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Genetic analysis of *hedgehog* signalling in the *Drosophila* embryo

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SUMMARY

The segment polarity genes play a fundamental role in the patterning of cells within individual body segments of the *Drosophila* embryo. Two of these genes *wingless* (*wg*) and *hedgehog* (*hh*) encode proteins that enter the secretory pathway and both are thought to act by instructing the fates of cells neighbouring those in which they are expressed. Genetic analysis has identified the transcriptional activation of *wg* as one of the targets of *hh* activity: here we present evidence that transduction of the *hh*-encoded signal is mediated by the activity of four other segment polarity genes, *patched*, *fused*, *costal-2* and *cubitus interruptus*. The results of our genetic epistatsis analysis together with the molecular structures of the products of these genes where known, suggest a pathway of interactions leading from reception of the *hh*-encoded signal at the cell membrane to transcriptional activation in the cell nucleus. We have also found that transcription of *patched* is regulated by the same pathway and describe the identification of *cis*-acting upstream elements of the *ptc* transcription unit that mediate this regulation.

Key words: segment polarity genes, *wingless*, *hedgehog*, *patched*, *fused*, ci^D, cell interactions

INTRODUCTION

During early development of the *Drosophila* embryo, cells become allocated to parasegments, metameric units with the properties of secondary embryonic fields, by the activity of pair-rule genes such as *fushi tarazu* and *even-skipped* (reviewed by Ingham and Martinez Arías, 1992). This process occurs at the transition from the syncitial phase of embryonic development, when positional information is generated through intracellular gradients of transcription factors (St. Johnston and Nüsslein-Volhard, 1992), to the multicellular phase, when positional information depends upon cell-cell communication. Amongst the many genes involved in this latter phase of development, those of the segment polarity class (Nüsslein-Volhard and Wieschaus, 1980) play a fundamental role in the patterning of cells along the anteroposterior body axis of the embryo.

The establishment of parasegments is marked by the activation of the segment polarity genes *wingless* (*wg*; Baker, 1987) and *engrailed* (*en*; DiNardo et al., 1985; Fjose et al., 1985; Kornberg et al., 1985), the interfaces of whose expression domains define the parasegmental boundaries (van den Heuvel et al., 1989). Although these expression patterns are initiated by the pair-rule genes during the blastoderm stage (DiNardo and O'Farrell, 1987; Howard and Ingham, 1986; Ingham et al., 1988), both genes subsequently come to depend upon the activity of each other for their maintenance (Martinez Arias et al., 1988). Expression of *wg* is maintained only in cells immediately adjacent to the *en* domain, whereas *en* expression persists in a stripe one to two cells wide immediately posterior to the *wg*-expressing cells. In the absence of *wg* activity, *en* expression ceases soon after gastrulation (DiNardo and O'Farrell, 1987; DiNardo et al., 1988; Martinez Arias et al., 1988). This requirement for *wg* activity is very transient, *en* expression becoming independent of *wg* within 5 hours of fertilisation, at stage 9 (Bejsovec and Martinez Arías, 1991; DiNardo and O'Farrell, 1987; DiNardo et al., 1988; Heemskerk et al., 1991; Martinez Arias et al., 1988). Since *wg* is the orthologue of the mammalian proto-oncogene *Wnt-1* (Rijsewijk et al., 1987), encoding a secreted glycoprotein, it has been postulated to act as the signalling molecule that directly mediates maintenance of *en* transcription. This interpretation is supported by immunolocalisation studies that reveal that Wg protein is found 1-2 cells away from its source (Gonzalez et al., 1991; van den Heuvel et al., 1989), corresponding to the width of the *en* domain under *wg* control.

Once *en* expression becomes independent of *wg* activity, the parasegment boundaries and hence the metameric organisation of the embryo, can be considered to be stable. Expression of *wg* persists at each boundary, however, and is subsequently required for the correct specification of cell identity within each parasegment (Bejsovec and Martinez Arías, 1991; Dougan and DiNardo, 1992). The dependence of *wg* transcription upon *en* activity implies a reciprocal interaction between the two cell populations at each parasegmental boundary (Martinez Arias et al., 1988). The best candidate for a signal mediating this interaction is the product of the *hedgehog* (*hh*) gene, a protein with a single putative transmembrane domain (Lee et al., 1992; Mohler

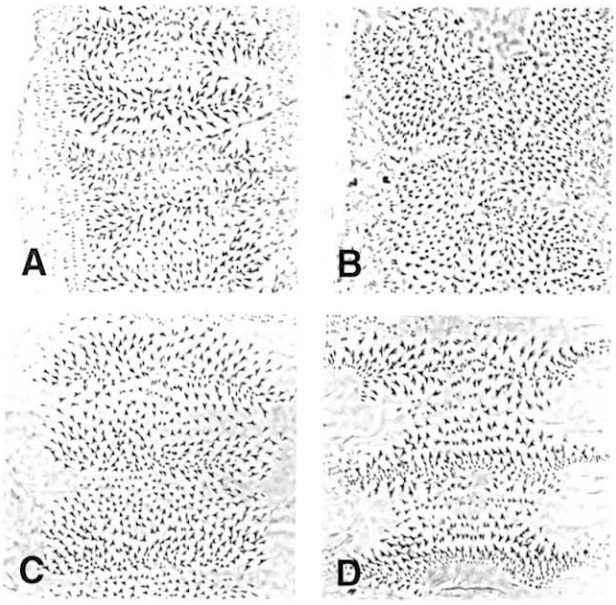

Fig. 1. Ventral cuticular patterns of (A) *wg*, (B) *hh*, (C) ci^D and (D) *fu* pharate larvae viewed under phase contrast illumination. Note the absence of naked cuticle, characteristic of the posterior half of each segment in wild-type larvae. In each case, the entire segment is covered by denticles; however, in *fu* and ci^D, there is greater diversity of denticle type than in *wg* and *hh*.

and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993) . Transcription of *hh* is coincident with *en* (Tabata et al., 1992) and expression of *wg* in neighbouring cells decays during stage 9 in *hh* mutant embryos (Hidalgo and Ingham, 1990; Ingham and Hidalgo, 1993). Genetic analysis suggests that the *hh* signal is transduced in an unusual manner, acting in some way to antagonise the activity of the segment polarity gene *patched* (*ptc*), which functions to repress the transcription of *wg* (Ingham and Hidalgo, 1993; Ingham, 1991).

The consequences of removing either *wg* or *hh* activity as revealed by their terminal cuticular mutant phenotypes are superficially very similar: in both cases, the precise segmental pattern of anterior denticle rows and posterior smooth or naked cuticle seen on the ventral surface of the newly hatched larva is replaced by a fairly homogeneous lawn of denticles (Baker, 1988; Jürgens et al., 1984; Nüsslein-Volhard et al., 1984; Mohler, 1988; see Fig. 1 for details). Given that *hh* mutants lack *wg* expression, this similarity is not unexpected; moreover, the *hh* mutant phenotype is almost totally suppressed by the restoration of *wg* expression that occurs in embryos doubly mutant for *hh* and *ptc* (Ingham et al., 1991). Yet while *wg* is required for the maintenance of *en* transcription along the entire parasegment boundary, in *hh* mutant embryos *en* expression persists in certain regions of the border (DiNardo et al., 1988). This finding suggests that the regulation of *wg* transcription by *hh* commences only *after* the expression of *en* has become *wg*-independent. That *en* expression does eventually decay in *hh* mutants implies a seperate role for *hh* in the maintenance of *en* and helps explain the similarity between the terminal phenotypes of *wg* and *hh* mutants.

If maintenance of *wg* transcription and maintenance of *en* in a specific sub-set of *en*-expressing cells are two distinct and independent functions of *hh*, we reasoned that mutations in genes downstream of the *hh* signalling pathway, responsible for maintenance of *wg* transcription, should abolish *wg* transcription without eliminating *en* expression. Amongst the known segment polarity mutations we have identified two in particular, *fused* (*fu*; Limbourg-Bouchon et al., 1991; Martinez-Arias, 1985) and *cubitus interruptus Dominant* (ci^D; Orenic et al., 1990), that fulfil this criterion for components of the *hh-wg* pathway.

Since the maintenance of *ptc* transcription in narrow stripes of cells that flank each *en* domain has previously been shown to require *hh* activity (Hidalgo and Ingham, 1990), we have also compared the effects of *hh*, *fu* and ci^D mutations on *ptc* transcription. The results suggest that transcriptional control of both *ptc* and *wg* by *hh* is mediated by the same signal transduction pathway.

RESULTS

Distribution of the Hedgehog protein is consistent with a role in cell-cell signalling

Like its transcript, the protein product of the *hh* gene is distributed in a series of stripes located around the anterior boundaries of each parasegment (Fig. 2B). Whereas the *hh* transcription domain occupies about one quarter the width of each parasegment, coinciding with the domain of expression of *en* (Tabata et al., 1992), Hh protein, however, appears to be more widely distributed than the En protein (compare Fig. 2A and B) (Taylor et al., 1993). The protein is first clearly detectable some time after the onset of gastrulation (stage 8) with levels peaking during stage 10 and becoming almost undetectable again by stage 12 (Taylor et al., 1993). This expression profile is consistent with the known requirements for *hh* function, expression of *wg* disappearing in *hh* mutant embryos by stage 10 (Ingham and Hidalgo, 1993).

At the sub-cellular level, Hh protein displays a distinctive non-uniform distribution; antibody staining is excluded from the apical region of ectodermal cells (see Fig. 2C) and the protein has a "capped" appearance accumulating in discrete patches which are almost invariably juxtaposed to similar accumulations in adjacent cells (Fig. 2D). In addition, the protein appears to accumulate in "dots", similar to those previously seen with Wg-specific antibodies (Gonzalez et al., 1991; van den Heuvel et al., 1989). This is reflected in the highly particulate appearance of the staining in whole embryos.

fused and ci^D are required for normal *wg* transcription

In wild-type embryos *wg* is activated during the blastoderm stage in dorsoventrally continuous stripes at the posterior margin of each parasegment; this expression is maintained until the beginning of stage 10 when lateral expression is lost so that each parasegment has separate dorsal and ventral stripes of *wg*-expressing cells (see Fig. 3). This later phase of *wg* expression in the ventral ectodermal cells has been shown to be specifically required for the differentiation of

naked cuticle in the posterior part of each segment (Bejsovec and Martinez Arías, 1991; Dougan and DiNardo, 1992). Embryos mutant for the segment polarity genes ci^D and *fu* display cuticular phenotypes similar to that resulting from the late loss of *wg* activity (see Fig. 1). In ci^{DR50} homozygous embryos, a reduction in the level of *wg* expression can first be seen at stage 8, and by the end of stage 9 ectodermal expression in the trunk region of the embryo is lost from all but 6 neuroblasts in each parasegment (see Fig. 3). As in wild-type embryos, this neuroblast expression fades as embryogenesis proceeds. During stage 10, ectodermal expression is activated in segmental patches along the dorsal edge of the embryo. Such dorsal activation is also observed in *hh* mutants (Ingham and Hidalgo, 1993) but in both ci^D and *hh* mutants this expression never reaches the levels observed in wild-type embryos and is lost during dorsal closure.

We have also re-examined the expression pattern of *wg* in embryos lacking wild-type *fu* activity, first reported by Limbourg-Bouchon et al. (1991). Complete loss of *fu* activity is lethal, and some alleles cause ovarian tumours and therefore female sterility. Accordingly, we used females of the adult viable genotype fu^1/fu^{V22} that have non-tumorous ovaries and crossed these to fu^1 males to generate embryos with reduced *fu* activity (hereafter referred to as fu^- embryos). The pattern of *wg* transcription in these embryos is essentially identical to that seen in ci^D and *hh* homozygotes (see Fig. 3).

fused and ci^D act downstream of *patched* to regulate *wg* transcription

Previous studies have shown that in *ptc* mutant embryos *wg* transcription is *hh* independent, leading to the notion that *hh* acts by antagonising *ptc* activity in some way (Ingham and Hidalgo, 1993; Ingham et al., 1991). A *ptc* mutation can therefore be thought of as being equivalent to a *hh* gain of function mutation, at least with respect to its effect on the regulation of *wg* transcription. Accordingly, if *fu* and ci^D are required downstream of *hh* they should both be epistatic to *ptc*. To investigate this possibility, we constructed fu^- *ptc* and *ptc* ci^D double mutants and assayed them for *wg* expression. In both $ptc^{G12};ci^{DR50}$ homozygous embryos and in fu^- embryos homozygous for ptc^{G12} the expression of *wg* is indistinguishable from that observed in fu^- or ci^{DR50} single mutant embryos (Fig. 4); similarly, the cuticular phenotypes of the double mutants are identical to those of the single mutants alone (data not shown). Thus both genes are required for the maintenance of *wg* expression after stage 9, irrespective of the presence or absence of *ptc*, indicating that they act downstream of *ptc* and, by extension, *hh*.

Cis-acting control elements drive *ptc* expression specifically in cells flanking the *hedgehog* domain

Although the spatial distribution of *ptc* transcript is initially quite distinct from that of *wg* (Hooper and Scott, 1989; Nakano et al., 1989), by stage 10 of embryogenesis the two genes share the characteristic of being expressed in cells adjacent to those expressing *en*, and hence *hh*. Whereas expression of *wg* is confined to cells anterior to each *hh* domain, *ptc* is transcribed in stripes of cells flanking each *hh* stripe (see Fig. 5). This results from repression of transcription in the middle of each broad stripe of *ptc*-expressing cells, and the maintenance and further activation of transcription in cells that are adjacent to those expressing *en* (Fig. 5)

Using promoter deletion analysis, we have separated regions of the *ptc* 5′ DNA that are required for this later expression, from those required for the initial pattern of broad stripes (Y. N., A. J. F. and P. W. I., unpublished observations). Reporter genes containing 12 kb of *ptc* upstream sequence give an expression pattern which is indistinguishable from that of the endogenous gene from gastrulation onwards. Broad segmental stripes appear during stage 8 which then resolve into two narrow stripes showing dorsoventral modulation typical of *ptc* (Fig. 5B,C). By contrast, smaller constructs containing 3.2 kb or 2.5 kb of upstream sequences show only the later part of this expression pattern. In wild-type embryos these small constructs are first activated in segmental pairs of narrow stripes from early stage 10 onwards (Fig. 5D). These narrow stripes are maintained through germband extension although they become less well defined during stage 11 due to dorsoventral modulation of expression within each stripe. Thus 2.5 kb of *cis*-acting sequences are sufficient to activate transcription in cells flanking the *hh* domain.

Maintenance of transcription adjacent to the *hh* domain requires *hh* activity

In the absence of *hh* activity *ptc* transcription fails to be maintained or activated in the cells bordering those expressing *en* and *hh* (Hidalgo and Ingham, 1990). In embryos homozygous for the strong loss of function allele, hh^{IJ}, this is first obvious at stage 10. In wild-type embryos at this stage, expression within the broad stripe has faded sufficiently dorsally for the maintenance of pairs of narrow stripes to be observed at the dorsal edge of the embryo (Fig. 6A). These narrow stripes do not however appear in *hh* mutants (Fig. 6B). Similarly, expression of the 3.2 and 2.5 kb reporter constructs fails to be activated in *hh* mutants (data not shown).

The disappearance of *ptc* transcription in *hh* mutants occurs with the same time course, and in the same pattern as the disappearance of midstripe expression in wild-type embryos. Expression is lost in a dorsal to ventral direction, leaving only a ventral posterior triangle of transcription in stage 11 embryos. This corresponds to the region of strongest expression within the broad stripes of stage 9 embryos, and is the last region of midstripe expression to be lost in wild-type embryos.

During stage 11 in *hh* mutants, *ptc* transcription is reactivated transiently in the tracheal placodes. This seems to correspond to the strong dorsal patch of activation, observed in the anterior narrow stripe in wild-type embryos. In *hh* mutants this activity and the remaining ventral posterior expression is completely repressed by stage 12 when all ectodermal staining has disappeared. Mesodermal expression of *ptc* appears to be independent of *hh* activity as transcription is activated normally during germband contraction in *hh* mutant embryos.

Transcriptional control of *ptc* is mediated by *fu* and ci^D

During early extended germband (stage 9-10) the pattern of

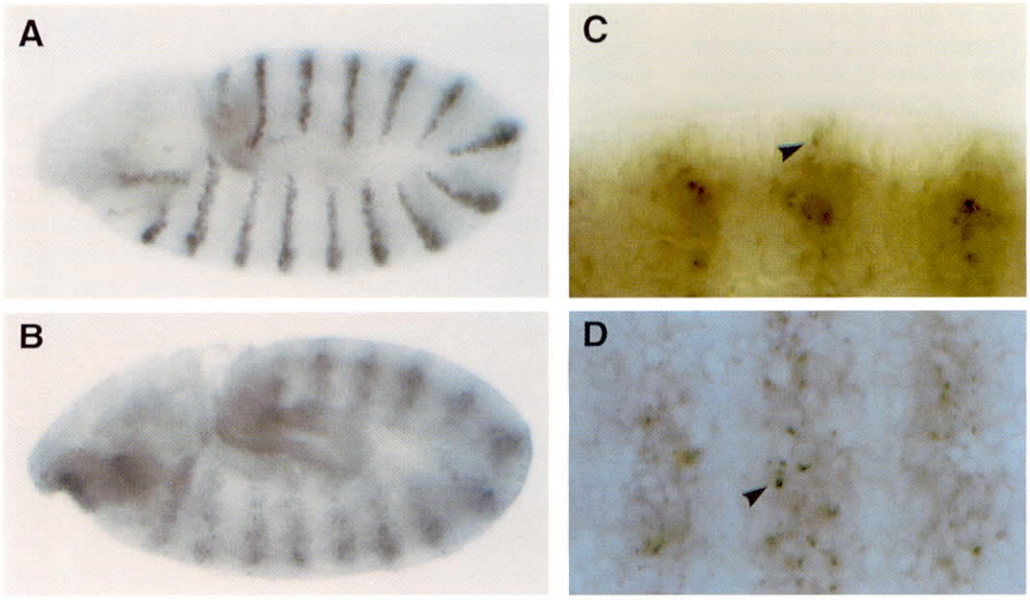

Fig. 2. Distribution of En (A) and Hh (B) protein in early stage 10 embryos. (C) Optical transverse section of a stage 10 embryo stained with an Hh antibody showing the subapical restriction of the protein (arrowhead). (D) View along the ventral midline of three adjacent parasegments of a stage 10 embryo stained with Hh antibody showing the "dotty" and "capped" (arrowhead) distribution of the protein.

ptc expression in *fu*$^{-}$ embryos is very similar to that observed in *hh*^{1J} mutants. Narrow stripes fail to be activated dorsally as the broad stripes of expression start to disappear (Fig. 6C). Fading of the broad stripes in *fu*$^{-}$ embryos continues during stages 10-11 and, as in *hh*^{1J}, the strong ventral-posterior patch of expression remains and activation takes place around the forming tracheal pits. However, in contrast to *hh*^{1J} mutants, during stage 11 expression also begins to be activated dorsally and anterioventrally, in a pattern that resembles wild-type embryos at this stage. Although this activation is weaker than in wild-type, by the time the germband starts to contract narrow stripes of expression can be distinguished bordering the *en* domains. As the germband contracts these narrow stripes of low level expression are maintained with even weaker expression throughout the broad domain between them.

In *ci*^{DR50} homozygous embryos, the pattern of *ptc* expression in early extended germ bands similarly resembles that observed in *hh* mutants. As the broad stripes of *ptc* expression fade, transcription fails to be maintained or activated in the cells bordering the *hh/en* domain (Fig. 6D). Expression is lost completely dorsally but remains at low levels throughout the broad stripe ventrally (see Fig. 6). During stage 11, *ptc* transcription is activated around the tracheal placodes but in contrast to *hh* mutants, it is also reactivated at low levels in broad segmental stripes that

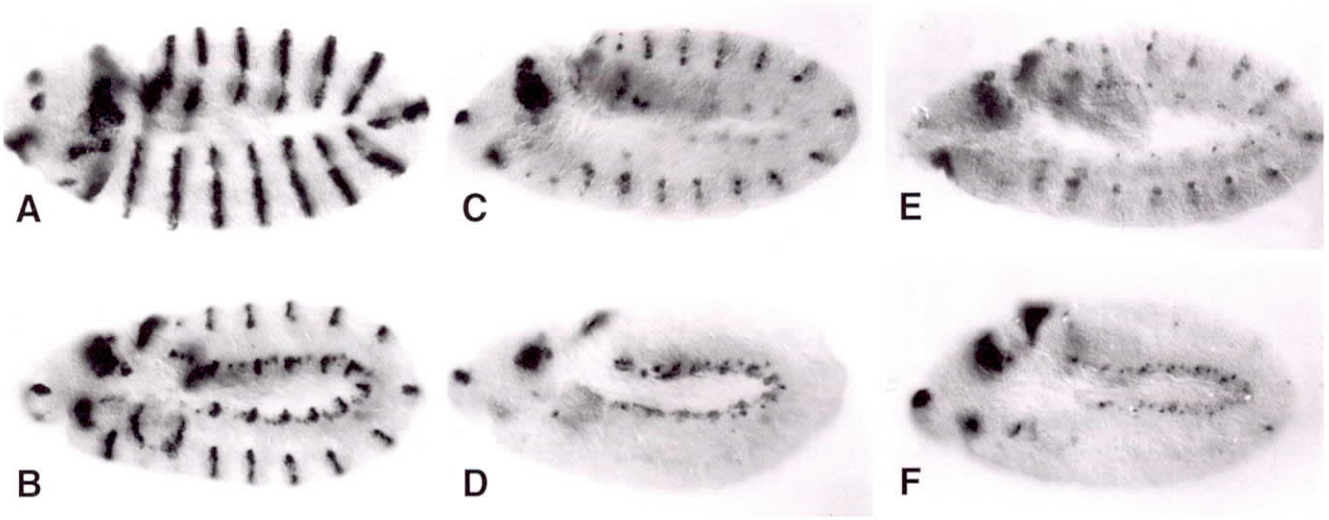

Fig. 3. *wg* expression in wild-type (A,B), *ci*^{DR50} (C,D) and *fu*^{1}/*fu*^{v22}(E,F) embryos. (Upper panels) Stage 9 embryos hybridised with a *wg*-specific digoxigenin-labeled RNA probe, lower panels are stage 11 embryos similarly stained. In wild-type embryos during stage 10 the dorsoventrally continuous stripes of *wg* expression disappear and are replaced by a dorsal patch and ventral stripe within each segment (A and B). In the absence of either *ci*^{D} or *fu* activity, *wg* expression disappears from the ectoderm during stage 9, expression remaining in 6 neuroblasts within each segment (C,E). By stage 11 this neuroblast staining is lost and *wg* expression is completely absent ventrally but it is activated in patches around the dorsal edge of the embryo (D and F).

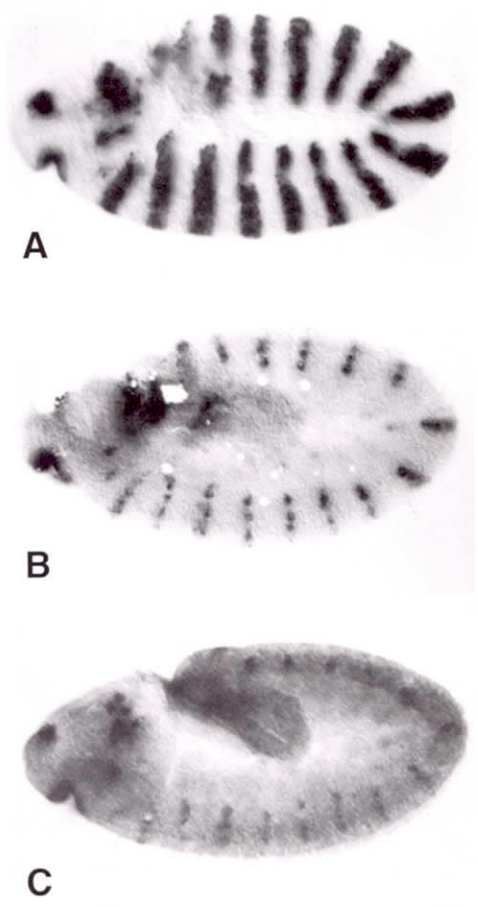

Fig. 4. *wg* expression in *ptc*, *fu;ptc* and *ptc;ci*^{D} embryos. (A) A stage 9 homozygous *ptc*^{G12} embryo hybridised with a *wg*-specific RNA probe. In the absence of *ptc* activity the *wg* expression domain broadens to fill about half the segment. (B,C) Stage 10 *fu*^{1}/*fu*^{V22};*ptc*^{G12} and *ptc*^{G12};*ci*^{DR50} embryos, respectively; in both cases, *wg* expression is lost from the ectoderm in the same way as in *ci*^{D} and *fu* single mutants (Fig. 3).

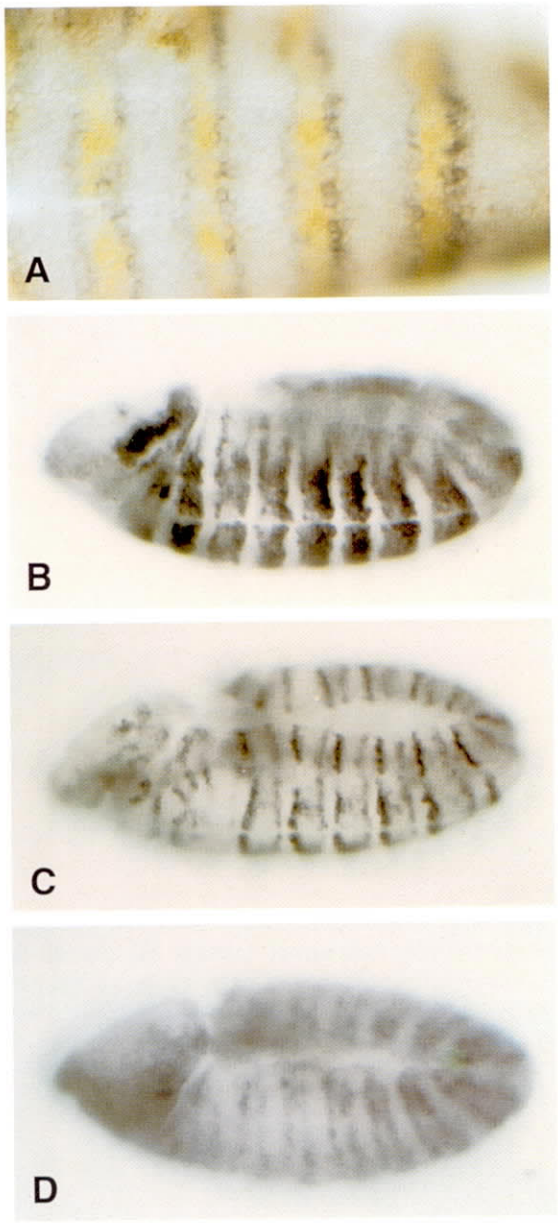

Fig. 5. (A) Ventral view of a stage 11 embryo hybridised with *ptc* DIG-labelled probe (blue) and subsequently stained with En antibody (brown) showing that expression of *ptc* is activated and maintained only in cells bordering the En domain by this stage. B and C show the expression of the 12.5 kb upstream *ptc-lacZ* fusion construct in early stage 10 and stage 11 embryos respectively. This construct is activated in broad segmental stripes after gastrulation (B) which then resolve into narrow stripes flanking the En domains during stages 10-11. (D) Expression of the 2.5 kb upstream *ptc-lacZ* fusion construct in an embryo at the same stage as that shown in B. This construct is never expressed in broad segmental stripes but is activated in pairs of narrow stripes during early stage 10.

exclude only the *en* domain. These stripes are modulated dorsoventrally such that expression is lowest laterally; however, no signs of increased levels of transcription in cells adjacent to the *en* domain are observed. These broad stripes of weak expression are maintained throughout germ band contraction. Loss of *ci*^{D} activity also has an effect on mesodermal expression of *ptc*, the transcript being present at abnormally high levels from stage 11 through germband contraction.

ci^{D} acts at the level of transcription to regulate *wg* and *ptc* expression

To confirm that the changes in *ptc* and *wg* transcript accumulation seen in *ci*^{D} mutant embryos reflect changes at the level of transcriptional control, we analysed the effects of the *ci*^{DR50} mutation on the expression of *lacZ* in two enhancer trap lines in which the reporter gene is under the control of enhancers of either *ptc* or *wg*. Expression of the *wg-lacZ* enhancer trap fades from the ectoderm during stage 8-9 leaving only neuroblast expression, which in turn disappears (Fig. 7); expression is then activated at the dorsal edge of the embryo in the same pattern as the endogenous gene at stage 10. Similarly, expression of the *ptc-lacZ*

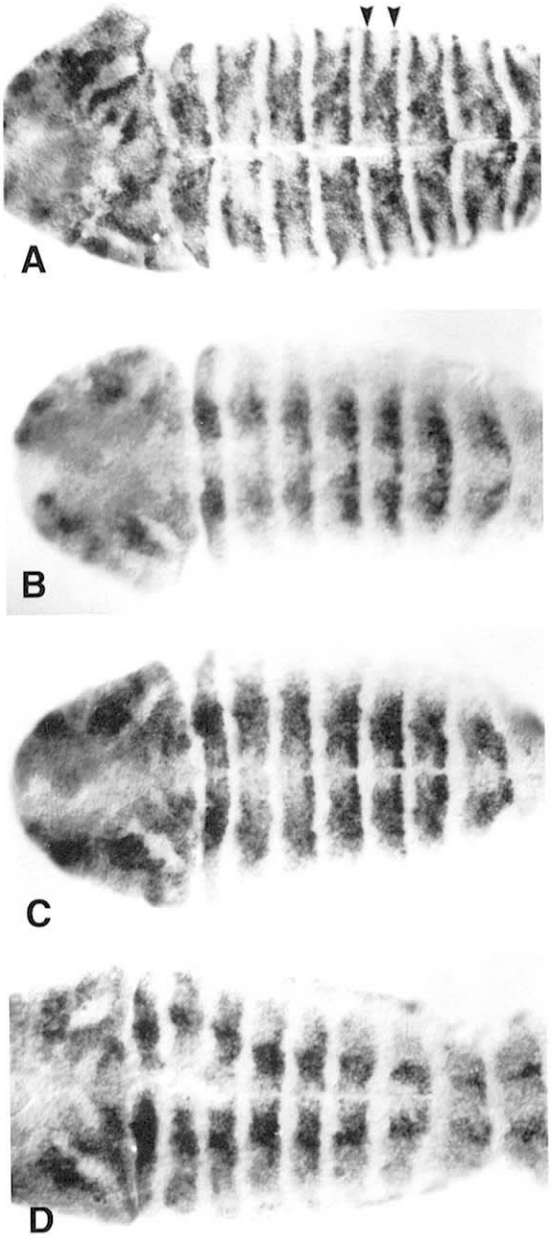

Fig. 6. (A) Ventral view of a wild-type embryo hybridised with *ptc*-specific DIG-labeled RNA probe. The repression of *ptc* expression within the broad stripes, and its maintenance in pairs of narrow stripes is first observed in early stage 10 embryos. Midstripe expression disappears first dorsally and expression remains only in cells bordering the *en* domain; two such stripes are indicated by arrowheads. (B-D) Similar aspects of *hh*, *fu* and ci^{D} embryos respectively at the same developmental stage. In all three mutants *ptc* fails to be maintained or activated in the narrow stripes of cells characteristic of wild-type.

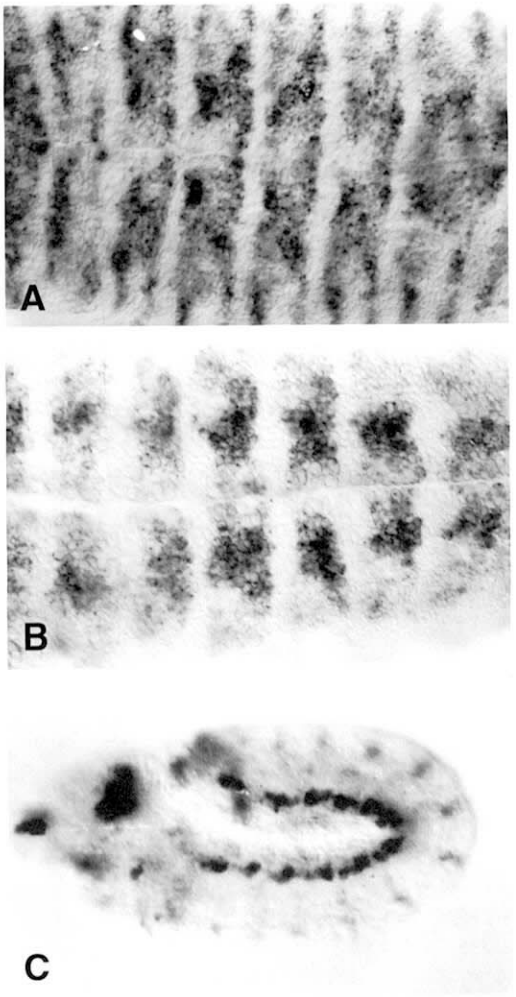

Fig. 7. Expression of *wg-lacZ* and *ptc-lacZ* in ci^{D} mutants. (A,B) Ventral views of *ptc-lacZ* expression in stage 10 wild-type and ci^{DR50} embryos respectively; (C) *wg-lacZ* expression in a stage 10 ci^{D} mutant. In the absence of ci^{D} activity both *wg-lacZ* and *ptc-lacZ* are expressed in the same patterns as the endogenous genes. *wg-lacZ* expression disappears from the ventral ectoderm by stage 9 (A); expression of *ptc-lacZ* is not maintained in stripes bordering the *en* domain in extended germband embryos (C).

reporter line H84 fails to be maintained in the cells bordering the *en* domain as the broad stripes of expression fade in stage 9-10 embryos (Fig. 7) and during stage 11 low level activation occurs throughout the non-*en*-expressing region of the segment. Like the endogenous gene, the *ptc-lacZ* reporter is also expressed at elevated levels in the mesoderm during germband contraction.

The *cos-2* gene negatively regulates *ptc* and *wg* transcription

Embryos lacking both maternal and zygotic wild-type activity of the *costal-2* (*cos-2*) gene develop a larval cuticular phenotype that resembles that of *ptc* mutant larvae (Grau and Simpson, 1987). In both cases the denticle rows in the posterior part of each ventral belt are eliminated and replaced with denticles of more anterior character. In contrast to *ptc* mutants, however, there is no duplication of the segment boundary or of the anterior row of small denticles in *cos-2* mutant larvae and no reversal of polarity of the remaining large denticles.

To investigate whether the phenotypic similarity between *cos-2* and *ptc* mutations is based on similar effects

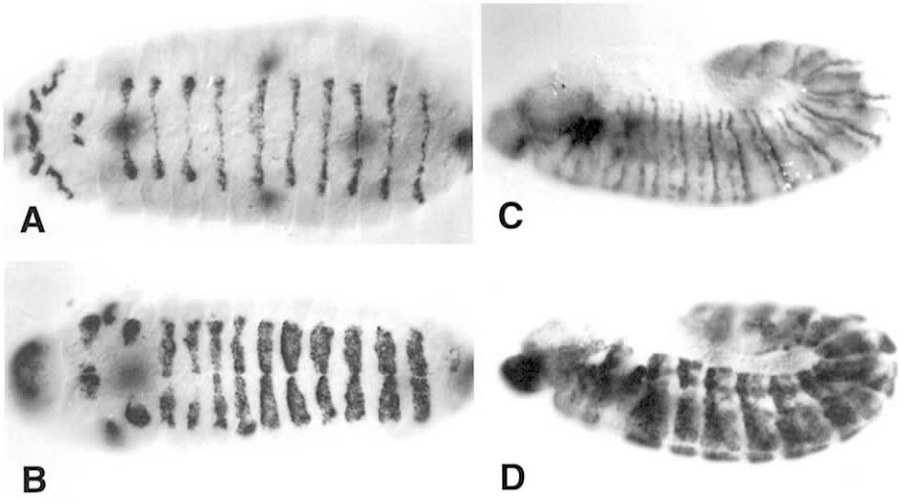

Fig. 8. The expression of *wg* and *ptc* in embryos derived from *cos-2* mutant germlines. (A,B) Ventral views of stage 13 embryos heterozygous and homozygous for *cos-2*^{5} respectively. Both embryos came from the same mother with a mutant germline and were hybridised with a *wg* DIG-labeled RNA probe. When *cos-2* activity is removed from both germline and embryo the domain of *wg* expression broadens anteriorly (B); this occurs during germband retraction. (C,D) Stage 12 embryos hybridised with a *ptc* probe. The embryo in C is heterozygous for cos-2 from a mutant germ line, that in D is homozygous. Loss of *cos-2* activity results in the failure of *ptc* repression. As a consequence *ptc* is overexpressed throughout the segment, excluding the *en* domain.

at the cellular level we analysed the expression of *ptc* and *wg* in *cos-2* mutant embryos. Females with *cos-2* mutant germ lines were generated by pole cell transplantation and crossed to males heterozygous for the *cos-2*^{5} allele. By germband extension (stage 9) the level of *ptc* transcript in *cos-2*$^{-}$ embryos as judged by in situ hybridisation is much higher than in their heterozygous siblings; each broad stripe of *ptc* expression persists through stage 12, failing to resolve into the two narrow stripes that characterise wild-type embryos at this stage (Fig. 8). Both these effects resemble the changes in the pattern of *ptc* transcription seen in embryos that lack a functional copy of the *ptc* gene itself (Hidalgo and Ingham, 1990). There are, however, some subtle differences between *ptc* and *cos-2*$^{-}$ embryos. In the absence of *ptc* activity the broad stripes of *ptc* expression do eventually split to give a narrow stripe of non-expression in the middle of the segment. It has been suggested that this is due to the repression of *ptc* in cells expressing *en* ectopically (Hidalgo and Ingham, 1990). In *cos-2* embryos in which there is no ectopic *en* expression (Forbes, 1992), such splitting of the *ptc* expression domain is not observed.

In *cos-2*$^{-}$ embryos there is also a dorsoventral modulation in the intensity of *ptc* expression not observed in *ptc* embryos. This modulation is first apparent in the broad stripes in stage 9 extended germbands. Expression fades laterally during stages 10 and 11, then as the germband contracts two regions of strong expression, both at the anterior of the broad stripe become clear in the ventral-lateral, and dorsal-lateral regions of each stripe. As in the wild-type *ptc* pattern, expression from the late extended germband (stage 11) to the end of development is strongest at the anterior of the segment i.e.: adjacent to the anterior segment boundary.

In contrast to the early effects on *ptc* transcription, expression of *wg* initially appears normal in *cos-2*$^{-}$ embryos. At stage 11, however, the *wg* domain becomes significantly broader than in wild type (Fig. 8). This ectopic expression of *wg* persists ventrally until the end of embryogenesis. By contrast, expression of *wg* fades dorsally during dorsal closure (stage 13-14).

The broadened expression of *wg* in *cos-2*$^{-}$ embryos from stage 11 is similar to the pattern of *wg* observed in *ptc* mutants; in both cases the ventral expression domain of *wg* expands to fill about half of each segment however, in *cos-2*$^{-}$ embryos this change in *wg* expression occurs much later than in *ptc* mutants in which the *wg* domains broaden in stage 9 embryos. The irregularity of some of the abdominal *wg* stripes in *ptc* mutants compared to *cos-2* may partly be due to the ectopic segment boundaries which form in the former, but not in the latter mutant embryos. A further difference between these mutants is in the late maintenance of the dorsal domain of *wg* expression; dorsal expression fades in *cos-2*$^{-}$ embryos, while in *ptc* mutants, as in wild type, *wg* continues to be expressed dorsally until the end of embryogenesis.

DISCUSSION

The *wg* gene has at least two temporally distinct functions in the development of each parasegment, acting first to consolidate the parasegment boundaries by maintaining *en* expression (DiNardo et al., 1988; Martinez Arias et al., 1988) and subsequently regulating the differentiation of individual cells such that they secrete naked cuticle rather than denticles (Bejsovec and Martinez Arías, 1991; Dougan and DiNardo, 1992). A particular level of *wg* activity appears to be required to specify naked cuticle: in its absence, cells of the ventral ectoderm produce a lawn of denticles, whereas if *wg* is ectopically expressed either under the control of a heat shock promoter (Nordemeer et al., 1992), or through the effects of other mutations such as *naked* and *ptc* (Dougan and DiNardo, 1992; Martinez Arias et al., 1988), denticle differentiation is supressed. The maintenance of *wg* in a single narrow stripe therefore seems essential for determining the proportion of each parasegment that will secrete naked cuticle. Here, we have

presented evidence that the ci^D and *fu* genes are specifically required to maintain *wg* expression after *en* has become independent of the *wg* signal, and suggest that they do so by transducing the *hh*-encoded signal.

The transcription pattern of *wg* in *fu* and ci^D mutant embryos is essentially indistinguishable from that seen in *hh* mutants. In each case *wg* expression is lost from the ectoderm during stage 9. Since the maintenance of *wg* at this stage is known to depend upon *wg* activity, it could be that both *fu* and ci^D act downstream of the *wg* signal, perhaps regulating the transcription or activity of *en*, which in turn feeds back on *wg* transcription via the activity of *hh*. Two considerations argue against this possibility: first, the phenotypic consequences of *fu* and ci^D mutations are significantly less severe than those caused by loss of *wg* function; second, the expression of En protein in the stage 10 embryo is relatively unaffected by the absence of activity of either *fu* (Limbourg-Bouchon et al., 1991) or ci^D (A. J. F. and P. W. I., unpublished observations).

Of course the possibility remains that either gene might post-translationally modulate the activity of *en*, thus influencing its ability to regulate the expression of the putative signal (Limbourg-Bouchon et al., 1991). Although we have not analysed the expression of *hh* in either mutant it is clear from the results of our epistasis analysis that both genes act downstream of the *hh* signal. It has been proposed previously that *hh* acts by antagonising the activity of *ptc* (Ingham et al., 1991) and since *ptc* encodes an integral membrane protein (Hooper and Scott, 1989; Nakano et al., 1989), one implication being that it acts as a receptor for the *hh* signal. In embryos doubly mutant for *ptc* and *hh*, *wg* is ectopically expressed (Ingham and Hidalgo, 1993), indicating that in the absence of *ptc*, *wg* transcription becomes independent of the signal. By contrast, *wg* transcription ceases at late stage 9 in both *ptc;*ci^D and *fu;ptc* embryos. Thus *fu* and ci^D are required for *wg* expression irrespective of the activity of either *ptc* or *hh*, implying that both genes act as downstream components of the *hh* signalling pathway.

Since *en* expression is largely unaffected in stage 10 ci^D and *fu* embryos, the loss of *en* expression observed in *hh* embryos at the same stage (DiNardo et al., 1988) implies an additional role for *hh* that must be mediated by a different pathway. Several other lines of evidence have suggested that regulation of *wg* expression is not the only role of *hh*; for instance, the *hh* cuticular phenotype is stronger than that of *wg* (Fig. 1); the cuticular phenotype of *ptc;hh* more closely resembles that of *ptc en* than of *ptc* alone (Hidalgo, 1991); and ubiquitous expression of *wg*, driven by a *hs-wg* construct, while able to partially rescue *wg* mutants fails to rescue the *hh* mutant phenotype (Sampedro et al., 1993).

Previous studies have implicated *hh* in the maintenance of *ptc* transcription, in addition to its roles in regulating *wg* and *en* expression, in the stage 10 embryo. In normal development, the pattern of *ptc* transcription resolves from a broad stripe, occupying the posterior three quarters of each parasegment, into two narrow stripes of cells that flank the *hh*-expression domain (Hooper and Scott, 1989; Nakano et al., 1989). Deletion analysis of the *ptc* 5′ region has allowed us to separate regions of DNA responsible for later Ci^D-dependent expression in segmental pairs of narrow stripes from those necessary for early expression in broad stripes. While a fragment containing 12 kb 5′ of the *ptc* transcription start site gives the complete post-blastoderm pattern of expression, smaller fragments of 3.2 kb and 2.5 kb direct expression only in pairs of narrow stripes flanking the *hh* domain in extended germband embryos. That this symmetrical expression of *ptc* depends upon the activity of *hh* suggests that the *hh* signal is not polarised, but rather that the anterior and posterior cells have differing competences to respond to the same signal. Since the activity of *ptc* represses its own transcription as well as that of *wg* (Hidalgo and Ingham, 1990), the maintenance of *ptc* transcription by *hh* is probably mediated in a similar manner, *hh* antagonising the activity of *ptc* in responding cells. Thus, the difference in competence between the two cells expressing *wg* and those expressing *ptc* seems unlikely to be at the level of signal reception. Another possibility is the existence of distinct signal transduction pathways such that the *hh* signal can elicit different outcomes in different cells; for instance one such pathway might operate in cells anterior to each *hh* domain, transducing the signal to activate *wg* transcription, whilst a distinct pathway could mediate the activation of *ptc* transcription in cells on either side of *hh*-expressing cells. Our finding that both *fu* and ci^D are required for the activation of *ptc* transcription in cells flanking the *hh* domain, strongly suggests, however, that the same factors transduce the *hh* signal to regulate both *wg* and *ptc* transcription.

The molecular structures of both *fu* and ci^D fit well with their proposed roles as intracellular transducers of the *hh* signal. ci^D encodes a zinc finger protein homologous to the mammalian proto-oncogene GLI (Orenic et al., 1990) suggesting that it may regulate *ptc* and *wg* expression at the level of transcriptional control. This interpretation is supported by our finding that the effects of the ci^D mutation on expression of *wg-lacZ* and *ptc-lacZ* reporter genes mirror those on the endogenous genes. Thus ci^D is exerting its control via the *cis*-acting regulatory regions of both genes, suggesting that the Ci^D protein binds directly upstream of the *wg* and *ptc* promoters to activate transcription. At present nothing is known about the distribution of the Ci^D protein during embryogenesis; however transcription of ci^D is not limited to those cells in which it activates *wg* and *ptc* transcription but occurs throughout most of each parasegment, in all cells except those expressing *en*. If the Ci^D protein is similarly distributed, it follows that its activity must be regulated at the post-translational level. In this regard it is interesting that the product of the *fu* gene is a serine threonine kinase (Preat et al., 1990), raising the possibility that ci^D activity may be modulated by phosphorylation.

In embryos lacking wild-type activity of the *cos-2* gene we have found an effect on *wg* and *ptc* expression reciprocal to that seen in *hh*, *fu* and ci^D mutants. As in *ptc* mutant embryos, *wg* and *ptc* are both ectopically expressed when *cos-2* activity is reduced; in contrast to *ptc* mutants, however, expression of *en* is unaffected. Thus *cos-2* most likely acts downstream of *ptc* to repress the transcription of both *wg* and *ptc*. If, as we have suggested, *fu* activates Ci^D by phosphorylation, one possible role for *cos-2* could be to inhibit this activation, by interacting either with Fu or with Ci^D itself. In the former case, *cos-2* might simply inactivate Fu. Alternatively, *cos-2* might encode a phosphatase whose

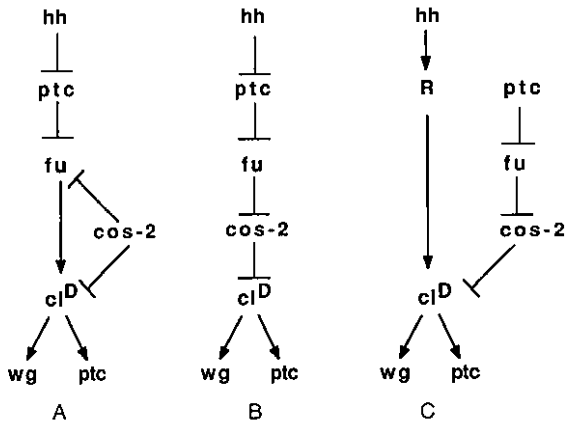

Fig. 9. Possible pathways mediating the regulation of *wg* and *ptc* transcription by *hh*. In the first two schemes (A and B), Hh is envisaged as repressing *ptc* activity, perhaps by binding directly to the Ptc protein. Activity of the latter in turn inactivates the Fu protein. Fu may function to activate Ci^D directly, perhaps by phosphorylation (A); in this case the role of *cos-2* could be to reverse this activation, perhaps by dephosphorylating Ci^D or by negatively regulating Fu itself. Alternatively (B), Fu could inactivate *cos-2*, which in turn may repress ci^D activity. In the third scenario (C), we envisage Ptc as acting in parallel to Hh, attenuating the activity of Ci^D protein via *fu* and *cos-2* . In this case *hh* would act to counterbalance the effects of *ptc*, stimulating the activity of Ci^D via an as yet unidentified receptor and intracellular transduction pathway. As in the schemes shown in A and B, *hh* activity would be redundant in the absence of *ptc* activity (see also Hooper and Scott, 1992).

activity could reverse the phosphorylation of the Ci^D protein. It is, of course, equally possible that *cos-2* is itself the substrate for *fu* activity in a linear pathway of negative regulation leading from the cell surface to the nucleus (Fig. 9). According to this scheme the function of *ptc* would be to inhibit the activity of Fu, whose activity in turn inhibits the activity of Cos-2. The latter might repress *ptc* and *wg* transcription by inactivating the Ci^D protein or perhaps sequestering it, preventing its entry into the nucleus.

An analagous pathway of negative interactions between the genes that regulate sex determination in *C. elegans* has previously been proposed on the basis of the results of genetic analysis (Kuwabara and Kimble, 1992). Intriguingly, not only are there similarities between the two pathways at the genetic level, but also in the molecular structures of some of the gene products involved. Thus the nematode *her 1* gene, like *hh*, encodes a product with the characteristics of a secreted protein (Kuwabara and Kimble, 1992); *tra-2* encodes an integral membrane protein with multiple transmembrane domains showing gross topological similarity, though only marginal sequence similarity, to *ptc* (Kuwabara et al., 1992). Finally, *tra*-1, the most downstream component of the nematode pathway, encodes a zinc finger protein with significant homology to ci^D (Zarkower and Hodgkin, 1992).

Although the different signalling pathways outlined in Fig. 9 are consistent with the results of our epistasis analysis, such analysis cannot distinguish between linear and parallel pathways. It is equally possibile that *hh* acts via an entirely separate pathway to activate Ci^D, independent of the *ptc/cos-2/fu* pathway. In this case the balance between activation and inactivation of Ci^D by these two parallel pathways would regulate the transcription of *wg* and *ptc*.

While the expression of both *wg* and *ptc* in embryos lacking wild-type *cos-2* activity resembles their expression in *ptc* mutants by stage 12, the ectopic activation of *wg* in the former is significantly delayed. Thus while *ptc* is expressed at elevated levels from stage 8 onwards, the domains of *wg* expression only broaden after stage 11. Since the *cos-2* allele that we have analysed is not a complete loss of function mutation, it may be that residual *cos-2* activity is able to maintain repression of *wg* during the early stages of embryogenesis. In terms of our models, this could be taken to mean that activation of *wg* transcription requires a higher threshold level of ci^D activity than does activation of *ptc*. In this regard, it is interesting that transcription of *ptc* is reinitiated in stage 12/13 embryos lacking wild-type *fu* activity. Like the $cos\text{-}2^5$ allele, the *fu* alleles that we have analysed necessarily cause only the partial inactivation of the *fu* gene. Thus it is possible that such mutant embryos retain sufficient activity to activate transcription of *ptc*, but not of *wg*. Again, in terms of our model, this would be consistent with different threshold levels of ci^D being necessary for the activation of the two genes. Why these threshold requirements should differ with developmental stage is unclear; however, it is almost certain that additional transcription factors co-operate with ci^D to regulate the expression of *ptc* and *wg* at different stages. The segment polarity gene *gooseberry*, for instance, is known to be required for transcription of *wg*, though not of *ptc* (Hidalgo and Ingham, 1990), in the stage 11 embryo. Clearly, the transcriptional control of both genes is likely to be complex; a detailed analysis of their *cis*-acting regulatory regions will be needed to provide a clearer understanding of this complexity.

Our analysis has focussed on the mechanisms controlling the expression of two segment polarity genes, *ptc* and *wg*, at a particular stage of embryogenesis. Both genes are subject to complex transcriptional regulation, a complexity reflected in their rapidly evolving patterns of expression in the developing embryo. In the case of *wg*, we know that these different controls underpin the multiple roles of *wg* as development proceeds. The functional significance of this later phase of *ptc* transcription is unclear but it suggests that *ptc* may also have late functions independent of its early role in regulating *wg* and *ptc* transcription. Differences between the patterns of *en* expression in *ptc* and *cos-2* mutants (Forbes, 1992) and in embryos in which *hh* is ectopically expressed (P.W.I., unpublished results) imply that *ptc* may have a role in limiting the extent of the *en* domain. The activty of *ptc* may have a repressive effect on the activation of *en* in response to *wg*, possibly by inhibiting the reception of the *wg* signal, or by altering the competence of cells to respond. Alternatively, *ptc* may act in some way to prevent *en*-expressing cells from mixing with their (*ptc*-expressing) neighbours during the cell movements of germband contraction, thus helping to maintain the integrity of the en domain. The expression of *ptc* on both sides of the *en* domain means it is in the correct intrasegmental location for both these suggested functions.

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