were analyzed to detect β-gal activity and ptc expression as described above. The same treatment was applied to study the phenotypic rescue.

Generation and identification of ptc mutant clones

For the generation of ptc mutant clones, *cn bw* ptc^{IIW} *dpp−lacZ sp/SM5* or *pr* ptc^{6P} *dpp−lacZ sp/SM5* females were crossed to $M(2)c^{33a}/bw^{V32g}$ males and the progeny X-irradiated with 1000 rad at 24−48 h after egg laying. Wing imaginal discs from wandering third instar larvae were dissected in PBS and stained with anti-ptc antibody. After development of the DAB reaction, discs were washed in PBS, fixed in 0.5% glutaraldehyde in PBS on ice for 2 min, and X-Gal was detected as described above. *ptc* mutant clones were identified in the wing discs as follows: in the case of ptc^{IIW} *dpp−lacZ* clones, by the absence of ptc staining (and this is more clearly detected when the clone includes cells of the stripe of maximal ptc expression, causing a visible gap in this stripe). In the case of ptc^{6P} *dpp−lacZ* clones, they are identified by the strong expression of the ptc mutant protein.

As a control, *dpp−lacZ/dpp−lacZ* females were crossed to $M(2)c^{33a}/bw^{V32g}$ males and larvae were irradiated as described above. We never observed de-repression of *dpp−lacZ* in wing imaginal discs from this experiment.

In both crosses, one-half of the larvae are *Minute*$^{+}$. Since these larvae pupate earlier than the *Minute* ones, they were discarded by dissecting only the larvae remaining after pupation of a significant fraction of the total larvae. One-half of these dissected larvae have the correct genotype for clone production. In different experiments, we obtained an average of one *ptc* mutant clone expressing *dpp−lacZ* from 20 dissected discs expressing the *dpp−lacZ* construct.

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