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Positional signaling by *hedgehog* in *Drosophila* imaginal disc development

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SUMMARY

We describe a dominant gain-of-function allele of the segment polarity gene *hedgehog*. This mutation causes ectopic expression of *hedgehog* mRNA in the anterior compartment of wing discs, leading to overgrowth of tissue in the anterior of the wing and partial duplication of distal wing structures. The posterior compartment of the wing is unaffected. Other imaginal derivatives are affected, resulting in duplications of legs and antennae and malformations of eyes. In mutant imaginal wing discs, expression of the *decapentaplegic* gene, which is implicated in the *hedgehog* signaling pathway, is also perturbed. The results suggest that hedgehog protein acts in the wing as a signal to instruct neighboring cells to adopt fates appropriate to the region of the wing just anterior to the compartmental boundary.

Key words: *Drosophila*, imaginal disc, *hedgehog*, *Moonrat*, segment polarity gene, cell signaling

INTRODUCTION

Signaling between adjacent cells often occurs where cells must adopt appropriate cell fates relative to each other. During *Drosophila* embryogenesis, within each developing segment, signaling between adjacent cells coordinates proper activity of genes of the segment polarity class, several of which are directly implicated in those signaling events (Baker, 1987, 1988; Hooper and Scott, 1989; Nakano et al., 1989; van den Huevel et al., 1989; Phillips et al., 1990; Bejsovec and Martinez Arias, 1991; Peifer et al., 1991; see Hooper and Scott, 1992, for review). The secreted product of the *wingless* (*wg*) gene is necessary to maintain the expression of the *engrailed* (*en*) gene in neighboring, posteriorly adjacent cells (DiNardo et al., 1988; Martinez Arias et al., 1988). In turn, *wg*-expressing cells require a signal, the product of the *hedgehog* (*hh*) gene, originating from the adjacent *en*-expressing cells to maintain *wg* expression (Martinez Arias et al., 1988; Ingham et al., 1991). The activity of these genes is often considered to constitute part of a code of 'positional information' that the developing organism interprets to establish proper spatial allocation of cell fates. Molecular analyses identify the product of the *hh* gene as a transmembrane protein that may be secreted (Lee et al., 1992; Taylor et al., 1993). Evidence is mounting that hh protein acts as a morphogen in the embryo, that is, it diffuses several cell diameters from those cells producing it and affects target cells in a dose-dependent manner (Tabata and Kornberg, 1994; Heemskerk and DiNardo, 1994). hh is thus a critical component in a pathway by which cells set up positional identity relative to each other and subsequently differentiate appropriately.

In addition to its role in embryos, the product of the *hh* gene plays a role in local positional signaling in imaginal tissues. In the imaginal eye disc, hh signals to cells in the morphogenetic furrow to express the product of the *decapentaplegic* (*dpp*) gene (Ma et al., 1993; Heberlein et al., 1993), a secreted TGF-β homologue (Padgett et al., 1987). Both *hh* and *dpp* are required for progression of the morphogenetic furrow and subsequent proper differentiation of the eye (Ma et al., 1993; Heberlein et al., 1993). During development of other imaginal structures (the imaginal wing, leg, and haltere discs), *hh* is expressed in the posterior compartment (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992). Genetic analysis suggests that *hh* is required non-autonomously in the wing primordium (Mohler, 1988). In the wing disc, the anterior limit of *hh* expression is adjacent to cells expressing *dpp* in a stripe nearly coincident with the anterior-posterior compartment boundary (Raftery et al., 1991; Lee et al., 1992). This paper and other recent work (Tabata and Kornberg, 1994; Basler and Struhl, 1994) show that hh in the wing disc also may signal to cells in the vicinity to express *dpp*, similar to what occurs in the developing eye. To define further the role of *hh*, we have characterized a gain-of-function allele of *hh* that acts during imaginal development.

MATERIALS AND METHODS

Isolation and characterization of the *Moonrat* mutation

The *Moonrat (Mrt)* mutation arose spontaneously in a cross between two different *T(Y;2)*-bearing strains. Based on analysis of 211 recombinants between e^s and *ca*, we placed *Mrt* at meiotic map location 79.7. To determine whether *Mrt* was a loss-of-function allele, an antimorph, or a neomorph (Muller, 1932), we examined *Mrt*/+/+ flies. Since *Mrt* mapped between *e* and *Pr* (at salivary chromosome subdivisions 93D and 96F, respectively), we generated a series of segmental duplications from *Y*-autosome translocations that spanned

from 92DE to 97B (Lindsley et al., 1972). The translocations used were *T(Y;3) L111, T(Y;3) D100, T(Y;3)R13, T(Y;3) B197,* and *T(Y;3) G75* (Lindsley et al., 1972; Lindsley and Zimm, 1992). None of these duplications altered the *Mrt* phenotype, suggesting that *Mrt* is a neomorphic mutation.

Drosophila strains

For quantification of the wing phenotype, either *Mrt*/*TM3, Sb* or a marked *ru h th st cu sr e^s Mrt ca*/*TM3, Sb* strain was used. For some experiments *Mrt* larvae had to be unambiguously identified. For these, a *TM6B* balancer chromosome marked with *Tb* was used; *Tb* is a larval marker. The *TM6B* balancer also enhances the *Mrt* phenotype. The brm^{23} mutation was isolated as an extragenic suppressor of the *Mrt* wing phenotype by A. L. F. (unpublished).

The P30 *hh* enhancer detector strain was obtained from John Lee (Lee et al., 1992); disc staining experiments were performed on progeny of a P30×*Mrt*/*TM3* or P30×*Mrt*/*TM6B* cross, at 20°C to maximize *Mrt* expressivity. Experiments using the *dpp* reporter strain, which carries the *dpp-lacZ* fusion construct P [(ry^+, *lacZ*) BS 3.0] H1-1 (Blackman et al., 1991; Raftery et al., 1991) mobilized to the 3rd chromosome, were carried out under the same conditions. The discs bearing the en^{Xho25} reporter (Hama et al., 1990) were derived from a cross of en^{Xho25}/*CyO*×*Mrt*/*TM6B* also under conditions to maximize *Mrt* expressivity.

Suppression assays

ru h th st cu sr e^s Mrt ca/*TM3* males were crossed to either *In (2L) dpp^{d12}/CyO, dpp^{s1} $dpp^{d\text{-}ho}$*, or to Oregon R virgin females at 25°C in the same incubator. Progeny bearing both *Mrt* and *dpp* mutations were scored for the penetrance of the *Mrt* phenotype. The penetrance was compared with that in the control cross. Similar tests were performed to examine possible effects of *patched (ptc)* [also called *tufted (tuf)*] gene dosage on *Mrt* expressivity, using the tuf^9 allele.

Histology

β-galactosidase staining of discs was carried out according to the method of Hama et al. (1990). Digoxigenin-labeled mRNA probes were derived from *hh* cDNA 11 (Lee et al., 1992), using digoxigenin-UTP (Boehringer-Mannheim). In situ hybridizations to embryos were done according to the method of Tautz and Peifle (1989) with the following modifications: proteinase K digestion was in a 100 μg/ml solution for 2 minutes and embryos were hybridized for 16 hours at 55°C in hybridization solution (50% formamide, 100 μg/ml boiled sonicated salmon sperm DNA, 100 μg/ml tRNA, 50 μg/ml heparin, and 0.1% Tween) containing the labeled RNA probe. In situ hybridization to imaginal tissues were performed according to the method of Masucci et al. (1990), but all steps were carried out in baskets (ten larvae per basket) constructed of truncated 1 ml Pipetteman tips with nitex mesh melted over the smaller opening. Hybridization was carried out for 36 hours at 55°C in the hybridization solution above.

RESULTS

The *Mrt* mutation is a gain-of-function allele of *hh*

We have isolated a spontaneous dominant mutation named *Moonrat (Mrt)*. The *Mrt* mutation primarily affects the wing (Fig. 1). The most commonly observed defect is an expansion of the distal, anterior region of the wing (Fig. 1B), with accompanying defects in anterior wing veins L2 and L3. We initially suspected that the mutation was a gain-of-function allele of *hh* for two reasons. First, it maps close to *hh.* Second, the wing defects are only observed in the anterior compartment. *hh* is normally expressed only in the posterior compartment (Lee et al., 1992) and loss of *hh* function affects only the posterior compartment (Mohler, 1988). Thus, a gain-of-function allele might be expected to perturb only anterior compartment structures. We named this mutation *Moonrat* after the closest mammalian relative of the hedgehog.

Based on the hypothesis that *Mrt* is a neomorphic *hh* allele, we examined *hh* expression in *Mrt* imaginal wing discs (Fig. 2). *hh* is normally expressed in the posterior of the wing disc (Lee et al., 1992; this work, Fig. 2A,C). Imaginal wing discs from *Mrt* larvae were overgrown to varying extents. In *Mrt* wing discs, *hh* expression was not restricted to posterior regions (Fig. 2B). Variable amounts and patterns of staining were seen in the overgrown, anterior portions of the disc destined to form the wing blade. The posterior expression pattern of *hh* remained, but was often physically distorted by the overgrowth of the anterior. The ectopic, anterior *hh* hybridization signal often appeared stronger than the normal posterior signal. Although the ectopic expression of *hh* in *Mrt* imaginal wing discs often overlapped the prospective wing margin, we did not find that it was confined to that location.

We used a transgenic *Drosophila* strain containing an enhancer trap reporter construct in the *hh* gene (Lee et al., 1992) to show that the *Mrt* mutation causes ectopic expression of the *hh* gene only in *cis*, and not in *trans*. Wing discs from this strain express β-galactosidase in the posterior compartment of the disc in the normal *hh* expression pattern (Fig. 2C). When the *Mrt* mutation was on the homologue, in *trans* to the enhancer detector, no ectopic β-galactosidase expression was observed (Fig. 2D) even when the anterior of the disc was overgrown. This demonstrates that the ectopic expression of *hh* message in *Mrt* wing discs is due to misregulation of *hh* by the *Mrt* mutation in *cis*, but not in *trans*.

We have obtained several dominant extragenic suppressors of the *Mrt* phenotype (unpublished). One of these is an allele of *brahma* (*brm*), a positive transcriptional regulator of homeotic gene expression (Tamkun et al., 1992). In wing discs mutant for both the brm^{23} allele and *Mrt,* ectopic *hh* expression was suppressed (Fig. 2E). This result suggests that *brm*, in addition to its role in regulating homeotic genes, is also a positive transcriptional regulator of *hh* in imaginal development. It also shows that suppression of the ectopic transcription of *hh* suppresses the *Mrt* wing phenotype, and supports the idea that the ectopic expression of *hh* is responsible for the *Mrt* mutant phenotype.

Genetic tests confirm that *Mrt* is a dominant gain-of-function allele of *hh*. We induced two *Mrt* revertants (out of 4220 chromosomes tested) with ethyl methane sulfonate (*Mrt-rv1* and *Mrt-rv2*). Both revertants completely suppressed the *Mrt* phenotype (Table 1). We obtained no recombinants between

Table 1. Suppression of *Mrt* by *hh* alleles in *cis* but not *trans*

	Severity of *Mrt* phenotype (number of flies)*		
Genotype	I	II	III-V
Mrt/+	5	3	218
Mrt-rv1/+	43	0	0
Mrt-rv2/+	85	0	0
Mrt/hh^3	2	9	103
Mrt/hh^4	2	8	97

*Refer to Fig. 1 for description of the classes of *Mrt* phenotypes.

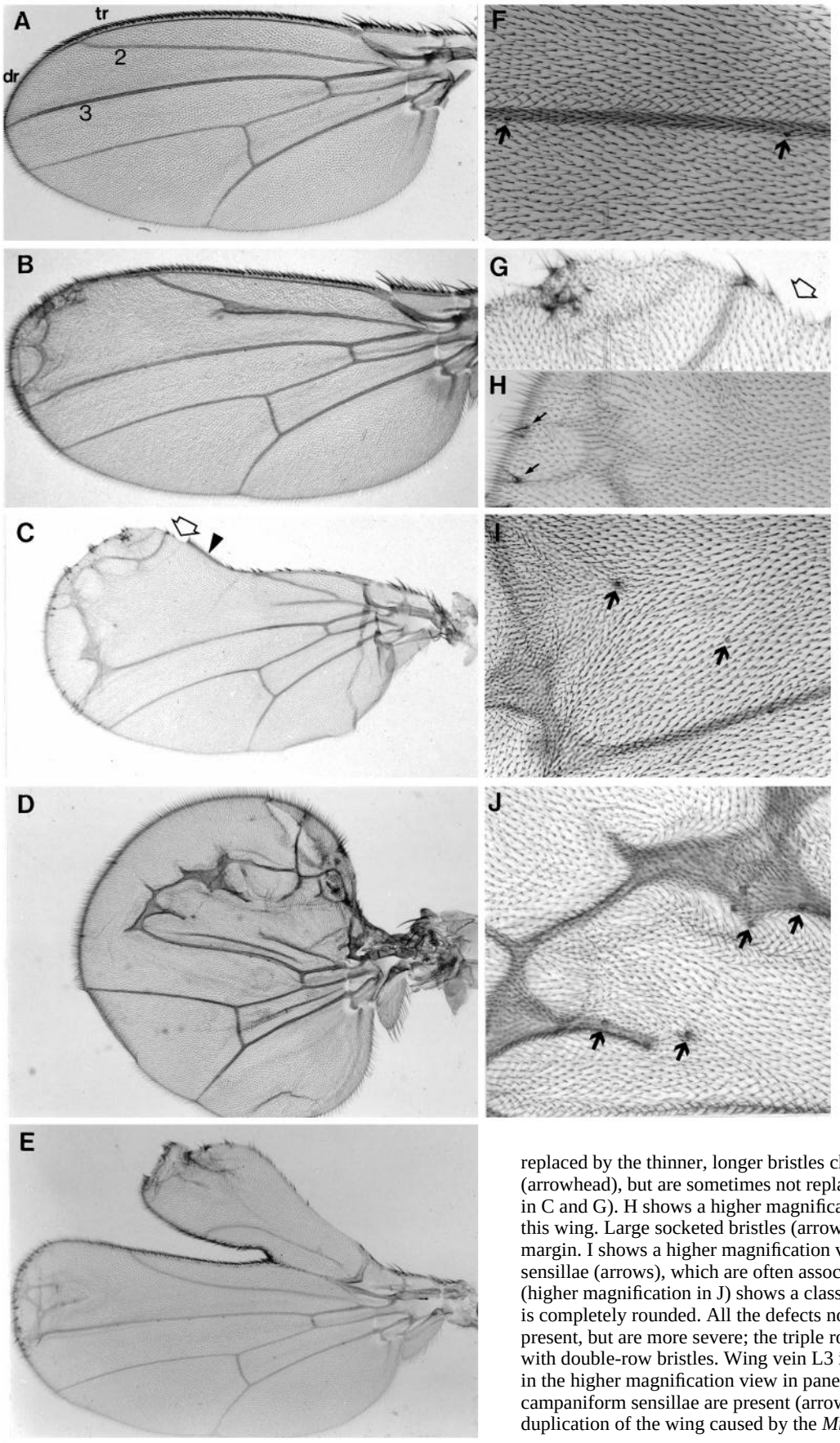

Fig. 1. *Mrt* wing phenotypes. A shows a wild-type wing with longitudinal wing veins L2 and L3 indicated. Note the large, thick bristles of the triple row (tr) at the anterior edge and the thinner, longer bristles of the double row (dr) at the distal edge. F shows a higher magnification view of the wing blade. Arrows indicate the campaniform sensillae characteristically associated with L3 in the wing blade. B-E and G-J show wings of heterozygous *Mrt* flies. We assigned the Roman numerals I through V to indicate differing classes of severity of the *Mrt* phenotype consistently distinguishable in affected wings. A class I wing appears wild type. A class II wing has a barely discernible defect, such as a thickened wing vein (data not shown). B shows an example of a class III wing. The distal anterior region of the wing is slightly expanded, with wing vein L2 partially duplicated. The extent of the vein duplication is variable in this class, and occurs in the distal half of the wing blade. The triple row of bristles is rarely disrupted. C (and higher magnifications in G,H, and I) shows a class IV wing. The distal anterior region of the wing is expanded and rounded. L2 is duplicated proximally, and absent distally. Wing vein L3 is extremely broadened distally. Gaps appear in the triple row of bristles. The triple-row bristles are sometimes replaced by the thinner, longer bristles characteristic of the double row (arrowhead), but are sometimes not replaced with any bristles (open arrows in C and G). H shows a higher magnification view of the distal margin of this wing. Large socketed bristles (arrows) differentiate near the distal margin. I shows a higher magnification view of the ectopic campaniform sensillae (arrows), which are often associated with ectopic wing veins. D (higher magnification in J) shows a class V wing. The anterior compartment is completely rounded. All the defects noted for the wing depicted in C are present, but are more severe; the triple row of bristles is completely replaced with double-row bristles. Wing vein L3 is elaborately broadened, as shown in the higher magnification view in panel J, and numerous ectopic campaniform sensillae are present (arrows). E shows a mirror-image duplication of the wing caused by the *Mrt* mutation.

Mrt and the two *cis* revertants out of 1105 and 1427 flies respectively, indicating that the new mutations responsible for suppressing the *Mrt* phenotype are likely to be intragenic. Both of these revertants are lethal alleles of *hh*. Two *hh* alleles tested in *trans* to *Mrt* do not suppress the phenotype. Thus, *hh* mutations suppress the *Mrt* phenotype in *cis* but not *trans*, confirming that *Mrt* is a *hh* allele.

The ectopic expression of *hh* causes a change in the differentiation of anterior wing structures

Fig. 1 illustrates the variable *Mrt* wing phenotype. To simplify quantification of the variation of the phenotype, we arbitrarily assigned Roman numerals to designate classes of flies displaying the mutant wing phenotype with differing severity (see Fig. 1). In the less severe cases (Fig. 1B), only the anterior, distal portions of the wing were expanded, and in the anterior compartment, longitudinal wing vein L2 was duplicated to varying extents, while the distal end of L3 was slightly plexate and often broadened. In more severely affected individuals (Fig. 1C), distal expansion was more obvious and the anterior of the wing blade was rounded. The triple row of bristles at the anterior wing margin was often missing in gaps and often, but not always, replaced with a double row of bristles more characteristic of the more distal, posterior wing margin. In other instances, the gaps in the anterior triple row had no bristles characteristic of any area of the wing margin replacing them (compare open arrows in Fig. 1C,G). Large socketed bristles were evident near the distal edge (Fig. 1H). Longitudinal wing vein duplication was more elaborate; not only were longitudinal veins L2 and L3 duplicated, but extra vein cells differentiated to form broad, plexate structures in the wing (Fig. 1I). Ectopic campaniform sensillae were often associated with the extra venation (Fig. 1I, arrows). In wild-type wings, campaniform sensillae in the distal wing blade (Fig. 1A,F) are normally associated only with vein L3, the wing vein just anterior to the compartment boundary. In many cases, even in the absence of obviously differentiated vein structures, ectopic campaniform sensillae appeared on *Mrt* wing blades. In the most extreme wing phenotype commonly seen (Fig. 1D), the wing was completely rounded, the entire anterior triple row was absent and replaced with bristles more characteristic of the distal margin, and extensive ectopic venation occurred. Ectopic campaniform sensillae were more numerous (Fig. 1J). More rarely, a complete mirror image duplication of the wing was present (Fig. 1E). In all cases, these defects only involved structures in the anterior compartment. Even in the most severely affected heterozygous individuals, the posterior wing

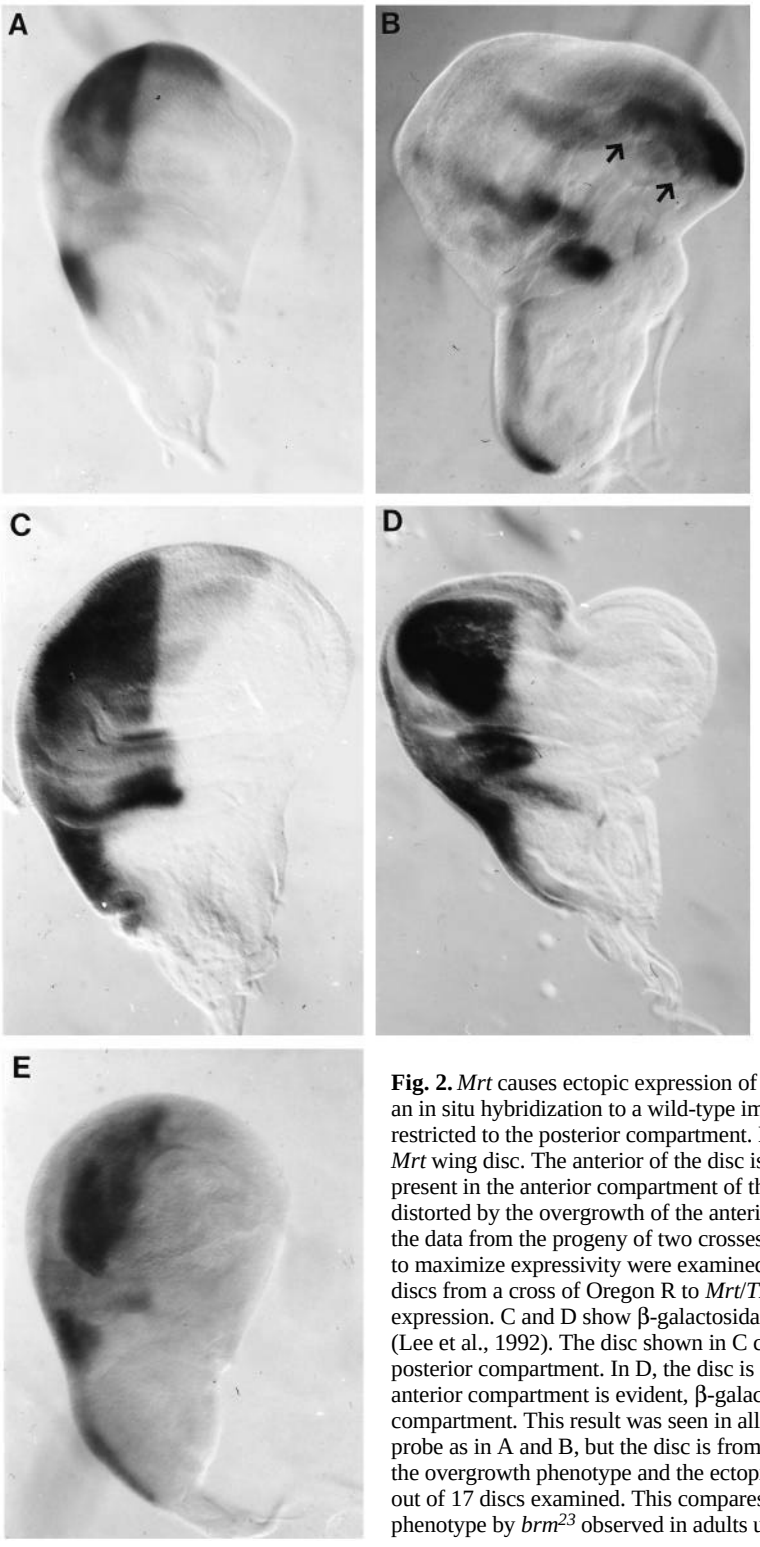

Fig. 2. *Mrt* causes ectopic expression of *hh* in *cis* but not *trans.* Posterior is to the left in all panels. A shows an in situ hybridization to a wild-type imaginal wing disc using a digoxigenin-labeled *hh* probe. *hh* RNA is restricted to the posterior compartment. B shows the result of a similar probe hybridized to a heterozygous *Mrt* wing disc. The anterior of the disc is overgrown and extensive ectopic expression of *hh* (arrows) is present in the anterior compartment of the future wing blade. The posterior of this disc is physically distorted by the overgrowth of the anterior. For in situ experiments examining *hh* expression in *Mrt* discs, the data from the progeny of two crosses were pooled. In the first, discs from *Mrt*/*TM3* flies raised at 20°C to maximize expressivity were examined; 7 out of 14 discs displayed ectopic *hh* expression. In the second, discs from a cross of Oregon R to *Mrt*/*TM6B* were examined; 3 out of 4 discs displayed ectopic *hh* expression. C and D show β-galactosidase expression in wing discs carrying the P30 *hh* enhancer detector (Lee et al., 1992). The disc shown in C carries only the P30 insert. As in A, expression is restricted to the posterior compartment. In D, the disc is from a *Mrt*/P30 fly. Even though extensive overgrowth of the anterior compartment is evident, β-galactosidase expression remains restricted to the posterior compartment. This result was seen in all 41 discs examined. E shows in situ hybridization using the same probe as in A and B, but the disc is from a *Mrt*/*brm*^{23} fly. The presence of the *brm* mutation suppresses both the overgrowth phenotype and the ectopic transcription of *hh.* The same strong suppression was seen in 16 out of 17 discs examined. This compares favorably with the frequency of suppression of the *Mrt* wing phenotype by *brm*^{23} observed in adults under these conditions (data not shown).

Table 2. Conditions affecting penetrance and expressivity of *Mrt*

Conditions	Number [and %] of flies in each class (I-V) of Mrt phenotype*				
	I	II	III	IV	V
Temperature (°C)†					
20	3 [2]	5 [4]	108 [78]	19 [14]	4 [3]
25	37 [15]	43 [18]	157 [64]	7 [3]	0
Paternal effect‡					
Mrt/TM3 father	5 [2]	3 [1]	111 [49]	83 [37]	24 [11]
Mrt/TM3 mother	2 [1]	10 [5]	158 [85]	15 [8]	0

*Refer to Fig. 1 for description of classes I-IV of *Mrt* phenotypes. I is wild-type in appearance, V most mutant. Percentages are approximate.

†*ru h th st cu sr e Mrt ca/TM3* males with class III wings were crossed to Oregon R females and the progeny raised at 20°C or 25°C throughout development. Significantly more flies do not display any visible phenotype (class I) when raised at 25°C ($P<0.005$). At 20°C, more class IV and V flies were found ($P<0.005$ for the pooled classes). Significance calculated using a χ-square test for comparing frequencies.

‡The paternal effect was determined by reciprocal crosses of *Mrt/TM3* flies (either males or females) to Oregon R flies at 25°C. The effect of paternal inheritance is readily observed by comparing the pooled numbers of class IV and V flies: significantly more progeny were class IV or V when the mutation was inherited paternally ($P<0.005$) Significance calculated using a chi-square test for comparing frequencies.

pattern was normal. These pattern duplications indicate that ectopic expression of *hh* in the wing causes some cells within the anterior compartment of the disc to differentiate with features characteristic of more distal cells, as evidenced by the intermittent replacement of the anterior triple row of bristles with double-row bristles. In addition, ectopic expression of *hh* in the wing also causes anterior structures to assume fates characteristic of more posterior structures normally found in the vicinity of vein L3, just anterior to the compartment boundary.

The *Mrt* mutation is unusual in several respects. The *Mrt* wing phenotype is temperature dependent; both penetrance and expressivity were lower at 25° and higher at 20° (Table 2). The temperature dependence may be linked to the rate of development; *Minute* mutations that prolong development also enhance the *Mrt* phenotype (data not shown). *Mrt* also displays a strong paternal effect (Table 2). The wing phenotype expressed in the progeny was more extreme when the *Mrt* chromosome was inherited from the father rather than from the mother. The paternal effect raises the intriguing possibility that the *hh* gene can be regulated by imprinting. Paternal imprinting has been proposed to explain two other cases of paternal effects in *Drosophila*, *E(var)3-93D* (Dorn et al., 1993) and the *Uab*^{1} mutation in the bithorax complex (Kuhn and Packert 1988), however, nothing is known about how this proposed imprinting might occur.

Mrt affects development of other imaginal discs

Other imaginal structures display defects in *Mrt* flies. *Mrt* homozygotes die during late pupal stages as they attempt to eclose, and these phenotypes are readily observed in homozygous pharate adults that have been dissected from their pupal cases. Duplications of the distal leg were observed frequently (Fig. 3B,C). Duplications in legs resulted in an expansion of bristle pattern elements normally associated with ventrolateral structures, such as duplicated sex combs and transverse bristles (Fig. 3B,C). The haltere (Fig. 3D) and antenna (Fig. 4D) also

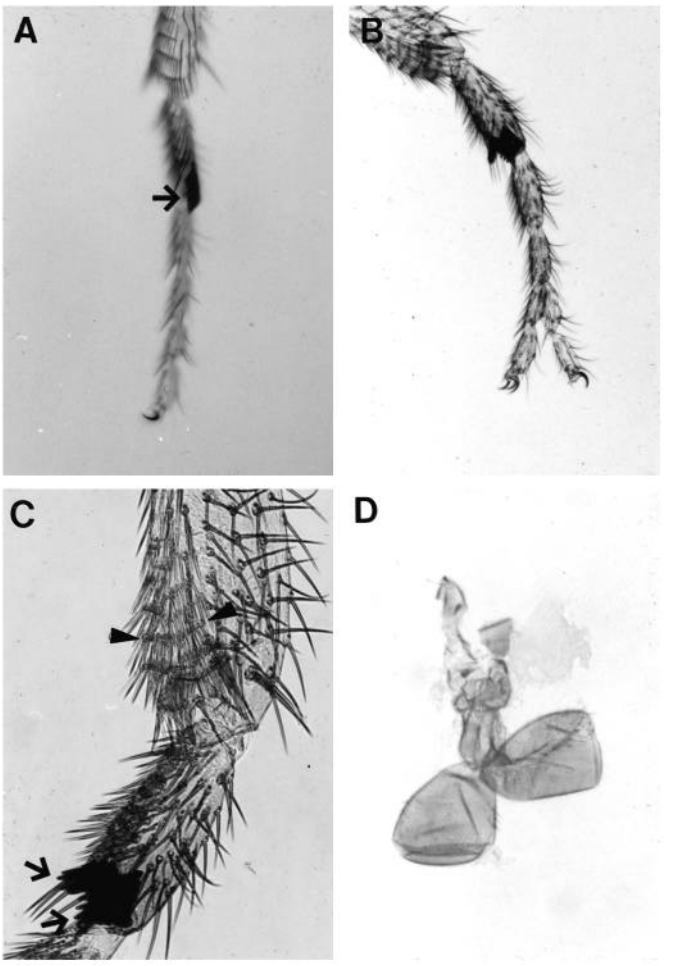

Fig. 3. The *Mrt* mutation causes pattern duplications in distal legs and halteres. A shows the distal portion of a first leg from a wild-type male. The arrow indicates the sex comb. B shows the first leg from a homozygous *Mrt* pharate adult. Note duplication of distal tarsal segments. C is a higher magnification of the leg in B; the arrow indicates a duplicated sex comb and the arrowheads indicate duplicated rows of transverse bristles on the tibia. D shows a haltere from a *Mrt* heterozygote in which the capitellum is duplicated.

were frequently duplicated. We believe the pattern duplications seen in the leg, haltere and antenna may result from ectopic *hh* expression in the anterior compartments of these imaginal discs, as shown above for the wing. The fate of tissues ectopically expressing *hh* may be dependent on precisely which genes in each disc respond to the *hh* signal.

The effect of *Mrt* on the eye is more complicated than the effect on the wing. The eye phenotypes were only seen in *Mrt* homozygotes; in heterozygotes the eye morphology was normal. In some cases, (Fig. 4B) the eye was enlarged and misshapen, and often had a characteristic ‘rough’ phenotype indicating that the ommatidia were disrupted. In other cases, the eye was reduced in size. (Fig. 4C). Expression of *hh* was also abnormal in *Mrt* eye discs (Fig. 5). These results are consistent with recent observations (Ma et al., 1993; Heberlein et al., 1993) establishing a role for *hh* in signaling positional information across the morphogenetic furrow in the eye disc, by analysis of loss-of-function *hh* alleles. The precise devel-

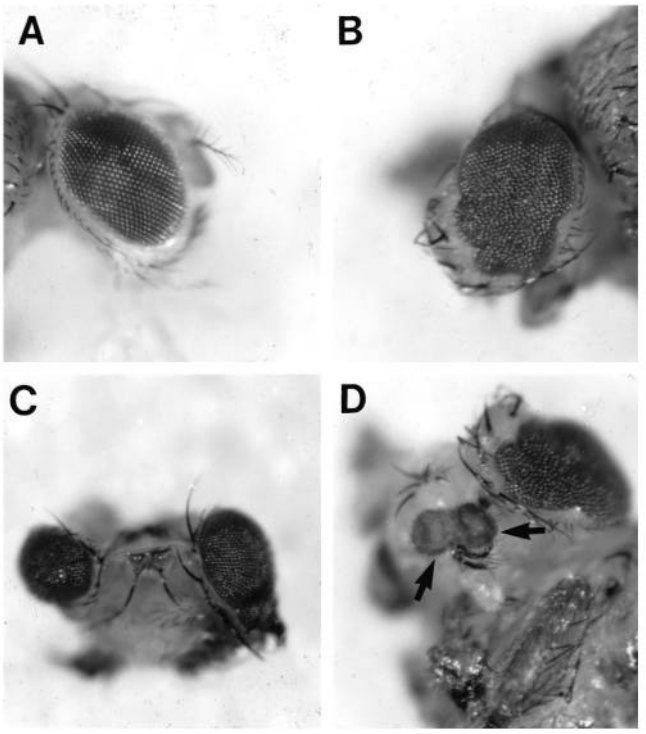

Fig. 4. Eye and antennal phenotypes of *Mrt.* A shows a side view of a wild-type eye. B shows a side view of an enlarged eye from a homozygous *Mrt* pharate adult. C is a head-on view of another example of a *Mrt* homozygote with one eye reduced in size. D shows duplicated antennal structures (arrows) in the same fly shown in B.

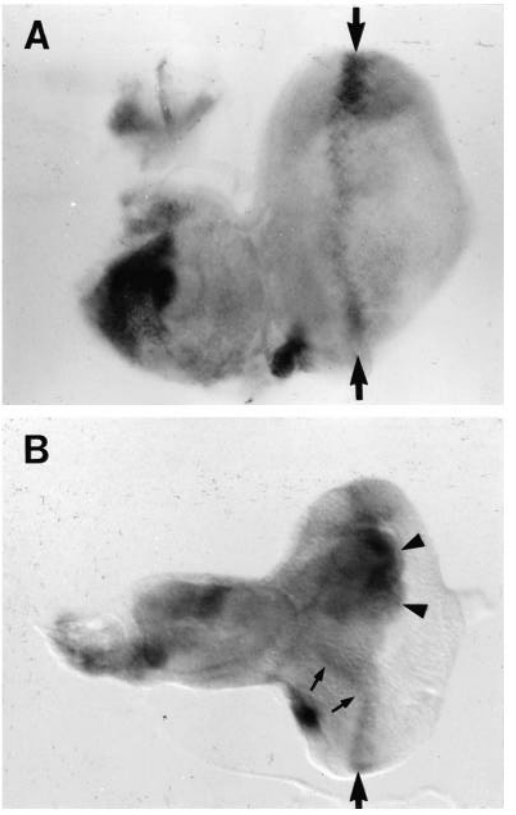

Fig. 5. *Mrt* causes abnormal *hh* expression in imaginal eye discs. A and B show in situ hybridizations to imaginal eye discs using a *hh* cDNA probe. In the wild-type eye disc (A), *hh* is expressed in a well-defined stripe (between arrows) posterior to the morphogenetic furrow. An eye disc from a *Mrt* homozygote (B) is malformed and overgrown, with extra folds of imaginal disc tissue associated with abnormal *hh* expression (arrowheads). A separate, more normal region of *hh* expression can be seen in a stripe in another part of the disc (arrows). Similar results were observed in four out of eight *Mrt* eye discs.

opmental consequences of misexpression of *hh* in the eye remain to be determined.

Mrt does not affect embryonic stages

We examined embryos and larval cuticle to determine whether *Mrt* affected development at early stages. We detected no early differences in embryonic *hh* expression in embryos from a cross between *Mrt* heterozygotes (data not shown). In third-instar larvae, the pattern of abdominal denticle belts in *Mrt* homozygotes was indistinguishable from that of wild type (data not shown). The defects observed in adult *Mrt* flies are thus the consequence of ectopic *hh* expression specifically in imaginal tissues.

Interactions with other genes in imaginal wing discs

The *Mrt* wing phenotype suggests that expression of other genes that play a role in wild-type wing development may be altered. We have examined two of these. The product of the *dpp* gene is normally expressed in the wing disc in a narrow stripe of cells along the anteroposterior compartment border (Raftery et al., 1991; also Fig. 6A). We tested whether ectopic *hh* expression altered *dpp* expression, monitoring *dpp* activity with a *dpp-lacZ* fusion construct (Blackman et al., 1991). In heterozygous *Mrt* larvae, ectopic *dpp* expression appeared in the anterior of the wing disc, often in the general region of the future wing margin (Fig. 6B). This result agrees with the work of Tabata and Kornberg (1994). Supporting this result, two chromosomes bearing *dpp* mutations [dpp^{d12} and a dpp^{s1} $dpp^{d\text{-}ho}$ double mutant chromosome], partially suppressed the penetrance of the *Mrt* phenotype. For the dpp^{d12} allele, 48 of 117 flies were class I. For dpp^{s1} $dpp^{d\text{-}ho,}$ 78 of 266 were class I. These proportions are significantly different ($P<0.005$, by a χ-square test for homogeneity) from the proportion of class I progeny in a control cross (16 of 223).

We also examined whether ectopic *hh* expression affected expression of the *en* gene. The en^1 loss-of-function mutation, even when homozygous, has no effect on the *Mrt* phenotype (data not shown), implying that *en* is not a critical component in the transformation caused by ectopic expression of *hh.* In order to test whether ectopic *hh* expression in the wing activates ectopic *en* expression in *Mrt* wing discs, we monitored *en* expression with an *en-lacZ* reporter construct (Hama et al., 1990). In heterozygous *Mrt* wing discs, *en* was not ectopically expressed (Fig. 6C,D). Anterior wing disc cells do not respond to hh by expressing *en,* and the transformation of anterior wing caused by *Mrt* is not dependent on *en* expression.

Other gene products have been identified that may participate in the *hh* signaling pathway in imaginal discs. For

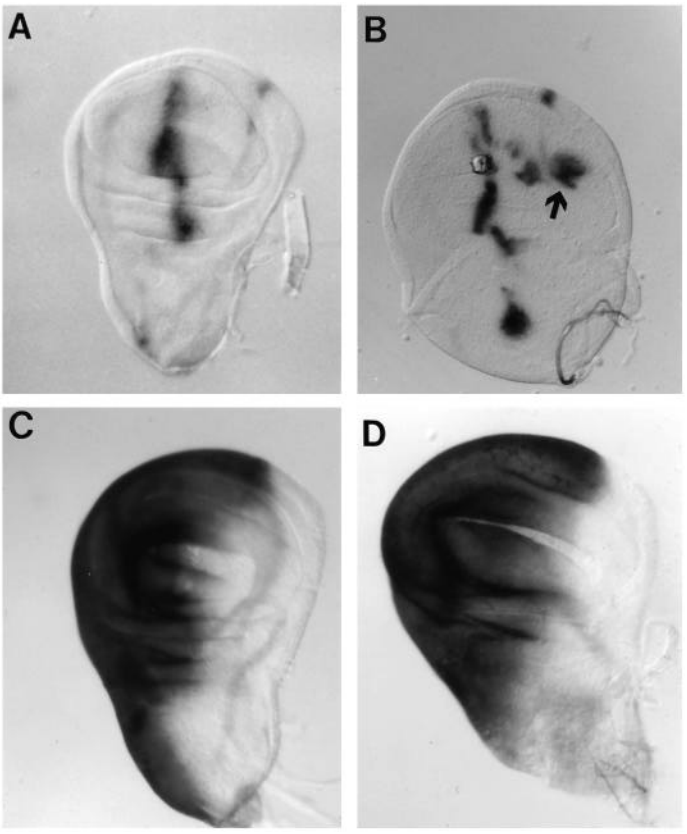

Fig. 6. Ectopic *hh* expression in the wing disc affects the distribution of *dpp* expression, but not *en* expression. A shows β-galactosidase expression in a wild-type wing disc carrying a *dpp* reporter construct (Blackman et al., 1991). The normal pattern of *dpp* expression is in a line nearly coincident with the anteroposterior compartment boundary. B shows expression from the same *dpp* reporter but in a heterozygous *Mrt* wing disc. In addition to the normal stripe of *dpp* expression, there is also ectopic expression (arrow) in the anterior compartment in the wing pouch near the prospective wing margin. Similar results were seen in all of ten discs examined. C and D show β-galactosidase expression in wing discs carrying the en^{Xho25} reporter construct. C shows normal *en* expression in the posterior compartment. D shows a disc from a heterozygous *Mrt* fly; no ectopic *en* reporter expression was seen in 23 discs examined.

instance, the *ptc (or tuf)* gene is involved in signaling in discs (Phillips et al., 1990) and its expression is altered in imaginal discs in which *hh* expression is altered (Tabata and Kornberg, 1994). However, we found no obvious effect of lowering the dosage of *ptc* on the *Mrt* phenotype (data not shown).

The suppression of *Mrt* by *brm* is interesting. The *brm* gene is a member of a group of genes, the trithorax group, that are thought to be positive regulators of homeotic gene expression (Kennison, 1993). Alleles of several other genes in this group also suppress the *Mrt* phenotype, including osa^1, osa^2, $kohtalo^1$, *Df(3L)kohtalo2*, $moira^1$, $moira^2$, $trithorax^{B16}$ and $trithorax^{B17}$ (data not shown). This suggests that at least some of the trithorax group genes are involved in transcriptional regulation of genes in the *hh* signaling pathway during imaginal development. However, we cannot exclude the possibility that this presumptive transcriptional regulation is specific only to the *Mrt* gain-of-function allele. Members of another class of genes, the Polycomb group, are negative regulators of homeotic gene function (Paro, 1990), and might be expected to act in this pathway as well. However, two strong mutant alleles of Polycomb group genes, $Polycomb^4$ and $Polycomb\text{-}like^{11}$, have no effect on the *Mrt* phenotype (data not shown), suggesting that the Polycomb group genes are not involved in regulation of genes in the *hh* signaling pathway.

DISCUSSION

Mrt is a gain of function allele of *hh* affecting imaginal development

We conclude that *Mrt* is a gain-of-function allele of *hh*, based on two lines of evidence. First, *hh* mutations in *cis* to *Mrt* revert the phenotype. In contrast, *hh* alleles in *trans* to *Mrt* do not suppress the *Mrt* phenotype. Second, *Mrt* causes ectopic expression of *hh*, but only from the copy of the *hh* gene in *cis* to the *Mrt* mutation. We have renamed the mutation hh^{Mrt}; to avoid confusion we will continue to refer to the mutation as *Mrt* for the remainder of this discussion. The *Mrt* mutation appears to cause ectopic *hh* expression only in imaginal tissues. Embryonic *hh* expression is normal even in *Mrt* homozygotes. The molecular nature of the *Mrt* mutation is unknown.

Some trithorax group genes may regulate expression of the genes that participate in positional signaling during imaginal development. A *brm* (Tamkun et al., 1992) mutation suppressed both the ectopic transcription of *hh* and the *Mrt* phenotype. Because *brm* mutations directly suppress ectopic *hh* mRNA expression, *brm* may be a transcriptional regulator of *hh* in wing discs, though this effect may be specific to the *Mrt* allele. The mode of action of the other trithorax group genes we examined is less defined. It will be interesting to test directly whether trithorax group genes regulate the normal *hh* expression pattern.

Tabata and Kornberg (1994) found that ectopic *hh* expression in *Mrt* imaginal wing discs was limited to the region of the future wing margin. In our analysis we found that ectopic *hh* was not always confined to this area. The difference between our observations and those of Tabata and Kornberg may be due to the variability in expressivity of the phenotype. We chose experimental conditions maximizing expressivity, and believe that we were looking at discs with higher levels or larger extents of ectopic *hh* expression.

The effects of ectopic *hh* expression in the imaginal wing disc on other genes in the signaling pathway

In both embryos and imaginal wing discs, *en* and *hh* are expressed in the same cells in the posterior compartment (Kornberg et al., 1985; Brower, 1986; Lee et al., 1992; Tabata et al., 1992). In the embryo, *en* and *hh* are involved in a reciprocal signaling pathway (DiNardo et al., 1988; Martinez Arias et al., 1988; Bejsovec and Martinez Arias, 1991). In the imaginal wing disc, however, ectopic hh does not cause ectopic *en* expression, nor does it indirectly induce activation of the *hh* gene itself, based on the expression of the *en* and *hh* reporter constructs in *trans* to *Mrt*. This indicates that hh does not signal to cells in the anterior compartment to express either *en* or itself indirectly, as it does in the early embryo (via *wg*), and may not participate in a reciprocal signaling pathway of any kind in the wing disc. These results are consistent with the notion that *hh*

signaling pathways in the wing disc and embryo are not completely homologous, an idea underscored by the observation that the product of the *wg* gene in the wing disc is not distributed in a pattern adjacent to *en*- or *hh*-expressing cells (Baker, 1988) as it is in embryos. We note, however, that ectopic hh in the embryo does not induce ectopic expression of *en* (Ingham, 1993), possibly as the result of overriding repression of *en* expression in those ectopic locations.

Ectopic hh in the wing disc causes ectopic *dpp* expression. This *dpp* expression appears to be required for maximal penetrance of the *Mrt* wing phenotype. This implies that hh in the wing disc, as has been suggested in the eye disc (Ma et al., 1993; Heberlein et al., 1993), directs cells to express the *dpp* gene. The expression of other genes involved in positional signaling is probably also affected; for instance, expression of the *ptc* (or *tuf)* gene is altered in imaginal wing discs in which *hh* expression is altered (Tabata and Kornberg, 1994). We cannot say whether hh signals directly or through several intermediate steps to cause neighboring cells to express *dpp* in the wing. The *hh* signaling pathway appears to be slightly different in the imaginal leg disc. Ventrolateral pattern elements are duplicated in *Mrt* legs. Recent work has established that *wg* is involved in determining ventrolateral cell fates in the leg (Struhl and Basler, 1993), and that ectopic hh in the leg causes ectopic expression of both *wg* and *dpp* (Basler and Struhl, 1994). Thus, *wg* activity may mediate the *Mrt* leg phenotype.

Mrt in imaginal eye discs

Although the *hh* signaling pathway in the wing disc appears to be different from that in the embryo, the role of hh in the eye and wing discs may be more similar. The eye phenotype of *Mrt* homozygotes is consistent with the proposed role of *hh* in eye discs; the eye is severely misshapen and can be either larger or smaller than normal. In the wild-type eye disc, diffusing *hh* signals to adjacent cells to express *dpp* within the morphogenetic furrow, where cells differentiate into neuronal cell types (Ma et al., 1993; Heberlein et al., 1993). Just anterior to the furrow, the product of the *hairy* gene is expressed (Carroll and Whyte, 1989). In the wing disc, *hh* also signals to neighboring cells to produce *dpp*. *hairy* is also expressed in a stripe of cells prefiguring the wing vein, just anterior and parallel to the compartment boundary and the domain of *dpp* expression (Carroll and Whyte, 1989). The campaniform sensillae, sensory neural structures associated with wing vein L3, differentiate in this region. It may be significant that this cascade of cellular signaling occurs, in the case of the wing disc, across the compartment boundary, and in the case of the eye disc, across the morphogenetic furrow. It may be possible to identify further parallels between these two structures.

The normal role of hh in imaginal tissues

A growing body of evidence establishes that hh acts as a diffusible morphogen (Tabata and Kornberg, 1994; Heemskerk and DiNardo, 1994), signaling to cells over short distances in a dosage-dependent way. How do the cells that receive the *hh* signal respond in the wing disc? Based on our analyses of ectopic *hh* expression in *Mrt* wing discs and the phenotype of *Mrt* mutant wings, we propose that *hh* signals to neighboring cells in the anterior compartment that they are near the anteroposterior boundary. Note that the *hh*-expressing cells probably do not have a posterior identity conferred on them by the *hh* expression endogenous to those cells, they merely send an inappropriate signal. The neighboring cells respond to this information by expressing *dpp* (and possibly other genes). This leads to the eventual differentiation of structures appropriate to tissues normally located just forward of the anterior boundary of *hh* expression, such as wing vein L3 and campaniform sensillae in the wing blade, and double-row bristles at the margin. These structures may be precluded from differentiating in the posterior compartment by the suppression of *dpp* expression in the posterior compartment by *en*-expressing cells (Raftery et al., 1991). In this model, hh, via dpp, indirectly induces campaniform sensillae and wing vein L3 differentiation in the neighboring cells of the anterior compartment only. This model explains why the ectopic structures produced in *Mrt* wings are from the vicinity of the anteroposterior compartment boundary, and why only the anterior compartment is affected.

The observation that *Mrt* causes ectopic activation of *dpp* also leads to a possible explanation of why anterior wing structures are often replaced with distal ones in *Mrt* wings. It has been suggested that *dpp* is part of a signaling center that determines proximodistal position (Posakony et al., 1991). Some *dpp* mutant alleles delete distal tissue (Spencer et al., 1982). However, *dpp* is not localized to, or required in, distally confined areas of the disc, but rather is required in a stripe of cells near the anteroposterior border (Masucci et al., 1991, Posakony et al., 1991, Raftery et al., 1991). How does a gene expressed in a narrow stripe dividing the disc along the anteroposterior axis affect structures along the proximodistal axis? Meinhardt (1983) suggested that compartment boundary intersections might act as sources of positional signaling. Such an intersection occurs at the center of the wing disc, where the anteroposterior and dorsoventral compartment boundaries intersect. The expression of *dpp* in a stripe along the anteroposterior boundary may act as one component of such a coordinate signal, where it intersects the dorsoventral boundary at the future wing margin (Posakony et al., 1991). Tabata and Kornberg (1994) extended this proposal. They suggested that where ectopic *dpp* overlaps the normal domain of *wg* expression at the wing margin, it mimics the region of orthogonally overlapping *wg-dpp* expression found at the center of the wild-type wing disc. The result is the formation of a second proximodistal axis, ultimately causing the differentiation of distal structures (in this case, the double row bristles) in the anterior of the wing. We find this explanation of the ectopic differentiation of distal structures in the anterior of the *Mrt* wing an attractive one. Although some details of our data differ from those described by Tabata and Kornberg (1994), they do not conflict with the model. Distallization might occur where ectopic *dpp* expression overlaps *wg* expression; if ectopic dpp does not overlap *wg* expression in every disc, it would explain the variability of the *Mrt* phenotype.

The overgrowth observed in *Mrt* wings probably also results from abnormal positional signaling. When different areas of a disc are experimentally juxtaposed by extirpation of part of the disc, either regeneration of the missing part or duplication of the intact parts (depending on the location and extent of the deleted area), results (Bryant et al., 1981). Juxtaposition of different areas of the disc in *Mrt* flies might result from misexpression of hh directly or as the consequence of cell death. Inappropriate signaling between cells would then stimulate regulative growth of local areas of the disc, causing local over-

growth. How the genes expressed during initial disc development act during this regulative regrowth remains unknown, but recent work (Brook et al., 1993) suggests an approach to resolving this question.

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