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Mechanisms of Development 51 (1995) 145–155

Differential requirements for segment polarity genes in *wingless* signaling

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Received 4 October 1994; accepted 7 December 1994

Abstract

The segment polarity genes *wingless* and *engrailed* are required throughout development of *Drosophila*. During early embryogenesis, these two genes are expressed in adjacent domains, in an inter-dependent way. Later, their expression is regulated by different mechanisms and becomes maintained by auto-regulation. To dissect the genetic requirements for the initial signaling between *wingless* and *engrailed* expressing cells, we have previously used a transgenic *Drosophila* strain that expresses *wingless* under the control of the heat shock promoter (HS-*wg*). Focusing on the later phases of *wingless* and *engrailed* regulation, we have now extended these studies, using embryos carrying various combinations of segment polarity mutations and the HS-*wg* transgene. We confirm some of the existing models of regulation of the expression of *wingless* and *engrailed*. In addition, we find that HS-*wg* embryos require *engrailed* for induction of ectopic endogenous *wingless* expression. Signaling from *engrailed* cells to this novel *wingless* expression domain is dependent on *hedgehog* but also on *porcupine*. We further demonstrate a novel requirement for *hedgehog* in maintenance of expression of *engrailed* itself.

Keywords: *Drosophila* embryogenesis; Segment polarity genes; *Wingless* gene expression; *Engrailed* gene

1. Introduction

A well studied example of pattern formation during embryogenesis is the establishment of the body plan of the fruit fly (reviewed by Lawrence, 1992; Bate and Martinez-Arias, 1993). After cellularization of the initial syncytium, epidermal cells become specified and organized in metameric units, the parasegments. The function of the segment polarity genes is essential in this process (Nüsslein-Volhard and Wieschaus, 1980). Their zygotic gene products are first detected at cellular blastoderm and encode diverse proteins: putative transcription factors, membrane associated proteins, protein kinases and secreted factors (reviewed by Hooper and Scott, 1992; Peifer and Bejsovec, 1992; Ingham and Martinez-Arias, 1992).

The products of the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*) are expressed on either side of the parasegmental border and are required for its formation (Dinardo et al., 1988; Martinez-Arias et al., 1988). After gastrulation, the expression of the two genes becomes inter-dependent (Heemskerk et al., 1991; Bejsovec and Martinez-Arias, 1991; Vincent and O'Farrell, 1992; Dougan and DiNardo, 1992). The secreted Wg protein is itself an important component of the signalling pathway maintaining *en* expression in the neighboring cell. Support for this comes from the observations that Wg protein can be detected in *en* cells (van den Heuvel et al., 1989; Gonzalez et al., 1991) and that Wg protein present in tissue culture cell medium is active in in vitro assays (Cumberledge and Krasnow, 1992; van Leeuwen et al., 1994).

The En homeodomain protein has been demonstrated to regulate transcription in vitro (Jaynes and O'Farrell, 1991) and is therefore unlikely to act directly in intercellular communication. The signal from the *en*-expressing cell to maintain *wg* is probably the Hedgehog (Hh) protein (Ingham et al., 1991; Ingham and Hidalgo, 1993;

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SSDI 0925-4773(95)00348-Q

Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992). Consistent with this are the observations that *wg* expression decays in a *hh* mutant embryo before *en* disappears, that *en* positively regulates *hh* (Tabata et al., 1992) and that the Hh protein can be detected in the adjacent *wg* expressing cells (Tabata and Kornberg, 1994).

Components of the *wg* signal transduction pathway have been identified among other segment polarity genes using epistasis analysis (Siegfried et al., 1994; Noordermeer et al., 1994; reviewed in Perrimon, 1994). These data together with other studies suggest a pathway in which *porcupine (porc)* is needed to secrete the Wg protein from the *wg* expressing cell (Van den Heuvel et al., 1993; Siegfried et al., 1994), while *dishevelled (dsh)* (Klingensmith et al., 1994; Theisen et al., 1994) and *armadillo (arm)* (Wieschaus and Riggleman, 1987; Riggleman et al., 1990; Peifer et al., 1990, 1994) are required for reception of the *wg* signal in the *en* cell. Transduction of the *wg* signal antagonizes the action of another component of this pathway, *zeste white3 (zw3)* (Siegfried et al., 1990, 1994; Bourouis et al., 1990), which mediates repression of *en* expression (Siegfried et al., 1992). Little is known about the biochemistry of these interactions.

Other studies have uncovered genes in the *hh* signalling pathway (reviewed in Perrimon, 1994). It was found that *hh* acts through, or parallel to, *patched (ptc)*, *fused*, *costal-2* and *Cubitus interruptus Dominant* to maintain *wg* (Forbes et al., 1993). The function of these genes in *hh* signaling is still poorly understood. However, Ptc, a putative transmembrane protein, whose activity can be antagonized by *hh*, acts as a constitutive repressor of *wg* (Ingham et al., 1991). It has been proposed that *hh* interacts with *ptc* at the cell surface (Taylor et al., 1993; Tabata and Kornberg, 1994). Another gene possibly acting in, or parallel to, the *hh* pathway to activate *wg* expression is *sloppy-paired (slp)* (Grossniklaus et al., 1992). By acting as a repressor of *en* and activator of *wg*, *slp* is involved in determining the competency of cells to express either *en* or *wg* (Cadigan et al., 1994).

Early in development the *hh/en* and *wg* signalling pathways are inter-dependent. Later, *en* expression enters a *wg*-independent auto regulatory phase (Heemskerk et al., 1991; Bejsovec and Martinez-Arias, 1991) and *wg* expression is maintained independently of *en* and *hh*. During this stage, *wg* expression is regulated by *gooseberry (gsb)*, presumably by a *wg/gsb*-auto regulatory loop (Li and Noll, 1993).

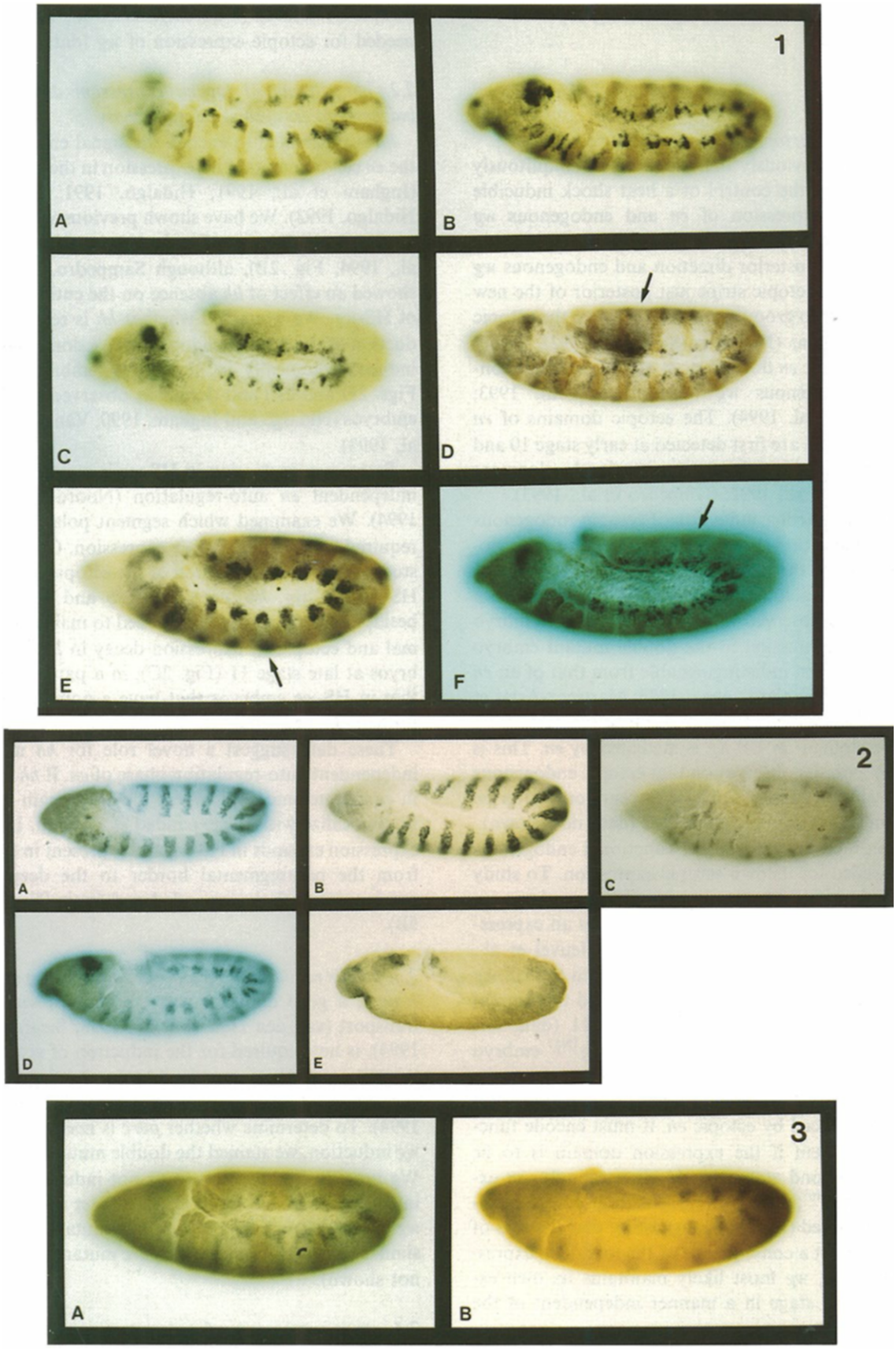
The mutual inter-dependence of *wg* and *en* expression has complicated the study of the role of a given gene product in the maintenance of either *wg* or *en* expression. We previously reported the use of a strain of flies that expresses *wg* under the control of a heat shock promoter (HS-*wg*; Noordermeer et al., 1992). Ubiquitous expression of *wg* by heat shock results in ectopic *en* expression whose initiation requires neither the endogenous *wg* nor *en* gene products (Sampedro et al, 1993, Noordermeer et al. 1994). It was therefore possible to determine which of several segment polarity gene products are specifically required to transduce the *wg* signal without the complication of the normal *en-wg* feedback loop (Noordermeer et al., 1994).

In addition to the expansion of the *en* domain following ubiquitous *wg* expression, endogenous *wg* is ectopically induced (Noordermeer et al., 1992). Here, we use HS-*wg* embryos to further investigate genetic requirements for *wg* and *en* regulation. We demonstrate that *slp* plays an essential role in the demarcation of the *en* and *wg* domains in HS-*wg*. Furthermore, *wg* ectopically activates *slp* expression, suggesting an interdependence of the two gene products. We show that besides a role for *hh* in *wg* activation, *hh* is also needed

Fig. 1. *En* and *wg* expression patterns in whole mount HS-*wg* stage 10/11 embryos in the absence of functional *en*, *wg*, *ptc* or *gsb*. The expression patterns of En protein and *wg* RNA are shown in wild type (A), HS-*wg* (B), *en*; HS-*wg* (C), wg^{IN67};HS-*wg* (D), *ptc*;HS-*wg* (E) and *gsb*; HS-*wg* (F) embryos. No ectopic endogenous *wg* expression is observed in HS-*wg* embryos that lack *en* expression (C), while in wg^{IN67};HS-*wg* is ectopically induced (D, the arrow indicates a segment with both the normal and ectopic *wg* stripe), but not maintained. In *ptc*;HS-*wg* *wg* is expressed in a broad domain, indicated by the arrow, in between two broad *en* stripes. In the absence of *gsb* expression *en* is induced ectopically (arrow) in HS-*wg*, while ectopic *wg* expression is induced but not maintained. Surface views of whole mount embryos are shown (anterior is to the left, dorsal is up). The embryos were double-labeled for *wg* RNA (in blue) and En protein (in red).

Fig. 2. *En* and *Wg* protein expression patterns in HS-*wg* embryos in the absence of *hh*. Embryos were double-labeled for En (blue) and β-galactosidase (brown) proteins (A–C) or for Wg (blue) and β-galactosidase (brown) proteins (D and E). (A) HS-*wg*, *hh*/TM3, *hb-lacZ* embryo with ectopic En expression. (B) HS-*wg*, *hh* embryo, showing the broadened En domain at stage 10, but lacking β-galactosidase expression indicating the absence of the TM3 marked balancer chromosome. (C) HS-*wg*, *hh* embryo at stage 12. Notice the loss of En expression at this stage. (D) HS-*wg*, *hh*/TM3, *hb-lac*Z embryo showing ectopic *wg* induction. (E) HS-*wg*, *hh* embryo that lack the ectopic Wg domain. Surface views of whole mount embryos are shown (anterior is to the left, dorsal is up).

Fig. 3. Wg protein expression pattern in HS-*wg* embryos in the absence of *porc*. Wg expression in HS-*wg* (A) and *porc*;HS-*wg* (B) embryos. Embryos were double-labeled for Wg (blue) and B-galactosidase (brown) proteins. (A) A *porc*/+;HS-*wg*/TM3, *ftz-lacZ* embryo showing ectopic induction of Wg expression. (B) In *porc*;HS-*wg* embryos Wg distribution resembles that of *porc* mutants and will decay later in development. Surface views of whole mount embryos are shown (anterior is to the left, dorsal is up).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for *en* expression during the *wg*-independent *en* auto-regulation phase. We also demonstrate that *porc* is needed for induction of ectopic endogenous *wg* expression in HS-*wg* embryos.

2. Results

2.1. Induction of ectopic endogenous wg requires en

We showed previously that when *wg* is ubiquitously expressed, under the control of a heat shock inducible promoter, the expression of *en* and endogenous *wg* changes dramatically (Figs. 1A and 1B). The *en* domain broadens in the posterior direction and endogenous *wg* is induced in an ectopic stripe just posterior of the new *en* domain. A deep groove is formed between the ectopic *en* and *wg* domains (Noordermeer et al., 1992). Induction of the ectopic *en* domain does not require functional *en* or endogenous *wg* (Sampedro et al, 1993; Noordermeer et al, 1994). The ectopic domains of *en* and *wg* expression are first detected at early stage 10 and are maintained throughout embryonic development (Noordermeer et al., 1992; Sampedro et al., 1993).

To examine whether induction of ectopic endogenous *wg* is dependent on the presence of the ectopic *en* domain, we studied the pattern of *wg* RNA in HS-*wg* embryos that lack a functional *en* gene. No ectopic *wg* expression was observed in the double mutant embryo (Fig. 1C). *wg* expression in the double mutant embryo decays in a pattern indistinguishable from that of an *en* mutant embryo (DiNardo et al., 1988; Martinez-Arias et al., 1988). We conclude that initiation of the ectopic *wg* expression domain in HS-*wg* is mediated by *en*. This is consistent with the observation that ectopic endogenous *wg* expression in HS-*wg* embryos appears only after *en* expression has maximally expanded (data not shown).

We then investigated whether functional endogenous *wg* was needed for its own ectopic expression. To study endogenous *wg* expression in a *wg*;HS-*wg* embryo we used an allele of *wg*, wg^{IN67}, which encodes an expressed but nonfunctional protein (van den Heuvel et al., 1993). wg^{IN67} RNA is ectopically induced in the HS-*wg* embryo (Fig. 1D), but both the ectopic and the normal stripe of *wg* expression decay at stage 11 (data not shown), as is seen in the single mutant wg^{IN67} embryo (van den Heuvel et al., 1993).

These results indicate that although endogenous *wg* is ectopically induced by ectopic *en*, it must encode functional Wg protein if the expression domain is to be maintained beyond stages 10–11. Interestingly, *en* expression in wg^{IN67};HS-*wg* embryos is induced ectopically and maintained (Fig. 1D), suggesting that the loss of ectopic *wg* is not a consequence of the loss of *en* expression. Therefore, *wg* most likely maintains its own expression at this stage in a manner independent of the ectopic *en* domain.

Since ectopic *en* expression is required for induction of ectopic endogenous *wg* expression, it is not surprising that *dsh* and *arm*, which are required for generating the ectopic *en* domain (Noordermeer et al., 1994), are also needed for ectopic expression of *wg* (data not shown).

2.2. hh is required for en expression during the wg-independent en auto-regulation phase

hh is postulated to encode the signal emanating from the *en* cell to maintain *wg* expression in the adjacent cells (Ingham et al., 1991; Hidalgo, 1991; Ingham and Hidalgo, 1993). We have shown previously that *hh* is not needed for induction of *en* in HS-*wg* (Noordermeer et al., 1994; Fig. 2B), although Sampedro, et al. (1993) showed an effect of *hh* absence on the cuticle phenotype of HS-*wg*. We examined whether *hh* is required for induction of the ectopic endogenous *wg* domain. *wg* is not induced ectopically in *hh*, HS-*wg* embryos (compare Figs. 2D and 2E) and decays as observed in *hh* mutant embryos (Hidalgo and Ingham, 1990; Van den Heuvel et al, 1993).

Ectopic *en* expression in HS-*wg* is maintained by *wg*-independent *en* auto-regulation (Noordermeer, et al. 1994). We examined which segment polarity genes are required for this phase of *en* expression. Of the mutants studied that allow induction of the ectopic *en* domain in HS-*wg* (*en*, *wg*, *hh*, *porc*, *ptc*, *gsb* and *slp*), only *hh*, besides the *en* gene itself, is needed to maintain *en*. Normal and ectopic *en* expression decay in *hh*, HS-*wg* embryos at late stage 11 (Fig. 2C), in a pattern similar to that in HS-*wg* embryos that have a non-functional En protein (Noordermeer et al., 1994).

These data suggest a novel role for *hh* in the *wg*-independent auto-regulation phase of *en*. If *hh* has a role in *en* maintenance, we expect the *hh* domain in HS-*wg* to co-localize with the expanded *en* domain. Indeed, *hh* expression expands in HS-*wg* and is present in the region from the parasegmental border to the deep groove, overlapping with the expanded *en* domain (Figs. 5A and 5B).

2.3. porc is required for ectopic endogenous wg expression

porc, a gene implicated in Wg protein secretion or transport (van den Heuvel et al., 1993; Siegfried et al., 1994), is not required for the induction of ectopic *en* in the HS-*wg* embryo, presumably due to the ubiquitous expression of the *wg* transgene (Noordermeer et al., 1994). To determine whether *porc* is needed for ectopic *wg* induction, we stained the double mutant embryos for Wg protein. Endogenous *wg* is not induced ectopically in *porc*;HS-*wg* embryos (Fig. 3B). Later in development *wg* expression is lost in the double mutant embryo in a similar pattern as in the single *porc* mutant embryo (data not shown).

2.4. ptc is a repressor of endogenous wg expression

It has been shown that *ptc* acts as a constitutive

repressor of *wg* (Ingham et al., 1991). The *wg* domain in HS-*wg*;*ptc* embryos spans the normal and ectopic *wg* stripe as observed in HS-*wg* embryos and in addition the domain between the two stripes, confirming a role for *ptc* as a repressor of *wg* (Fig. 1E). *en* is expressed in a similar domain as seen in the HS-*wg* embryo (Fig. 1E).

It has further been proposed that the repression of *ptc* can be relieved by *hh* (Ingham et al., 1991), possibly by direct interaction of the Ptc and Hh proteins at the cell surface. Based on this model, *hh* might be predicted to alleviate *ptc* repression of *wg* in HS-*wg* at the two interfaces between the *hh* and *ptc* expression domains. In HS-*wg*, *ptc* is present in all cells of the segment that do not express *en* (Noordermeer et al., 1992), while the novel *hh*

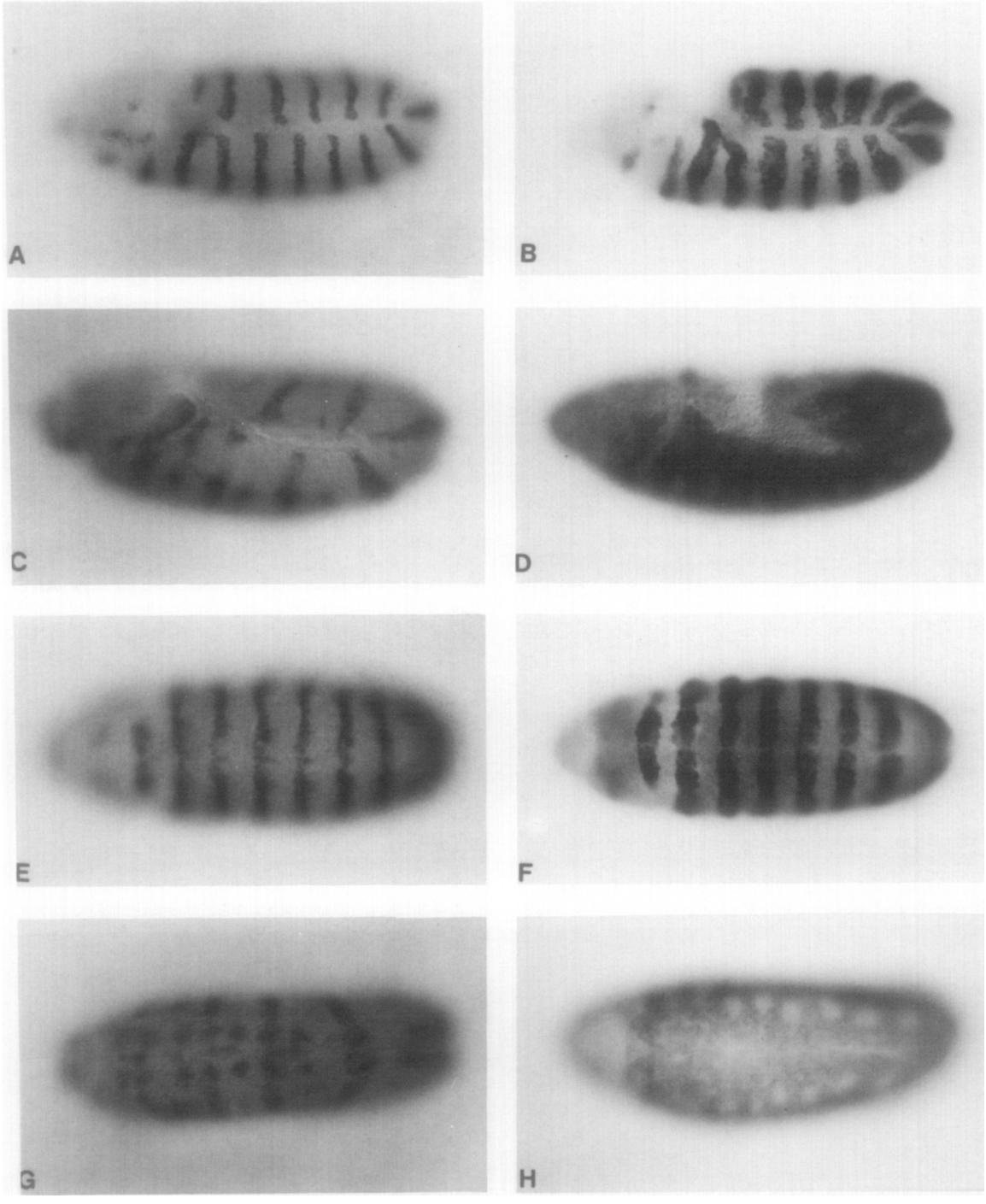

Fig. 4. En is uniformly expressed in the ventral epidermis of *slp*;HS-*wg* embryos. En protein distribution in wild type (A and E), HS-*wg* (B and F), *slp* (C and G) and *slp*;HS-*wg* (D and H) embryos during stage 11 of development. In *slp*;HS-*wg* embryos En is uniformly expressed except for segment 15 and some lateral patches. Anterior is to the left. A–D show lateral surface views, while E–H are ventral surface views.

expression domain is coincident with the expanded *en* domain (Fig. 5B). It is indeed at the novel border of the *ptc* and *hh* domains where one observes the ectopic stripe of endogenous *wg* expression (Fig. 1B).

2.5. *gsb plays a role in maintenance of ectopic wg expression*

gsb encodes a putative transcription factor and has been implicated in a pathway maintaining expression of both *gsb* itself and *wg* (Li et al., 1993). *gsb*;HS-*wg* embryos display a novel pattern of *wg* expression found in neither of the single mutants alone (Fig. 1F). In these embryos, *wg* is induced ectopically but the ectopic and normal domains are maintained only laterally and are lost from the ventral epidermis at stage 11. *en* expression in the double mutant embryo appears identical to that

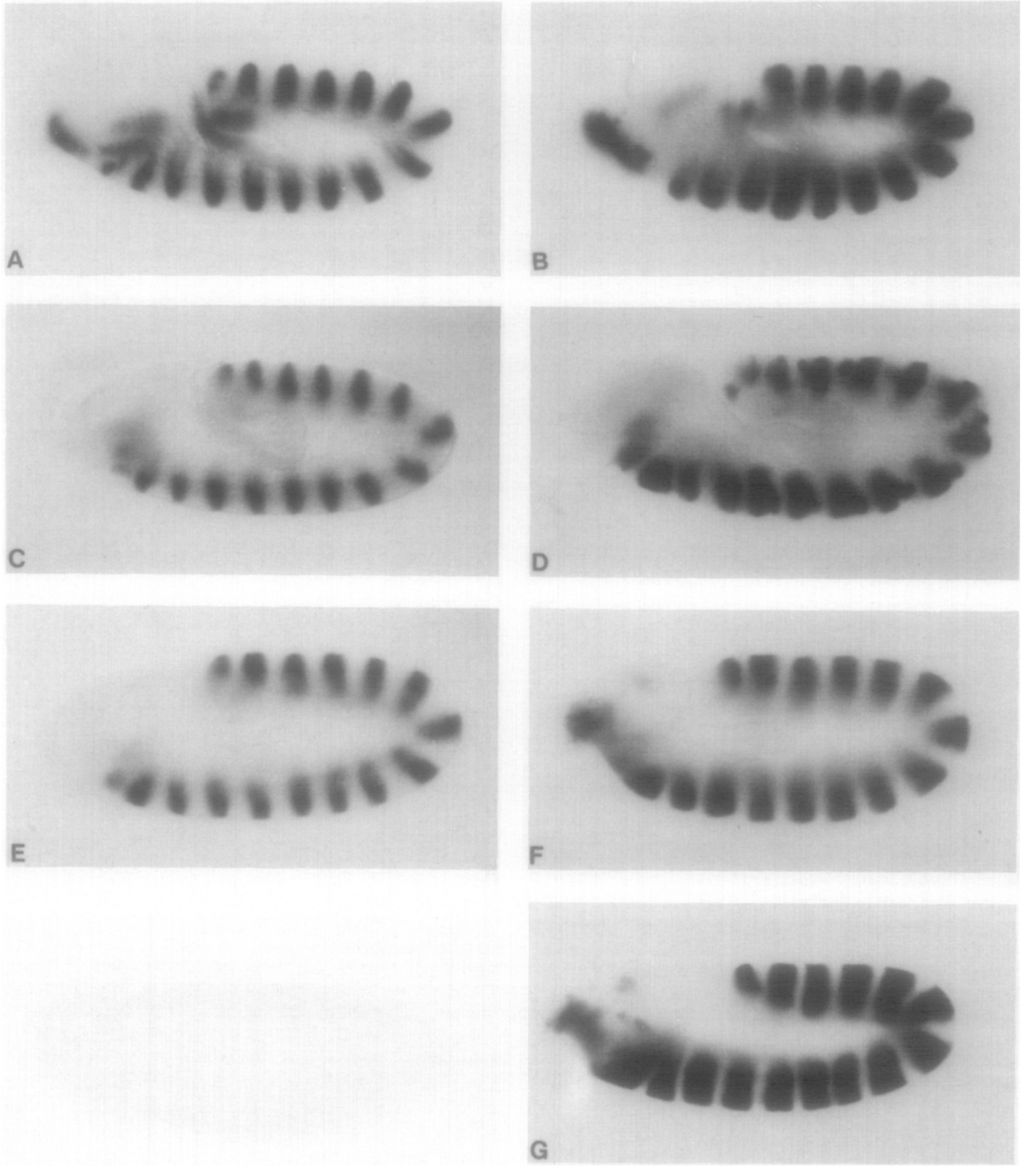

Fig. 5. Expression pattern of *hh*, *gsb* or *slp* RNA in whole mount HS-*wg* embryos. RNA distribution pattern of *hh* (A and B), *gsb* (C and D) and *slp* transcripts (E, F and G) in wild type (left column) and HS-*wg* embryos (right column). *hh* expression in HS-*wg* broadens and overlaps with *en* expression, the *gsb* domain expands and includes the ectopic endogenous *wg* domain and *slp* expression extends and spans the *wg* competent domain. Panels A through F show stage 10–11 embryos. In G, *slp* expression is shown in a stage 8 embryo, in which *slp* is expressed in a wider domain as in stage 10 (F). Anterior is to the left, dorsal is up.

seen in the HS-*wg* embryo (Figs. 1B and 1F). Presumably, *gsb* is required to maintain *wg* expression ventrally, independent of ectopic *en* expression.

We examined whether the expression pattern of *gsb* in HS-*wg* embryos is consistent with a role in maintenance of the ectopic *wg* domain. Indeed, *gsb* expression is expanded in the posterior direction relatively to the parasegmental border and deep groove present in HS-*wg* embryos (Noordermeer et al., 1992). The novel *gsb* expression domain encompasses the expanded *en* domain and the normal and ectopic *wg* expression domains (Figs. 5C and 5D). At stage 11, there is no expression of *gsb* in the dorsal part of the epidermis of wild type and HS-*wg* embryos, suggesting that *gsb* function is restricted to the ventral epidermis.

2.6. *slp prevents en from being expressed throughout the ventral parasegment in HS-wg*

Ubiquitous *wg* expression does not induce *en* expression throughout the segment (Noordermeer et al., 1992; Figs. 4B and 4F). Previous analyses of *en* and *wg* expression patterns in segment polarity mutants have led to the postulation of the existence of domains competent for the expression of either *wg* or *en*, but not both (Ingham et al., 1991). Recent data suggest that the putative transcription factors *slp1* and *slp2* (Grossniklaus et al., 1992) delineate the *en* and *wg* competence domains by activating *wg* and repressing *en* (Cadigan et al., 1994; Figs. 4C and 4G). In accordance with these models, we found that *en* is expressed in all cells of the ventral epidermis and is absent only in some lateral patches in *slp*;HS-*wg* embryos (Figs. 4D and 4H). This result suggests that the *slp* gene product prevents *en* from being induced throughout the ventral segment in the HS-*wg* embryo.

2.7. *slp is needed for induction of ectopic endogenous wg in HS-wg*

In addition to a role as repressor of *en* expression in HS-*wg*, *slp* is also required to induce ectopic endogenous *wg* expression. In *slp*;HS-*wg* embryos the normal *wg*

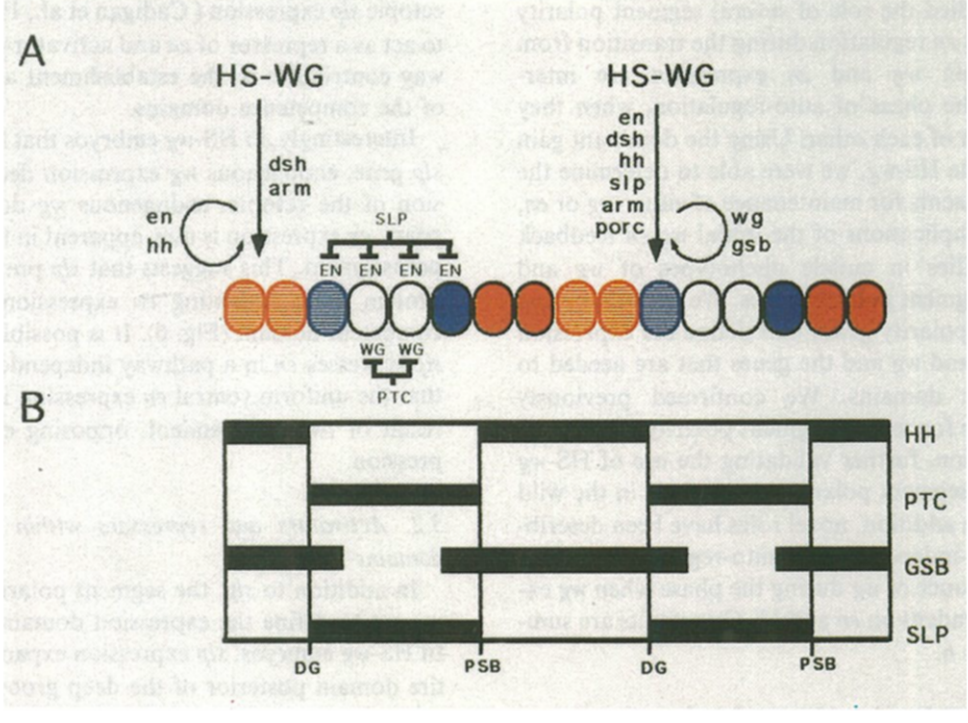

Fig. 6. Schematic representation of the genetic requirements for induction of ectopic *en* and endogenous *wg* expression in stage 10 HS-*wg* embryos. (A) Genetic requirements for induction of ectopic *en* and endogenous *wg* in a typical pair of parasegments of a stage 10, HS-*wg* embryo. At early stage 10 the parasegment is about 8 cells in width. The normal *en* expression domain (red) spans about 2 cells posterior of the parasegmental border (PSB) and the normal *wg* RNA domain (blue) one cell anterior of the border. In heat shocked, HS-*wg* embryos *en* expression is expanded in posterior direction to half the width of the parasegment (ectopic *en* cells indicated with red stripes). Endogenous *wg* is ectopically induced posterior of the ectopic *en* domain (ectopic *wg* cells indicated with blue stripes). The genetic requirements for induction for ectopic *en* and *wg* expression are indicated next to the straight arrow going down from HS-*wg*, while the requirements for their maintenance are shown next to the half circle at the side of their expression domains. For induction of *en* by HS-*wg* *dsh* and *arm* are needed, while *en*, *wg*, *porc*, *hh*, *gsb* and *ptc* are dispensable. For maintenance of ectopic *en*, *en* itself and *hh* are required. Induction of ectopic *wg* requires *en* (and therefore *dsh* and *arm*), *hh*, *slp* and *porc*. *gsb* and *wg* are needed for maintenance of the ectopic *wg* domain. Within the *wg* competent domain *ptc* acts as a repressor of *wg*, except there where Hh protein can antagonize this function in the cells flanking the parasegmental and deep groove (DG). *slp* represses *en* in the entire *wg* competent domain. (B) The expression domains of *hh*, *ptc* (Noordermeer et al., 1992), *gsb* and *slp* RNA in a stage 10 HS-*wg* embryo are indicated by the solid bars. The bars are shown relatively to the parasegmental (PBS) and deep grooves (DG) in (A). The *hh* expression domain overlaps with that of *en* and seems to cover the *en* competent domain. *ptc* and *slp* are expressed in the *wg* competent domain, while *gsb* is expressed in the entire domain of *en* and endogenous *wg*. Anterior is to the left, posterior to the right.

stripe decays in a pattern similar to that seen in the *slp* single mutant embryo (Cadigan et al., 1994; data not shown).

To better understand the role of *slp* in establishing the HS-*wg* phenotype, we studied the expression of *slp* in HS-*wg* embryos. At stage 8 the *slp* domain has expanded compared to the wild type embryo and *slp* is expressed in almost the entire parasegment (Fig. 5G). In subsequent hours, when the *en* domain expands, *slp* expression becomes confined to the domain between the deep groove and the parasegmental border (Fig. 5F), where *en* is not expressed. Endogenous *wg* is present in two stripes within the anterior and posterior borders of the *slp* expression domain in HS-*wg* (Noordermeer et al., 1992). We conclude that not only is *slp* needed to induce ectopic endogenous *wg*, but also that *wg* activates *slp* expression ectopically in HS-*wg* embryos.

3. Discussion

We have studied the role of several segment polarity genes in *wg* and *en* regulation during the transition from the phase when *wg* and *en* expression are interdependent to the phase of auto-regulation, when they are independent of each other. Using the dominant gain of function allele HS-*wg*, we were able to determine the genetic requirements for maintenance of either *wg* or *en*, without the complications of the initial *wg-en* feedback or the similarities in cuticle phenotypes of *wg* and several other segment polarity genes. We were interested in the segment polarity genes that define the expression domains of *en* and *wg* and the genes that are needed to maintain these domains. We confirmed previously postulated roles for several segment polarity genes in *en* and *wg* regulation, further validating the use of HS-*wg* as a model for segment polarity interactions in the wild type embryo. In addition, novel roles have been described for *hh* in *wg*-independent *en* auto-regulation and for *porc* in maintenance of *wg* during the phase when *wg* expression is dependent on *en* and *hh*. Our results are summarized in Fig. 6.

3.1. Defining the domains of en and endogenous wg expression in HS-wg embryos

Induction of ectopic *en* in HS-*wg* can first be observed at early stage 10, at which time *en* expression is dependent upon *wg* in the wild type embryo (Heemskerk et al., 1991; Bejsovec and Martinez-Arias, 1991). An intriguing question has been why HS-*wg* does not induce *en* expression in the whole parasegment. Presumably, other gene products are required, in concert with *wg*, to effect *en* expression and these factors might not be uniformly distributed. Alternatively, repressors may prevent ubiquitously expressed *wg* from inducing *en* expression in all cells across the segment. Here, we present additional evidence for the presence of at least one strong repressor of *en*, *slp* (Cadigan et al., 1994).

The wild type parasegment can be considered to consist of two equivalence groups, the *en* and the *wg* competence domains (Ingham and Hidalgo, 1993). The pair-rule genes establish the competence domains, while later the segment polarity genes maintain and refine the *en* and *wg* expression patterns. Cells in each domain are able to, but do not necessarily, express *wg* or *en*. The observed expression domains of *en* and *wg* are the result of the superimposition of both activator and repressor activities upon the competence domains.

The *slp* gene is thought to play a major role in defining the borders of the competence domains (Cadigan et al., 1994). During germband extension, *slp* is expressed over and just anterior to the *wg* expression domain, spanning one third of each segment. In a *slp* mutant embryo, *wg* expression is lost from the epidermis and the *en* domain expands anteriorly into the *wg* expressing domain. Based upon these observations and the study of ectopic *slp* expression (Cadigan et al., 1994), *slp* appears to act as a repressor of *en* and activator of *wg* and in this way contributes to the establishment and maintenance of the competence domains.

Interestingly, in HS-*wg* embryos that lack a functional *slp* gene, endogenous *wg* expression decays and expression of the ectopic, endogenous *wg* domain never appears. *en* expression is now apparent in the entire ventral parasegment. This suggests that *slp* prevents the HS-*wg* protein from activating *en* expression within the *wg* competent domain (Fig. 6). It is possible, however, that *slp* represses *en* in a pathway independent from *wg* and that the uniform ventral *en* expression in *slp*;HS-*wg* is a result of two independent, opposing effects on *en* expression.

3.2. Activators and repressors within the competence domains

In addition to *slp*, the segment polarity genes *hh* and *ptc* act to define the expression domains of *wg* and *en*. In HS-*wg* embryos, *slp* expression expands to fill the entire domain posterior of the deep groove, extending to the parasegmental border (Fig. 6). *ptc* is expressed in the same domain while *hh* is expressed in the adjacent *en* domain. Since *hh* is secreted to adjacent cells, it can alleviate the *ptc*-mediated repression of *wg* at the interface between the *hh*-producing cells and the *slp*-expressing cells. However, no Hh protein is present in the middle of the *wg* competent domain (Fig. 6), where *wg* remains repressed by *ptc*. Consistent with this is the observation that initiation of ectopic endogenous *wg* is dependent on both *hh* and *slp*. Furthermore, in *ptc*;HS-*wg* embryos *wg* is expressed in the region between the two *wg* stripes observed in HS-*wg* alone, suggesting that *ptc* acts to repress *wg* in this domain.

The *wg* competence domain in HS-*wg* is revealed in the *ptc*;HS-*wg* embryo and spans half the width of the segment, overlapping the expanded *slp* domain (Fig. 6). It has been observed that the *wg* competence domain in *ptc* coincides with the third of the segment where *slp* expression occurs (Cadigan et al., 1994). This correlation of *slp* expression and the *wg* competence domain in both the *ptc* and *ptc* ;HS-*wg* embryos, further suggests a role for *slp* in determining the potential of cells to express *wg*.

nkd and *zw3* have been shown to repress *en* within the posterior region of its competence domain. The two gene products are believed to antagonize *wg* function and to act as repressors of *en* auto-regulation (Heemskerk et al., 1991; Siegfried et al., 1992). In HS-*wg* embryos, ubiquitous *wg* expression overcomes *en* repression by *zw3* and *nkd*, such that *en* is induced as in single *nkd* and *zw3* embryos. No further expansion of *en* is seen in HS-*wg*, *nkd* or *zw3*;HS-*wg* double mutants (data not shown), indicating that these proteins repress the potential of *wg* to induce *en* within the *en* competent domain, but not outside that domain.

3.3. Early and late regulation of wg expression

Maintenance of the expression of *wg* during stage 9 through late stage 10 requires *en* and *hh* (reviewed in Perrimon, 1994). In this paper, we have shown that induction of ectopic endogenous *wg* in HS-*wg* embryos is a consequence of the induction of the ectopic *en* domain. In addition, *hh* is required for initiation of the ectopic *wg* domain in HS-*wg*, reflecting its postulated role in the wild type embryo.

We further show that another segment polarity gene, *porc* is required for ectopic induction of *wg*. Based upon the altered distribution of the Wg protein in *porc* embryos (Van den Heuvel et al., 1993; Siegfried et al., 1994), it has been proposed that *porc* plays a role in secretion of the Wg protein. Given that *hh* mediates the one known signaling pathway from the *en* cell to the *wg* cell, it is possible that *porc* is required for the secretion of the Hh protein. The loss of *wg* RNA at stage 11 in *porc* could then be, at least in part, a result of a failure to secrete Hh. We stained *porc* embryos with an antibody against the Hh protein (Tabata and Kornberg, 1994) and did not observe a change in distribution of Hh protein in stage 10 embryos (data not shown). The role of *porc* in signals sent from the *en* cell to the *wg* cell remains unclear.

After *wg* expression has been established in the wild type embryo by the combined action of *porc*, *en*, *hh* and *slp* (Dinardo et al., 1988, Hidalgo and Ingham 1990, Cadigan et al., 1994), the Gsb protein maintains *wg* in a *gsb-wg* auto regulatory loop (Li and Noll, 1993). These genetic requirements for the maintenance of the ectopic *wg* expression domain in the HS-*wg* embryo are identical to those seen for maintaining *wg* in the wild type embryo (Fig. 6). After stage 11, both endogenous *wg* and *gsb* are required for maintenance of ectopic *wg* expression, while ectopic *en* expression seems unaffected in the *wg*;HS-*wg* and *gsb*;HS-*wg* mutants, suggesting that this phase of *wg* maintenance does not require *en*.

3.4. Regulation of en expression

At late stage 10, *en* expression is maintained by auto-regulation independent of *wg* in the wild type embryo (Heemskerk et al., 1991). We have shown earlier that maintenance of the expanded *en* domain in HS-*wg* after late stage 10 does not require functional endogenous Wg protein (Noordermeer et al., 1994). We now find that *en* auto-regulation at this stage requires *hh*. As Hh is secreted from the cell where its function is required during *wg*-independent *en* auto-regulation, it seems likely that *en* and *hh* participate in an autocrine mechanism of *en* regulation. Consistent with a role for *hh* in *en* maintenance is the observation that *en* expression decays prematurely in a *hh* mutant embryo (Hidalgo and Ingham, 1990). However, since *hh* has an earlier function in maintaining *wg*, the loss of *en* expression at this stage might be an indirect effect of the loss of *wg* expression.

This later function of *hh* might also explain why the cuticle phenotype of the *ptc,hh* embryo is more like *ptc,en* than that of *ptc* alone (Hidalgo, 1991; Forbes et al., 1993). Furthermore, the differences between the cuticle phenotype of HS-*wg* and HS-*wg*, *hh* may similarly result from this novel role for *hh*. The HS-*wg*, *hh* embryo has an almost naked cuticle like the HS-*wg* embryo, but the HS-*wg*, *hh* embryo is smaller and apparently unsegmented (Sampedro et al., 1993; Noordermeer et al., 1994), possibly in part as a result of the failure to maintain the ectopic *en* domain.

4. Experimental procedures

4.1. Drosophila stocks

The HS-*wg*/TM3*Sb* stock is described in Noordermeer et al., (1992). The HS-*wg* P element insertion, containing the *wg* cDNA under the control of the *hsp*70 promoter, is located on the third chromosome at cytological location 66A/B.

All segment polarity strains that are used, were previously described as strong alleles, based upon cuticle phenotype or molecular lesion: D*f*(2R)*en*-E (Z.Ali and T.Kornberg, personal communication; Fjose et al., 1985), wg^{IN67} (Nusslein-Volhard et al., 1984), ptc^{P78} (Nakano et al., 1989; Hooper and Scott, 1989), hh^{G51} (Mohler, 1988, 1992; Tabata et al., 1992; Lee et al., 1992), the *slp* deficiency *CyO, D34B* (Grossniklaus et al., 1992), D*f*(2R)gsb^{zipper} (Nusslein-Volhard et al., 1984; Baumgartner et al., 1987).

arm (Riggleman et al., 1990; Peifer and Wieschaus, 1990), *porc* and *dsh* (Perrimon et al., 1987) are maternal

effect x-linked zygotic lethal mutations. To eliminate the maternal contribution of these genes, germline clones of strains carrying the mutant alleles *arm*^{XM19} or *dsh*^{V26} were generated by the FLP-DFS technique (Chou and Perrimon, 1992). *porc*^{PB16} germ line clones were induced by mitotic recombination after X-irradiation (Klingensmith et al., 1989) or with the FLP-DFS technique.

4.2. Identification of double-mutant embryos

For the study of the HS-*wg* phenotype in a segment polarity mutant background, we generated embryos that contain HS-*wg* and lack one of the following segment polarity genes: *en*, *wg*, *ptc*, *hh*, *gsb*, *slp*, *porc*, *arm* and *dsh*. Since HS-*wg* flies are weak, it was in most cases not possible to maintain stable stocks that contain the HS-*wg* P element over a TM3 balancer chromosome and a balanced segment polarity mutation. Only for *hh*, located on the third chromosome, a recombinant stock was obtained and balanced over TM3, marked with a P element carrying the β-galactosidase gene under the control of the *hunchback* promoter (G. Struhl, unpublished). β-galactosidase staining can be detected from stage 6 to 13. All staging was performed according to Wieschaus and Nusslein-Volhard (1986). In this way, we were able to identify embryos that carry the HS-*wg* P-element and are mutant for *hh*. In the case of *en*, *wg*, *gsb*, *slp* and *ptc*, mutations that are located on the second chromosome, crosses were performed between HS-*wg*/TM3 and one of the following strains: D*f*(2R)*en*-E/CyO, *wg*^{IN67b}*pr*/CyO, D*f*(2R)*gsb* ^{zipper}/Cy, D34, CyO/cn *Adh l*(2) and *ptc*^{P78}/CyO. F1 males and females, carrying the particular segment polarity mutation and one copy of the HS-*wg* P-element, were selected and F2 progeny collected and heat shocked (see below). Using a combination of RNA in situ hybridization and antibody labeling, in most cases embryos could be identified that showed a novel staining pattern for *wg* and *en* not present in the mutant or the HS-*wg* embryos alone. In this way we were able to identify unambiguously the double mutant embryos. In other cases we counted the ratios of the different classes of embryos and thereby determined the double mutant phenotype.

Germline clones were generated from the stocks *arm*, *dsh* and *porc* (Chou and Perrimon, 1992), and crossed with HS-*wg*/TM3 males that carry a FM7 balancer, marked with the *β-galactosidase* gene under the control of the fushi-tarazu-promoter (Kania et al., 1990). Embryos of these stages, that do not stain with anti-*β-galactosidase* antibody, lack the particular segment polarity gene on the first chromosome and half of these embryos carry the HS-*wg* P element. By double labeling for anti-*β-galactosidase* and anti-*wg* antibodies, the pattern of Wg protein in the double mutant embryos could be determined.

4.3. Heat shock procedure

Embryos from each individual cross described above were collected on plates for 5 h and aged for 1 h, transferred to eppendorf tubes and submerged in a water bath for 20 min at 36°C. After the first heat shock, embryos were placed on to apple juice plates and maintained at room temperature. Second and third heat shocks were carried out 2 and 4 h after the start of the first heat shock. After the last heat shock embryos were maintained for 3 h prior to dechorionation and fixation in 4% formaldehyde. Subsequently, stages 10 to 12 embryos were analyzed for their change in distribution of Wg and En proteins. As a consequence of the heat shock procedure, uniform Wg protein derived from the transgene is present in these embryos starting at the first heat shock at around stage 3/4 (approximately 2 h after egg laying) and stays around until 1.5 h after the last heat shock. The embryos are then around stage 9/10 (6.5 h after egg laying) and are allowed to develop another 1.5 h before fixation. Heat shocked segment polarity mutant embryos were used as a control for the general effects of the heat shock treatment.

4.4. Whole mount RNA in situ hybridization

RNA in situ hybridization was performed, largely as described (Tautz and Pfeiffle, 1989), with some modifications (Noordermeer et al., 1992). The following DNA-probes were used:

- a 2.9 kb *Bam*HI-fragment from the *wg* cDNA (Rijsewijk et al., 1987).
- a 2.7 kb *Eco*RI-fragment from the *gsb-d* cDNA (Baumgartner et al., 1987).
- a 2.4 kb *Eco*RI-*Hin*dIII-fragment from the *hh* cDNA (Lee et al., 1992).
- a 1.5 kb *Eco*RI-fragment from the *slp1* cDNA (Grossniklaus et al., 1992).

4.5. Whole mount immuno stainings

Fixation of embryos and single and double anti-body labelings were carried out as described (Lawrence et al., 1989). En antibody was used unpreabsorbed in a 1:4 dilution, rabbit polyclonal anti-*wg* was pre-absorbed as described (van den Heuvel et al., 1989) and used in 1:200 dilution, while monoclonal and rabbit polyclonal anti-*β-galactosidase* (Promega and Cappel, respectively) were pre-absorbed and used in a 1:8000 dilution.

4.6. Detection of wg RNA and En protein simultaneously in embryos

In order to detect *wg* RNA and En protein in the same embryo, RNA in situ hybridization was performed using a *wg* cDNA probe followed by an antibody staining with the *en* monoclonal antibody (Scott Dougan, personal communication). The RNA in situ hybridiza-

tion was done as described above, with the exception that incubation with proteinase K was 2 instead of 6 min. After the *wg* RNA was visualized as a dark blue precipitate using alkaline phosphatase, an antibody staining was performed. Embryos were incubated overnight with primary anti *en* antibody and the next day incubated with biotinylated secondary anti-mouse antibody. Using the Vectastain Elite System (Vector Labs) the HRP-conjugated anti-mouse complex was detected in the presence of 500 mg/ml DAB and H_2O_2, resulting in formation of a brown/red precipitate.

Acknowledgments

J.N.N. acknowledges L. Fradkin for advice, stimulating discussions and for making Fig. 6. We would like to thank N. Perrimon for providing several unpublished fly stocks, W. Gehring, R. Holgrem, P. Ingham, T. Kornberg, J. Mohler, C. Nüsslein-Volhard, G. Struhl and E. Wieschaus for fly stocks and K. Cadigan for the *slp1* probe. Thanks also to L. Fradkin, D. Lessing, K. Cadigan, K. Kozopas and A. Wodarz for critically reading the manuscript. Monoclonal *en* antibody was a gift of C. Goodman and Nipam Patel. K. Shaw is acknowledged for help with preparing the manuscript.

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