**engrailed and polyhomeotic interactions are required to maintain the A/P boundary of the *Drosophila* developing wing**

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*Accepted 18 May; published on WWW 9 July 1998*

**SUMMARY**

Engrailed is a nuclear regulatory protein with essential roles in embryonic segmentation and wing morphogenesis. One of its regulatory targets in embryos was shown to be the *Polycomb* group gene, *polyhomeotic*. We show here that transheterozygous adult flies, mutant for both *engrailed* and *polyhomeotic*, show a gap in the fourth vein. In the corresponding larval imaginal discs, a *polyhomeotic-lacZ* enhancer trap is not normally activated in anterior cells adjacent to the anterior-posterior boundary. This intermediary region corresponds to the domain of low *engrailed* expression that appears in the anterior compartment, during L3. Several arguments show that *engrailed* is responsible for the induction of *polyhomeotic* in these cells. The role of *polyhomeotic* in this intermediary region is apparently to maintain the repression of *hedgehog* in the anterior cells abutting the anterior-posterior boundary, since these cells ectopically express *hedgehog* when *polyhomeotic* is not activated. This leads to ectopic expressions first of *patched*, then of *cubitus interruptus* and *decapentaplegic* in the posterior compartment, except for the dorsoventral border cells that are not affected. Thus posterior cells express a new set of genes that are normally characteristic of anterior cells, suggesting a change in the cell identity. Altogether, our data indicate that *engrailed* and *polyhomeotic* interactions are required to maintain the anterior-posterior boundary and the posterior cell fate, just prior to the evagination of the wing.

Key words: *Drosophila*, *engrailed*, *polyhomeotic*, *Compartment boundary*, *Target gene*, *Wing development*

**INTRODUCTION**

Pattern formation involves numerous spatial and temporal decisions, including the global definition of position early in embryogenesis, followed by the segregation and elaboration of more specialized structures and organs within the developing animal. The pattern-formation system underlying appendage development in *Drosophila* involves the interplay of many genetic functions that also participate in the partitioning of the embryo. One of the important events in imaginal disc ontogeny is the formation of the anterior-posterior (A/P) compartment boundary (Garcia-Bellido and Santamaria, 1972; Garcia-Bellido et al., 1973). This boundary divides the disc into an anterior and a posterior compartment, with the posterior one being characterized by expression of the *engrailed* (*en*) gene (Lawrence and Morata, 1976; Hama et al., 1990; Tabata et al., 1995).

*en* encodes a homeodomain-containing transcription factor that controls the expression of several target genes (Desplan et al., 1985; Saenz-Robles et al., 1995; Serrano et al., 1995; Schwartz et al., 1995; Serrano et al., 1997). It has recently been shown that *en* is not only involved in the specification of posterior patterns, but also in creating the compartment border, whereas the *en*-related gene, *inverted*, only performs a discrete subset of the functions ascribed to *en* (Simmonds et al., 1995). *en* also plays a major role in the control of imaginal disc growth, by regulating the expression of *decapentaplegic* (*dpp*) in a stripe of cells in the anterior compartment (Sanciola et al., 1995), through regulation of the signaling protein Hedgehog (Hh; Tabata et al., 1992, 1995). *en* activity in the posterior cells directs them to express *hh* and, at the same time, blocks their ability to respond to Hh (Zecca et al., 1995). The absence of *en* activity in anterior cells allows them to be induced by Hh to express *dpp*, that in turn exerts a long-range organizing activity on growth and patterning of both compartments (Capdevila and Guerrero, 1994). Hh acts on anterior cells through the Patched (Ptc) receptor, whose expression is also activated by the Hh signal in cells abutting the A/P boundary (Marigo et al., 1996). *hh* is also required for *ptc* maintenance during the third larval instar, but not for the maintenance of *dpp* (Capdevila et al., 1994). *en* has therefore two separable roles: a cell autonomous role in defining the posterior compartment, by repressing genes that are expressed in the anterior compartment, such as *ci* (Eaton and Kornberg, 1990; Guillen et al., 1995), *ptc* and *dpp* (Sanciola et al., 1995), and a non-autonomous role in the anterior compartment through its
regulation of hh (Tabata et al., 1992). Surprisingly, during the third larval instar a low level of en expression appears in the anterior compartment, which is not associated with expression of hh (Blair, 1992). This en expression depends on the putative serine-threonine kinase protein fused (Sanchez-Herrero et al., 1996) and on the level of hh expression in the posterior cells abutting the A/P boundary, and so depends indirectly on en expression in the posterior compartment (Capdevila et al., 1994; de Celis and Ruiz-Gomez, 1995). The exact role of this late L3 anterior compartment en expression is still not understood.

In order to understand better how en maintains the posterior cell fate throughout development, we are currently studying its target genes. Using different approaches, all based on the En protein ability to bind DNA specifically, we have identified several target genes that had not been detected in previous genetic screens (Serrano et al., 1995, 1997). One of these targets is polyhomeotic (ph), a Polycomb group (PcG) gene. Early in development, segmentation genes, such as en, specify the segments and initiate the expression of the homeotic genes (Martinez-Arias and White, 1988; Mann, 1994), while the PcG genes take over the maintenance of the expression of homeotic genes and of en later on (Paro, 1990; De Camillis et al., 1992; Dura and Ingham, 1988; Moazed and O’Farrell, 1992). We have shown that, during embryogenesis, ph expression depends transiently on en expression during germ-band extension (Serrano et al., 1995). Later on, during germ-band retraction, ph maintains several sets of genes, including en (Dura and Ingham, 1988). The en/ph interaction detected at germ-band extension could be involved in this switch of regulation between segmentation genes and PcG genes that occurs at the retraction of the germ-band, just after en has activated ph.

In this report, we show that en also activates ph during wing morphogenesis. Indeed, transheterozygous flies, mutant for both en and ph, show a wing phenotype with a gap in the fourth vein. In the corresponding larval imaginal discs, we detect a decrease in ph-lacZ expression in cells adjacent to the A/P compartment border, compared to wild-type and single mutant discs. The region where ph expression is affected corresponds exactly to the domain of low en expression in the anterior compartment of late L3 larvae (Blair, 1992). Analysis of the expression patterns of different genes of the Hh signaling pathway that are normally expressed in this intermediary region showed that the segmentation gene patched is highly affected in ph/en mutant discs. The gap in the fourth vein can therefore be correlated with a misregulation of ptc in the posterior compartment. Interestingly, this ectopic ptc expression appears not only in the cells where ph is affected, but also in neighboring posterior cells. This ectopic expression of ptc progressively invades the posterior compartment during the third instar to fill the whole compartment in mature larvae. Genetic data indicate that the level of hh expression is involved in this phenomenon, suggesting that the progressive invasion of the posterior compartment by Ptc is due to an increased secretion of Hh by the cells of the anterior intermediary region, towards cells localized more posteriorly. As a consequence of this ptc misregulation, cubitus-interruptus (ci) and decapentaplegic (dpp) are activated in the posterior compartment, suggesting that the intermediary region, where dpp expression is normally confined, expands posteriorly. As a result of the absence of ph activation by En in cells abutting the A/P boundary, this boundary is not maintained at its normal position, but is progressively shifted posteriorly, while cells lose their posterior identity.

MATERIALS AND METHODS

Fly strains
enB88 is an EMS-induced allele with a translation stop at codon 104, isolated by Wieschaus and Nüsslein-Volhard (Gustavson et al., 1996). The en-lacZ strain corresponds to the ryxho25 transformed line, where lacZ expression follows the normal en expression pattern (Hama et al., 1990). The phiso strain, provided by M. O. Fauvarque and J. M. Dura, was induced by PlacW-element mutagenesis and corresponds to an insertion in the ph locus (Serrano et al., 1995). The ph+4 allele corresponds to an inversion that inactivates the proximal unit of the ph locus (Dura et al., 1987). The other ph mutants that were used are described in Dura et al. (1987). The dpp-lacZ, ptc-lacZ, hh-lacZ lines as well as the hs-hh and hh125 strains were kindly provided by T. Kornberg.

Antibodies
Two anti-Engrailed antibodies have been used, either a polyclonal antibody provided by F. Payne and A. Vincent (Serrano et al., 1997) or the 4D9D4 monoclonal antibody. Monoclonal antibody directed against β-galactosidase was obtained from Promega. Anti-Cr antibody is a polyclonal antibody, a gift from T. Kornberg (Aza-Blanc et al., 1997). Anti-Patched antibody is a monoclonal antibody from I. Guerrero (Capdevila et al., 1994).

β-galactosidase staining of larvae
In situ detection of β-galactosidase activity was carried out as described by Glaser et al. (1986).

Whole-mount immunostaining of imaginal discs
Dissected larvae were fixed for 20 minutes in 30 mM Pipes (pH 7.4), 160 mM KCl, 40 mM NaCl, 4 mM Na3 EGTA, 1 mM spermidine, 0.4 mM spermine, 0.2% β-mercaptoethanol, 0.1% Triton X-100 and 4% paraformaldehyde, washed four times in PBT (PBS + 0.1% Triton X-100), and blocked for 30 minutes in PBT, supplemented with 1% BSA. For double staining, first antibodies against Mouse and Rabbit were incubated together in PBT in the presence of 1% BSA overnight at 4°C. Discs were then rinsed and washed 2 times in PBT, blocked in PBT plus 1% BSA and incubated with secondary antibodies, which were fluorescein-conjugated anti-rabbit and rhodamin-conjugated anti-mouse. Discs were mounted in Citifluor AF1 and analysed by Confocal microscopy.

Confocal laser scanning microscopy
Confocal laser scanning microscopy was performed using a Leica confocal imaging system (TCS4D). Images were collected using an oil immersion lens (63×, NA 1.4 plan Apochromat). For fluorescein and rhodamin excitations, argon Krypton laser is used.

RESULTS

Genetic interactions between engrailed and polyhomeotic
In order to determine the functional relationships between en and ph during imaginal development, we generated transheterozygous flies bearing mutations in both loci. The ph locus is localized on the X chromosome and includes two genetic and molecular units whose functions are largely
redundant (Dura et al., 1987; Saget and Randsholt, 1994), although the proximal and the distal units might have different functions or could be expressed at different levels during development (Hodgson et al., 1997). Wild-type males have therefore two functional \( ph \) copies whereas females have four. Mutations inactivating a single genetic unit are hypomorphic and cause homeotic transformations but no lethality; inactivation of both genetic units (in the \( ph^{505} \) amorphic allele) results in a severe embryonic lethal phenotype (Dura et al., 1987). We analysed phenotypes of flies carrying various combinations of viable \( ph \) alleles and \( en \) mutations. Transheterozygous \( ph/en \) females, who have three copies of \( ph^+ \), normally do not show any wing phenotype, or only with low penetrance (Table 1; Fig. 1). Their male siblings, who have only two functional \( ph^+ \) copies and thus are the true transheterozygotes, all show a gap in the fourth vein (Table 1; Fig. 1), in addition to the numerous homeotic transformations normally present in \( ph \) mutants. The gap of the fourth vein was essentially found with \( ph \) alleles that affect the proximal unit (Table 1), suggesting a functional difference between the two units. Indeed, this phenotype was found in males carrying one copy of the hypomorphic \( ph^{410} \), \( ph^{lac} \) and \( ph^2 \) alleles or in females homozygous for those alleles, in combination with all the \( en \) mutants that we tested (Table 1; Fig. 1). With the thermosensitive \( ph^2 \) allele, the gap in the fourth vein is only detectable at 29°C (Dura et al., 1985). This wing phenotype is never observed in \( ph \) mutants and is only rarely detected in \( en^1 \) homozygous flies or in viable \( en^B86/en^1 \) heteroalleles (Hama et al., 1990).

These results show that the fourth vein defect is characteristic of flies with a reduction of both the \( ph \) and \( en \) functions and may reflect functional interactions between the two genes during wing morphogenesis. The dosage effect that we detect when varying the number of functional \( ph^+ \) copies (Table 1; Fig. 1. Compare males to females), suggests that the interaction might be direct, but does not predict its direction.

**Engrailed regulates polyhomeotic expression in imaginal discs**

To further characterize the \( ph/en \) interactions during wing morphogenesis, expression of both genes was analysed in mutant wing discs. We used the \( en-lacZ \) strain (Hama et al., 1990; Fig. 2A) to follow the pattern of \( en \) expression and the \( ph^{lac} \) strain to monitor \( ph \) expression (Serrano et al., 1995; Fig. 2B). In both lines, the \( en \) and \( ph \) patterns of expression are normal and these flies exhibit no wing phenotype. \( ph^{lac} \) is an enhancer trap insertion in the \( ph \) locus, which is homozygous viable and induces mild homeotic transformations (Fauvarque et al., 1995). \( ph^{lac} \) males carrying one mutated \( en \) copy exhibit a gap in the fourth vein (Fig. 1B2). The \( en-lacZ \) transgene inserted in the \( en \) locus causes an \( en \) mutation that is homozygous lethal, and \( en-lacZ/+ \) flies also exhibit a gap in the fourth vein in association with \( ph \) mutations (Fig. 1A3).

The \( \beta \)-galactosidase expression of \( en-lacZ \) discs is not affected by any of the viable \( ph \) alleles that we tested (Fig. 2C; data not shown): a posterior compartment, where \( en \) is expressed, and an anterior compartment, where \( en \) is not, are still present. Indeed, discs from \([ph^{410}/Y; en-lacZ/+]\) males, that show mutant wings (Fig. 1A3) and \([ph^{410}+/+; en-lacZ/+]\) females that have normal wings (Table 1), express \( en-lacZ \) in a pattern that is indistinguishable from that of \([en-lacZ/+]\) discs (compare Fig. 2C and A). We therefore observe, under these conditions, no apparent requirement of \( ph \) function for normal \( en-lacZ \) expression.

In contrast, the pattern of \( ph^{lac} \) expression is altered in \( en \) male discs. Normally, \( ph^{lac} \) shows a prominent expression in an intermediary region, in the middle of the disc (Fig. 2B). In combination with either \( en^B86 \) heterozygotes (Fig. 2D) or \( en^1 \) viable mutants (not shown), \( ph^{lac} \) discs present little \( \beta \)-galactosidase activity in this region, when compared to the wild-type background. This difference remains clear even when the staining is allowed to proceed for a long time. The dependence of \( ph \) on \( en \) function is also found in heat-shocked \([ph^{lac}; hsen]\) larvae. In such larvae, \( en \) is ectopically expressed throughout the wing disc and ubiquitous \( ph^{lac} \) driven \( \beta \)-galactosidase in the disc is also observed (not shown).

These results show that the fourth wing vein defect of transheterozygous adults is correlated with a misregulation of \( ph-lacZ \) expression in the wing discs. They also suggest that \( ph/en \) interactions occur in the intermediary part of the wing pouch and that \( ph \) activation in this region depends on regulation by \( en \).

![Fig. 1. Wing phenotypes of transheterozygous flies, affected both in engrailed and polyhomeotic. Transheterozygotes are generated by mating homozygous ph mutant females to en7CyO males. Note in A1 the slight wing phenotype that appears in 5% of females of this genotype.](image-url)
polyhomeotic and engrailed are both expressed in the intermediary region of the wing disc

We determined, by confocal microscopy, that the intermediary cells of the wing disc that highly express phi\textsuperscript{lac} also express en, at least for the anterior compartment cells abutting the A/P boundary (Fig. 3). The region of co-expression lies next to the posterior cells that express en at a high level and includes the anterior compartment cells in which en expression is activated at a low level during L3 (Blair, 1992). In fu\textsuperscript{l} mutant larvae where this late en expression never appears (G. Alves and D. Busson, personal communication), the activation of phi\textsuperscript{lac} in the intermediary region is also affected (data not shown). This suggests that the activation of phi in this region might depend, at least in part, on en regulation. The disc region, where this low level of en expression occurs, gives rise to the region between the third and the fourth veins in the proximal part of the wing (Blair, 1992), indicating that the wing phenotype of the phi/en transheterozygous flies could result from the abnormal low level of phi expression in the intermediary region of the larval wing disc. Since en expression in the anterior cells abutting the A/P boundary appears only during L3, we expect the activation of phi to also occur during L3 if it depends on en expression in these cells.

Development of the intermediary region of the wing pouch during L3

In early L3, when En is still confined to the posterior compartment, anterior cells receive the Hh signal from the en-expressing cells. In turn, Hh activates patched (ptc), as well as cubitus-interruptus (ci) and decapentaplegic (dpp) (Zecca et al., 1995; Sanchez-Herrero et al., 1996). The upper panel on Fig. 4 shows that, in early L3 discs, ptc and dpp are highly expressed in the same cells abutting the A/P boundary. This creates an intermediary region, corresponding to the anterior cells that receive the Hh signal. The intermediary region separates the en-expressing cells of the posterior compartment from the anterior compartment cells that do not receive the Hh signal and do not express en.

As the disc grows during L3, the intermediary region expands and gets divided into two regions, one that expresses dpp (and a weak level of ptc) (I1 on Fig. 9), and the other that highly expresses ptc (I2 on Fig. 9) (see the lower panel on Fig. 4). Because the number of cells in the stripes in early and late discs does not increase considerably, growth per se cannot account for the change in overlap of the dpp and ptc domains. The en activation that occurs during L3 in the I2 region is probably responsible for the dpp repression in these cells, since En is a strong repressor of dpp (Sanicola et al., 1995; Sanchez-Herrero et al., 1996). We verified that the low en expression, in the three rows of anterior cells abutting the posterior compartment, overlaps the cells that strongly express ptc (data not shown). The most anterior cell rows that highly express ptc, also express dpp (and appear in yellow on the lower panel on

Table 1. Penetration of the gap in the fourth vein phenotype in transheterozygous flies at 25°C and 29°C

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Gap in the 4th vein</th>
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<tbody>
<tr>
<td></td>
<td>25°C</td>
</tr>
<tr>
<td>phi\textsuperscript{505}/+; en\textsuperscript{B86}/+</td>
<td>10%</td>
</tr>
<tr>
<td>phi\textsuperscript{114}/+; en\textsuperscript{B86}/+</td>
<td>0%</td>
</tr>
<tr>
<td>phi\textsuperscript{410}/Y; en\textsuperscript{B86}/+</td>
<td>100%</td>
</tr>
<tr>
<td>phi\textsuperscript{410}/Y; en\textsuperscript{LA4}/+</td>
<td>100%</td>
</tr>
<tr>
<td>phi\textsuperscript{410}/Y; en\textsuperscript{SX11}/+</td>
<td>100%</td>
</tr>
<tr>
<td>phi\textsuperscript{410}/Y; en\textsuperscript{C}/+</td>
<td>100%</td>
</tr>
<tr>
<td>phi\textsuperscript{410}/Y; en\textsuperscript{lacZ}/+</td>
<td>0%</td>
</tr>
<tr>
<td>phi\textsuperscript{410}/Y; en\textsuperscript{SFX31}/+</td>
<td>100%</td>
</tr>
<tr>
<td>en\textsuperscript{C}/+</td>
<td>2%</td>
</tr>
<tr>
<td>en\textsuperscript{C}/+; phi\textsuperscript{lac}/+</td>
<td>5%</td>
</tr>
<tr>
<td>phi\textsuperscript{lac}/Y; en\textsuperscript{lacZ}/+</td>
<td>100%</td>
</tr>
<tr>
<td>phi\textsuperscript{lac}/Y; en\textsuperscript{en\textsuperscript{SFX31}}/+</td>
<td>100%</td>
</tr>
<tr>
<td>phi\textsuperscript{lac}/Y; en\textsuperscript{en\textsuperscript{C}}/+</td>
<td>20%</td>
</tr>
<tr>
<td>phi\textsuperscript{lac}/Y; phi\textsuperscript{410}/+</td>
<td>0%</td>
</tr>
<tr>
<td>phi\textsuperscript{lac}/Y; phi\textsuperscript{505}/+</td>
<td>0%</td>
</tr>
<tr>
<td>phi\textsuperscript{lac}/Y; phi\textsuperscript{LA4}/+</td>
<td>0%</td>
</tr>
<tr>
<td>phi\textsuperscript{lac}/Y; phi\textsuperscript{B86}/+</td>
<td>0%</td>
</tr>
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</table>

Transheterozygotes are generated by mating homozygous phi mutant females to heterozygous en\textsuperscript{CyO} males. ND indicates that the phenotype has not been tested at 29°C. phi\textsuperscript{505} and phi\textsuperscript{114} correspond to the amorphic allele, affected in both phi units. phi\textsuperscript{410} and phi\textsuperscript{B86} correspond to mutations that affect the proximal unit of phi, whereas phi\textsuperscript{la4} corresponds to mutations that affect the distal unit (Dura et al., 1987). en\textsuperscript{SFX31} corresponds to null alleles and are described in Gustavson et al. (1996).

Fig. 2. β-galactosidase stainings of phi\textsuperscript{lac} and en\textsuperscript{lacZ} in late L3 wing imaginal discs. (A) en\textsuperscript{lacZ}/+; wings of this genotype are normal. (B) phi\textsuperscript{lac} strain; wings of this genotype are normal. (C) phi\textsuperscript{410}/Y; en\textsuperscript{lacZ}/+ male; wings of this genotype show a gap in the fourth vein. (D) phi\textsuperscript{lac}/Y; en\textsuperscript{SFX31}/+ male; wings of this genotype show a gap in the fourth vein. The arrow indicates the region of the presumptive A/P boundary where phi\textsuperscript{lac} is highly expressed.
that express ph/en L3) and in late L3 (Fig. 5), after dramatic in mid L3 larvae (e.g. 24 hours after the beginning of region between early and mid L3. The effect gets more activated 5). In a transheterozygous become detectable in the intermediary region (Blair, 1992; Fig. 5). At that stage, expression level in the intermediary region, in wild-type, as since, by late L3, En expression and a higher expression of dpp compartment, except for the region of the D/V boundary that is unaffected (Fig. 5).

Fig. 4) suggesting that en, although at a lower level, must be indeed present in these cells, but barely detectable. Cells in the intermediary region are particular because they co-express genes that are specific of either the anterior or the posterior compartment. The cells forming the intermediary I2 region are the cells that strongly express phlac in late L3 larvae.

**Analysis of the expressions of the hedgehog target genes in ph/en transheterozygotes**

To identify the function of the activation of ph in the intermediary region, we analysed the behaviour of the different hh target genes that are normally expressed in this region. Genes of the hh signaling pathway were monitored in a transheterozygous ph/en mutant background, in conditions where phlac is not induced in the intermediary region (Fig. 9). Strains where lacZ is driven by different gene promoters (ci, dpp, hh and ptc) were used for this analysis. We observed that the expression of the segment polarity gene ptc is particularly affected (Fig. 5).

In early L3, when En is confined to the posterior compartment, phlac is ubiquitously expressed in the wing discs of both wild-type and transheterozygous ph/en mutant larvae (Fig. 5). At that stage, ptc is already established with a higher expression level in the intermediary region, in wild-type, as well as in transheterozygous mutant ph/en discs. Between early and mid L3, En expression and a higher expression of ph become detectable in the intermediary region (Blair, 1992; Fig. 5). In a transheterozygous ph/en mutant background, when ph is not normally activated in the intermediary region, ectopic expression of ptc appears in the posterior compartment (Fig. 5). ptc first begins to expand posteriorly from the intermediary region between early and mid L3. The effect gets more dramatic in mid L3 larvae (e.g. 24 hours after the beginning of L3) and in late L3 (Fig. 5), after en should have normally activated ph in the intermediary region.

Therefore, the maintenance of ptc during L3 appears to depend on the activation of ph in the intermediary region. Interestingly, ptc expression is not only misregulated in cells of the intermediary region, but also in neighboring posterior cells (Fig. 5). This phenomenon is also dynamic during L3 since, by late L3, ptc has invaded the entire posterior compartment, except for the region of the D/V boundary that is unaffected (Fig. 5). These results suggest that the misregulation of ptc in ph/en mutant discs is not cell autonomous. It might involve an intermediate molecule, controlled by ph in the I2 region, that is able to diffuse into neighboring posterior cells and to activate ptc. Since Hh is a secreted molecule, active during wing morphogenesis, we asked whether Hh could be this intermediate.

**polyhomeotic prevents hedgehog activation by engrailed in the intermediary region of the wing pouch**

Several lines of evidence show a role for hh in the observed vein gap phenotype and in the misregulation of ptc. Genetic arguments are summarized in Table 2 and show that a change in the level of hh expression affects the penetrance of the ph/en wing phenotype. In these experiments, we took advantage of the fact that most of the ph alleles are thermosensitive (Dura et al., 1985; Saget and Randsholt 1994). Transheterozygous [ph410/Y; enl+] males that present a gap in the fourth vein at 25°C, die at 29°C. In contrast, whereas [ph410/Y; enl+] females raised at 25°C all have normal wings, 5% of them show a wing phenotype at 29°C. The effect of hh on the fourth vein phenotype was then analysed in such transheterozygous females at 29°C, by introducing either a hs-hh gene (to increase the concentration of Hh) or a hhph12 gene (to decrease the concentration of Hh). The penetrance of the gap in the fourth vein is significantly increased when hh is overexpressed, whereas the phenotype is rescued when hh is defective (Table 2). The wing phenotype is therefore sensitive to the concentration of Hh. In addition, in the presence of higher amounts of Hh, ptc expands posteriorly in discs from [ph410/Y; enl+; hs-hh+] larvae (data not shown). This shows again that the gap in the fourth vein is correlated with an upregulation of ptc in the posterior compartment.

Since hedgehog is involved in the fourth vein gap phenotype and in the ectopic ptc expression, and since hh is normally not expressed in the I2 region, we wondered whether ectopic...

**Fig. 3.** Confocal microscopy of late L3 phlac imaginal discs. A phlac disc is double stained (A) with an anti-En antibody, secondary detected by rhodamin and (B) with an anti-β-galactosidase antibody, secondary detected by fluorescein. (C) Composite image of the rhodamin and fluorescein fluorescences shown in A and B. It reveals posterior orange cells that express both en and ph and yellow cells that express en and a high level of ph.

**Fig. 4.** Evolution of the intermediary region during L3. The upper panel shows early L3 imaginal discs. The lower panel shows late L3 imaginal discs. In both cases, dpp-lacZ larvae are double stained. Ptc is detected with an anti-Ptc antibody, secondary detected by rhodamin and dpp expression is detected with an anti-β-galactosidase antibody, secondary detected by fluorescein. Composite images of the rhodamin and fluorescein fluorescences are shown at both stages.
expression of hh is present in the I2 cells from ph/en transheterozygotes. Previous work has shown that ph is involved, all along wing morphogenesis, in maintaining the expression levels of Hh signaling pathway genes (en and hh but also ptc and dpp in the anterior compartment). Interestingly ph represses en in the anterior compartment and regulates the level of en expression in the posterior compartment (N. B. R., F. M, and P. Santamaria, unpublished data). Therefore, we asked whether the role of the increased of ph expression in the intermediary region is to maintain the level of en expression that occurs during L3 in these cells or whether the presence of Ph ensures that en does not activate hh in this region. To answer this question, we analysed the expression patterns of en and hh in mid L3 [ph410/Y; en+/+] larvae. If wild-type ph gene is only required to maintain the level of en expression in the intermediary I2 region, these anterior cells should still only express en in L3 [ph410/Y; en+] larvae, possibly at a higher level than in wild type. Alternatively, if the role of wild-type ph gene is to prevent hh activation by en in the intermediary I2 cells, these anterior cells that normally only express en should also express hh in [ph410/Y; en+] larvae. Fig. 6 shows that, in [ph410/Y; en+] larvae, except for the cells of the D/V boundary, all the en-expressing cells also express hh. These data indicate that ph can act as a repressor of hh expression. They show that, in the absence of Ph, en activates hh, confirming that en is an activator of hh (Tabata et al., 1992). They also suggest that ph is required to prevent hh activation by en in the intermediary region, but whether ph represses both the en and hh genes remains uncertain.

Table 2. Genetic analysis showing the involvement of hh in the fourth vein gap phenotype

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<tr>
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</tr>
<tr>
<td>ph410/Y; en+; hsHh/+</td>
<td>0%</td>
</tr>
<tr>
<td>ph410/Y; en+; hsHh/+; hh ts2/+</td>
<td>0%</td>
</tr>
</tbody>
</table>

The percentages were estimated from at least 200 flies for each genotype.

To visualize the consequences of the progressive misregulation of ptc in posterior cells of L3 [ph410/Y; en+] larvae, we analysed the identities of these cells by looking at expressions of Hh signaling pathway genes. Using confocal microscopy, we detected that late L3 [ph410/Y; en+] posterior wing disc cells that ectopically express ptc also express ci (Fig. 7A). Interestingly, the misregulation of ci follows the progressive misregulation of ptc, suggesting that ectopic ptc misregulation is a primary event. Similarly, a misregulation of dpp is detectable in the posterior compartment (Fig. 7B). The cells that ectopically express dpp are the same as those that ectopically express ci (Fig. 8C). These posterior compartment ci-expressing cells from [ph410/Y; en+] larvae show lower expression levels of en and hh than surrounding cells (Fig. 8A,B). All these results are summarized on a diagram shown in Fig. 9. In a [ph410/Y; en+] transheterozygous mutant background, where ph is not activated in the intermediary region, en is able to activate hh in I2. This leads to activation of ptc in the neighboring posterior cells that

Fig. 5. β-galactosidase stainings of wing discs at different times during L3, either in wild-type or in a transheterozygous mutant ph/en background, as indicated. (A) phlac is used to follow ph expression. This expression is analyzed in early L3 (3 days AEL), mid L3 (4 days AEL) and late L3 (5 days AEL). (B) ptc/lacZ is used to follow ptc expression. This expression is analyzed at different times after the beginning of L3 as indicated.
receive an increased amount of Hh signal. As shown on the diagram, ptc activation leads to ci activation, probably because of the existence of a feedback loop between ptc and ci (Hepker et al., 1997). Finally, except for the D/V boundary cells, which are unaffected, the intermediary region seems to expand posteriorly, suggesting that the increased expression of ph in the I2 region is required for the maintenance of the precise location of the A/P boundary in the wing disc (see model Fig. 9).

**DISCUSSION**

**engrailed and polyhomeotic interact in late L3 wing discs**

Gene expressions evolve in developing wing discs during L3. Until early L3, the Hh signal along the anterior-posterior boundary is responsible for organizing wing development, by inducing neighboring anterior cells to highly express ptc, ci and dpp (Basler and Struhl, 1994; Zecca et al., 1995; Alexandre et al., 1996). This defines an intermediary region of cells that do not express en and receive the Hh signal, separating the posterior compartment, where en is expressed, from the anterior compartment where there is no en expression. As development proceeds and the disc grows, this intermediary region is enlarged and gets divided into two distinct regions: the I2 region that highly expresses ptc and the I1 region that highly expresses ci and dpp (Fig. 9). This subdivision into two distinct regions might result from the activation of en that occurs, at that stage, in the I2 region and which is responsible for dpp repression (Blair, 1992; de Celis et al., 1995; Sanchez-Herrero et al., 1996).

The genetic interactions between ph and en in imaginal cells were revealed by experiments in which the relative dosage of ph and en was reduced. In Drosophila, the amount of gene product is usually proportional to the number of wild-type gene copies (Baillie and Chovnick, 1971; Kennison and Tamkun, 1988). However, if the number of functional copies of two genes is reduced and the expression of one gene depends on the other, expression of the dependent gene might be severely reduced. One consequence would be enhanced mutant phenotypes in double mutants. Our observation that ph mutants and heterozygous en flies have normal wings, but [ph/Y; en+/+] flies have vein defects in the posterior compartment of their wing blades suggests such a dependent regulatory interaction between en and ph. Moreover, the dosage effect that we detect, when comparing females to males, suggests a direct interaction between these two genes. Interestingly, only ph alleles that affect the proximal unit interact with en and not those that affect the distal unit, even though alleles affected in both units [ph<sup>505</sup>/++; en<sup>7</sup>/+] show higher penetrance of the wing phenotype (Table 1; Dura et al., 1987). As already suggested, the proximal and the distal ph units, although highly redundant, show slightly different functions during development (Hodgson et al., 1997). Analysis of different [ph<sup>505</sup>/Y; en<sup>7</sup>/+] wing discs, leading to a gap in the fourth vein of adult wings, reveals a lower phlac expression in a heterozygous en mutant background, whereas the en-lacZ expression remains unchanged in ph viable mutants. Altogether, these results...
suggest that en and ph interact during wing morphogenesis and that ph activation depends on en in the I2 region of the wing disc during L3.

**polyhomeotic prevents activation of hedgehog in the I2 region**

In this report, we show that ptc expression is normally established in a transheterozygous ph/en mutant background, but is not properly maintained. This misregulation of ptc not only affects the anterior cells where ph is misregulated, but also posterior cells. ptc misregulation progresses during L3. Indeed 12 hours after L3 starts, ptc begins to expand into a few posterior cells close to the I2 region, to finally invade the entire posterior compartment by late L3, except for the D/V boundary which is unaffected. Consequently, adult wings have a normal margin, but show abnormalities (a gap in the fourth vein) in the posterior compartment. Since ptc misregulation does not appear in all posterior cells at the same time, the posterior progression of the ptc misregulation cannot be easily explained by a direct effect of a defect of ph in the posterior compartment. It rather suggests that a secreted factor is involved. This factor must be activated in the I2 region of ph/en mutants and must be able to activate ptc by diffusing into more posterior cells. We show that Hh is this factor. We first show that the level of hh expression is involved in the penetrance of the gap in the fourth vein defect of transheterozygous ph/en flies. Indeed, in transheterozygous mutant females, a hs-hh transgene increases

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*Fig. 8. Analysis of expression patterns in late L3 imaginal discs of hh, en and dpp, compared to ci in wild-type and in a transheterozygous mutant ph/en background, as indicated. (A) hh-lacZ larvae double stained either with an anti-Ci antibody, secondary detected by fluorescein and with an anti-β-galactosidase antibody, secondary detected by rhodamin or with an anti-Ci antibody, secondary detected by fluorescein and with the 4D9 anti-En antibody, secondary detected by rhodamin. (B) dpp-lacZ larvae double stained with an anti-Ci antibody, secondary detected by fluorescein and with an anti-β-galactosidase antibody, secondary detected by rhodamin. Composite images of the rhodamin and fluorescein fluorescences are shown.*

*Fig. 9. Model for the role of the engrailed and polyhomeotic interactions in the intermediary region abutting the A/P boundary in late L3 imaginal discs. The model is deduced from the misregulations of different genes of the Hh signaling pathway, obtained in a transheterozygous mutant ph/en background compared to wild type. It shows that in the absence of ph activation in the I2 region, the A/P boundary is not maintained, but is shifted posteriorly.*
the penetrance of the wing phenotype at 29°C. In the wing discs of these females, we verified that ptc is misregulated and becomes expressed in the posterior compartment, expanding a few cells posteriorly from the I2 region (data not shown). Moreover, in a ph/en transheterozygous background, hh is activated in the I2 cells that normally only express en in wild-type. As Ph binds to the polytenic chromosomal localization of hh, one possibility would be that Ph directly prevents activation of hh by En (Franke et al., 1992). Interestingly, the posterior cells of the D/V boundary do not show any ptc misregulation, which explains why no transformation of the posterior margin into an anterior one has been detected in ph/en adult wings.

**Posterior cells are competent to respond to the Hh signal in ph/en flies**

That hh is responsible for the changes appearing in the posterior compartment of ph/en flies implies that posterior cells might become competent to respond to the Hh signal. Such competence could be attributed to the presence of a low level of ci which is present in a [ph+/en+/+] background (see Fig. 8). Indeed, ph has been shown to be a repressor of ci in the posterior compartment (N. B. R., F. M. and P. Santamaria, unpublished data). A mechanism involving en and ph, must be responsible for ci repression in the posterior compartment. Transcriptional repression of ci in the posterior compartment could be initiated by en and maintained by ph, the ph expression depending itself on en expression (Serrano et al., 1995). Indeed, posterior heterozygous en/+ cells do not show any phenotype unless they are also mutant for ph. One hypothesis could be a feedback loop involving en and ph to maintain the level of en expression and ci repression in the posterior compartment. Such feedback loop mechanisms to maintain factors at a particular level have already been described in several organisms (Thomas and Thieffry, 1995). If the basic level of en expression, in the posterior compartment, depends on both en and ph, we can expect en to be maintained at a lower level in a [ph+; en+/+] background. These cells might now produce enough C1 and Ptc to become competent to receive the Hh signal (Alexandre et al., 1996; Dominguez et al., 1996). If posterior cells are not competent to receive a Hh signal, higher amounts of Hh will not affect the posterior cells (Basler and Struhl, 1994).

Such a feedback loop mechanism between en and ph, maintaining the level of en expression, could also explain the lack of hypomorphic en mutants, since such mutants will be detectable only when ph is affected. Indeed, the only homozygous viable en mutant that has been isolated so far is en', which is neomorphic (Hidalgo, 1994).

**Late engrailed expression in the I2 region is a prerequisite for patched misregulation and fourth vein defect**

Our results show that ptc misregulation starts from the intermediary I2 region and suggest that it depends on the late en expression in this region. That en expression in the I2 region is essential in this phenomenon is shown by the analysis of wings in a fu'/ background. fused is a putative serine-threonine kinase protein that is involved in dpp activation by Hh in the intermediary region (Sanchez-Herrero et al., 1996). Indeed, although ph'ac is not normally activated in the I2 region of fu' mutant larvae (data not shown), we found that fu', [ph'acfu'/Y] or [ph'acfu'/Y; en'+/+] flies do not show a gap in the fourth vein. In such a context, posterior wing disc cells must however be as competent as [ph'ac/Y; en'+/+] cells to receive the Hh signal. Therefore, the late en expression in I2 is a prerequisite for induction of the fourth vein vein gap phenotype.

These results agree with our hypothesis of needs, on one hand, of ph to ensure that en expression in I2 does not activate hh and, on the other hand, of the competence of the posterior cells to respond to the HH signal from the I2 region, leading to the misregulation of ptc and to the gap of the fourth vein.

**Engrailed and polyhomeotic interactions are necessary to maintain the A/P boundary and the posterior cell identity of late L3 wing discs.**

ptc misregulation was the most obvious event observed in transheterozygous ph/en mutant wing discs. ptc expression is not only misregulated in cells of the intermediary region, where it gets lower, probably because of the ptc autorepression (Hepker et al., 1997), but ptc also gets higher in neighboring posterior cells (Fig. 5). Other genes of the Hh signaling pathway also appeared to be misregulated, but to a lower extent. Indeed, ci and dpp are both ectopically expressed in the posterior compartment, but in fewer cells than ptc, suggesting that ptc misregulation is the primary event that occurs once hh is activated by en in the I2 region. Finally the posterior cells that express ci also express less en and less hh in late L3 transheterozygous ph/en mutant wing discs, suggesting a change in the posterior cell identity in anterior one. Moreover, ph/en clones induced in an en mutant background expand either anteriorly or posteriorly, as ph' or Ptc clones do (data not shown; Busturia and Morata, 1988), suggesting that a lack of ph expression might lead to the loss of the restriction lineage and a shift of the A/P boundary. As shown on the diagram on Fig. 9, these results suggest that in the presence of en expression in the I2 region, but in the absence of ph activation in these cells, the intermediary region expands posteriorly. These results show that the interaction between engrafted and polyhomeotic is necessary to maintain the A/P boundary, just prior to the evagination of the wing.

This work shows that en and ph share 'particular relationships' during wing morphogenesis, as they do during embryogenesis, since they regulate each other at different times and in different cells. This confirms that ph is an important en target gene since its regulation by en is necessary to maintain the posterior cell fate and the position of the A/P boundary, a role assigned to en function. Such interactions between en and ph could also be involved in maintaining the posterior cell identity during embryogenesis, since ph is activated in the posterior compartment of germ-band-extended embryos, at a stage when en autoactivation occurs (Serrano et al., 1995; Heemskerk et al., 1991).

We wish to acknowledge Jean-Antoine Lepesant for helpful discussions during this work. We thank Richard Schwartzman for photographic assistance. We thank Georges Alves and Denise Busson for sharing results before publications. This work was supported by the Centre National de Recherche Scientifique (CNRS) and by grants from ACC SV4 No 9504211 and ARC 1370 to F. M.
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