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The *porcupine* gene is required for *wingless* autoregulation in *Drosophila*

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SUMMARY

The *Drosophila* segment polarity gene *wingless (wg)* is required in the regulation of *engrailed (en)* expression and the determination of cell fates in neighboring cells. This paracrine *wg* activity also regulates transcription of *wg* itself, through a positive feedback loop including *en* activity. In addition, *wg* has a second, more direct autoregulatory requirement that is distinct from the *en*-dependent feedback loop. Four gene products, encoded by *armadillo (arm), dishevelled (dsh), porcupine (porc)* and *zeste-white 3 (zw3)*, have been previously implicated as components of *wg* paracrine signaling. Here we have used three different assays to assess the requirements of these genes in the more direct *wg* autoregulatory pathway. While the activities of *dsh, zw3* and *arm* appear to be specific to the paracrine feedback pathway, the more direct autoregulatory pathway requires *porc*.

Key words: *Drosophila*, segment polarity, *porcupine*, *wingless*

INTRODUCTION

The segment polarity genes *wingless (wg)* and *engrailed (en)* are transcribed in adjacent, non-overlapping stripes of cells in each of the 14 developing segments of the *Drosophila* embryonic epidermis (Baker, 1987; DiNardo et al., 1988; Martinez-Arias et al., 1988). Each of these genes is required to maintain the expression of the other in neighboring stripes of cells (reviewed in Perrimon, 1994). *wg* encodes a secreted glycoprotein which is a member of the Wnt family (Baker, 1987; Rijsewijk et al., 1987; reviewed in McMahon, 1992; Nusse and Varmus, 1992). *wg* protein (Wg) is secreted and enters *en*-expressing cells; thus it is thought that Wg itself may be the paracrine signal leading to maintenance of *en* expression (van den Heuvel et al., 1989; Gonzalez et al., 1991, review by Siegfried and Perrimon, 1994). *wg* activity is also required in the specification of ventral naked cuticle and cell type diversity in the larva (Nusslein-Volhard and Wieschaus, 1980; Bejsovec and Wieschaus, 1993).

Other segment polarity genes have been postulated to encode crucial components of *wg* signaling (reviewed in Perrimon, 1994). *dishevelled (dsh)* encodes a novel protein which is conserved evolutionarily (Klingensmith et al., 1994; Thiesen et al., 1994; Sussman et al., 1994; Sokol et al., 1995), *zeste-white 3 (zw3)* encodes the *Drosophila* homolog of mammalian Glycogen Synthase Kinase 3 (Siegfried et al., 1992; Ruel et al., 1993), *armadillo (arm)* encodes *Drosophila* β-catenin (Riggleman et al., 1990; Peifer and Wieschaus, 1990) and the *porcupine (porc)* gene has not yet been molecularly identified. These genes have been ordered in an epistatic pathway (Siegfried et al., 1994; Noordermeer et al., 1994). In combination with clonal analyses (Klingensmith et al., 1994; Wieschaus and Riggleman, 1987), these epistasis experiments have led to a model in which the *wg* signal is received or transduced in neighboring cells via *dsh*, *zw3* and *arm* activity to regulate *en* expression.

In contrast, it is thought that *porc* is required for presentation of the *wg* signal (Siegfried et al., 1994; Noordermeer et al., 1994; Klingensmith, 1993). Like *wg* mutations, and unlike mutations in *dsh*, *zw3* and *arm*, *porc* mutations act in a non-cell autonomous manner (Baker, 1987; Klingensmith et al., 1994; Klingensmith, 1993). Since, in *porc* mutants, Wg appears to be abnormally confined to the cells where it is transcribed, it has been postulated that *porc* is required for normal secretion of the Wg protein (van den Heuvel et al., 1993a; Siegfried et al., 1994). It is also possible that the main role for *porc* could be in the regulation of *wg* transcription.

Because *wg* is required for maintenance of *en* transcription (DiNardo et al., 1988), and *en* is in turn required for that of *wg* (Martinez-Arias et al., 1988), *wg* may regulate its own transcription indirectly through a 'paracrine feedback loop' (Ingham and Hidalgo, 1993). However, *wg* also has a second, distinct autoregulatory role that may reflect an autocrine *wg* activity that is independent of signaling via *en* (Bejsovec and Wieschaus, 1993; Hooper, 1994; Yoffe et al., 1995). Since it appears that this second autoregulatory function of *wg* is required prior to *en* activity (Yoffe et al., 1995; this work), we will refer to it as 'direct autoregulation' for simplicity. As *wg* stripes fade in *porc*, *dsh* and *arm* mutant embryos (van den Heuvel et al., 1993b), it is possible that these genes are crucial components of direct autoregulation as well as paracrine signaling by *wg*. Alternatively, these genes may only be components of the latter, the loss of *wg* being a secondary result of the loss of *en*. To date the genetic components of the two

modes of *wg* autoregulation have been examined only indirectly, in mutants for the *patched (ptc)* gene (Hooper, 1994).

We have tested the genetic basis of *wg* maintenance by attempting to identify components that may be distinct to direct *wg* autoregulation versus the paracrine *wg* signaling. We have used three assays: (1) monitoring the timing of disappearance of *wg* versus *en* expression in *porc*, *dsh* and *arm* embryos, (2) determining the requirement for these three genes in *zw3* mutants, in which *wg* paracrine signaling is 'constitutive' (Siegfried et al., 1992) and (3) assaying the autoregulatory potential of exogenous Wg in the absence of the genes *porc*, *dsh* and *arm*. Our results suggest that *dsh*, *zw3* and *arm* are required specifically in the paracrine signaling, while *porc* is required for direct autoregulation but not paracrine signaling. Thus these two *wg* pathways appear to be genetically distinct.

MATERIALS AND METHODS

Fly strains

wg^{IG22} is a protein null allele of *wg* (van den Heuvel et al., 1993a). wg^{en11} is a null *wg* allele on the CyO balancer chromosome that expresses *lacZ* in the *wg* pattern (Kassis et al., 1992). en^{CX1} makes a nonfunctional En protein and behaves genetically as a null allele (Heemskerk et al., 1991). arm^{XM19} and dsh^{v26} have all been previously described as molecular and genetic nulls during embryogenesis (Peifer et al., 1991; Klingensmith et al., 1994). dsh^{75}, $zw3^{M11}$ and $porc^{PB16}$ have been described as being genetic nulls (Perrimon et al., 1989). *hGAL4* (also known as *1J3*) is an insertion of the *GAL4* construct pGawB at the *hairy (h)* locus on the third chromosome and has been previously described (Brand and Perrimon, 1993). *UASwg* was constructed to express the wg^{IL114} temperature-sensitive allele which is active at 16°C. The *UASwg* insertion is located on the third chromosome and is homozygous viable (Wilder and Perrimon, 1995).

Generation of embryos

arm^{XM19}, dsh^{v26}, dsh^{75}, $porc^{PB16}$, $zw3^{M11}$, arm^{XM19} $zw3^{M11}$, $zw3^{M11}$ dsh^{75} and $zw3^{M11}$ $porc^{PB16}$ mutant embryos were generated by heterozygous females having homozygous mutant germlines (germline clone females), as previously described (Siegfried et al., 1994). Using the *hGAL4* line, we can direct the expression of *UASwg* in a *h* 'pair-rule' stripe pattern (Brand and Perrimon, 1993). wg^{en11}*; hGAL4/UASwg* and en^{CX1}; *hGAL4/UASwg* embryos were generated as described in Yoffe et al. (1995). *hGAL4-UASwg (h-wg)* is a recombinant third chromosome which carries both the *hGAL4* and *UASwg* inserts, and was introduced into mutant embryos by crossing *h-wg/TM3* males to *dsh*, *arm* and *porc* germline clone females. Thus one half of the non zygotically rescued (e.g. *dsh*/Y) embryos bear the *h-wg* chromosome. dsh^{v26}; *h-wg* and arm^{XM19}; *h-wg*, embryos were identified by their predominantly 'seven stripe' expression patterns of endogenous *wg*. For each experiment, at least 100 progeny were examined. The percentage of rescued embryos matched the predicted number that should have been generated in the genetic cross (1 out of 4) in the *dsh* and *arm* mutant backgrounds. However, not all embryos are rescued in a perfect seven stripe pattern and the extent of rescue was often incomplete. $porc^{PB16}$; *h-wg* embryos were identified by the fading of *wg* transcription in embryos displaying seven broad (*h-wg*) Wg stripes. This analysis was accomplished through Wg antibody and endogenous *wg* mRNA double labeling experiments (Manoukian and Krause, 1992): Wg (in a *h* pattern) can be detected whereas endogenous *wg* transcription is lost. Hundreds of embryos were examined and *wg* transcription was never rescued in the epidermis of *porc*; *h-wg* embryos. All experiments were

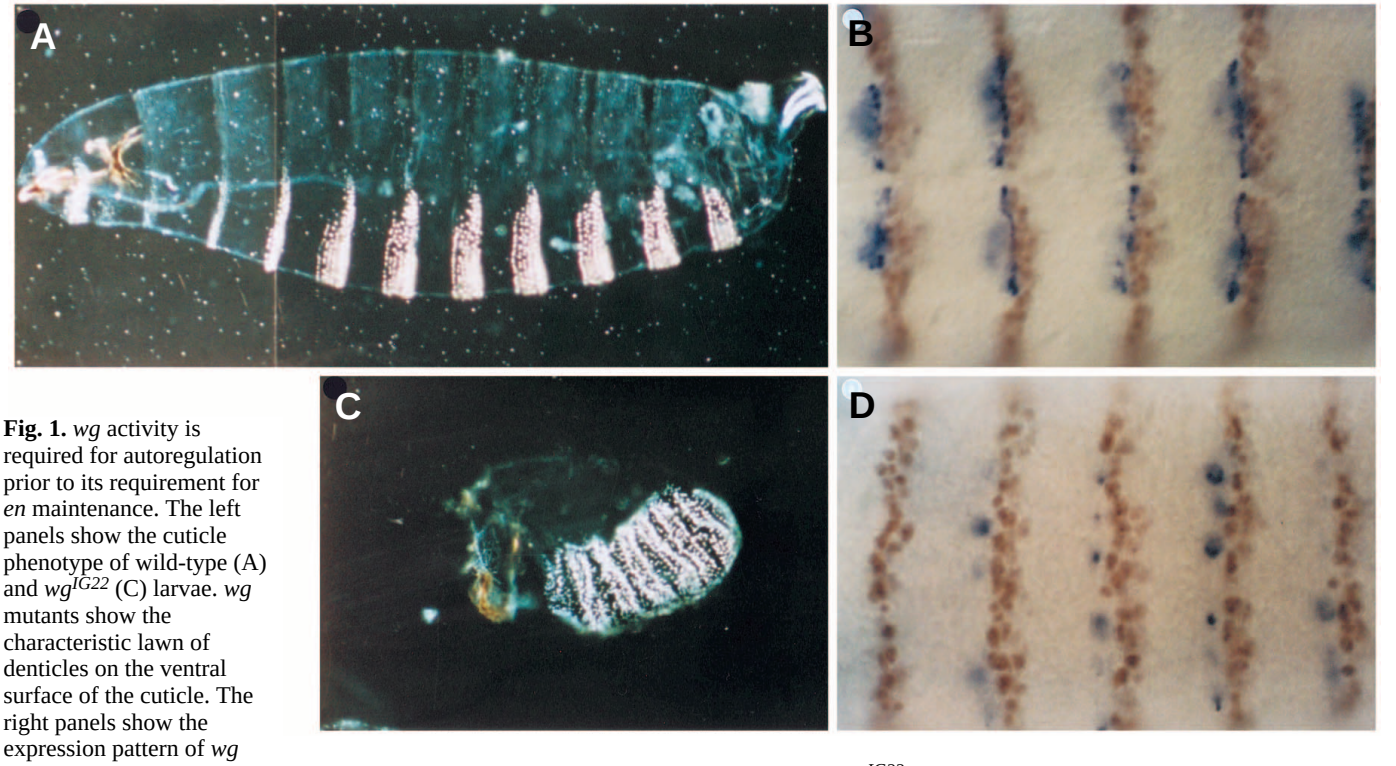

Fig. 1. *wg* activity is required for autoregulation prior to its requirement for *en* maintenance. The left panels show the cuticle phenotype of wild-type (A) and wg^{IG22} (C) larvae. *wg* mutants show the characteristic lawn of denticles on the ventral surface of the cuticle. The right panels show the expression pattern of *wg* transcripts (blue) and En protein (brown) in the ventral epidermis of wild-type (B) and wg^{IG22} (D) embryos. In wild-type embryos, *wg* is expressed in a series of 14 single-cell-wide stripes. In the absence of functional *wg* protein, these 14 stripes of expression fade starting at stage 9 before En has faded. This shows that *wg* is required for its own expression prior to that of *en* and thus exhibits direct autoregulation. All figures are oriented anterior to left, dorsal up unless otherwise specified.

carried out at 16°C, the permissive temperature of *UASwg* (see Wilder and Perrimon, 1995; Yoffe et al., 1995).

Cuticle preparations

Cuticles were prepared by clearing in Hoyer's medium (Struhl, 1989) and photographed under dark-field optics.

Embryo stainings

Fixation and hybridization and/or immunostaining of embryos and detection of expression patterns were as previously described (Manoukian and Krause, 1992; Yoffe et al., 1995). A digoxigenin-labeled probe that detects endogenous *wg* RNA but not exogenous *h-wg* transcripts was generated by PCR, using a 5′ *wg* untranslated specific sequence (Yoffe et al., 1995). En antibodies (Patel et al., 1989) were used at a 1:2 dilution, and Wg antibodies (van den Heuvel et al., 1989) were used at a 1:100 dilution.

RESULTS

porc is required for direct *wg* autoregulation

The cuticle phenotypes of *arm*, *dsh* and *porc* embryos are virtually identical to that of *wg*, having a uniform 'lawn' of ventral denticles (Perrimon et al., 1989; Figs 1C, 2A,C). In these embryos, both *wg* and *en* expressions fade (Peifer et al., 1991; van den Heuvel et al., 1993b). Previously it has been shown that, in *wg* mutant embryos (which produce *wg* RNA but no protein), stripes of endogenous *wg* fade during embryonic stage 9, before the disappearance of the En stripes (Yoffe et al., 1995; Fig. 1D). This result suggested that *wg* has a more direct autoregulatory activity than the *en*-dependent positive feedback loop. We therefore simultaneously studied the timing of disappearance of *wg* versus En expression in *porc*, *dsh* and *arm* mutant embryos in order to distinguish possible differences in temporal requirements for these three genes. We note a difference in the timing of the loss of *wg* transcription in *porc* versus *dsh* or *arm* mutants, as monitored using En expression as an assay. In the absence of *dsh* (Fig. 2B) or *arm* (not shown) activity, *wg* transcription fades after or simultaneously with En. As *dsh* and *arm* are indispensible for the regulation of *en* transcription by *wg* (Noordermeer et al., 1994), this result suggests that *dsh* and *arm* may not be components of direct *wg* autoregulation. Rather, they may be required for *wg* transcription only indirectly via the paracrine feedback loop. In contrast, *porc* appears to be required for direct *wg* autoregulation since, just as in *wg* mutant embryos, *wg* fades before En in *porc* mutants (Fig. 2D).

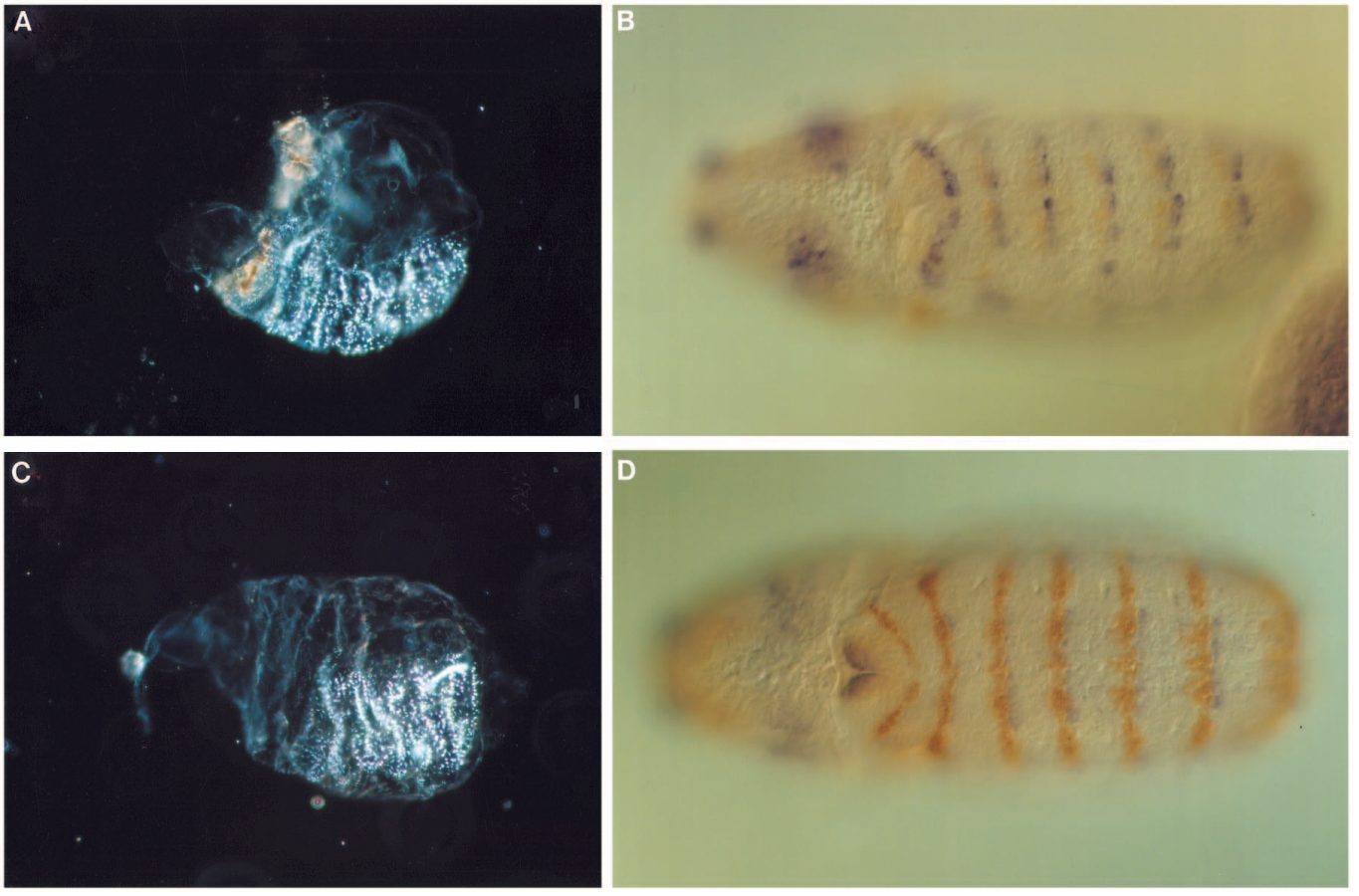

Fig. 2. The order of fading of *wg* RNA versus En protein in *dsh* and *porc* mutant embryos. Shown on the left are the cuticle phenotype of dsh^{75} (A) and $porc^{PB16}$ (C) larvae. Both *dsh* and *porc* mutants resemble *wg* mutants in phenotype (see Fig. 1). The right panels show expression of *wg* and En in dsh^{75} (B) and $porc^{PB16}$ (D) mutant embryos, oriented to show the posterior ventral epidermis. In *dsh* (b) and *arm* (not shown) mutant embryos, En fades before or simultaneously with *wg* transcripts. By contrast, in *porc* mutant embryos, *wg* transcription fades before En is affected. This is not due to the increased stability of En in *porc* mutant embryos, since *en* transcripts fade at the same stage as En in the absence of *porc* (not shown). These events occur mid stage 9 of embryogenesis.

porc is epistatic to *zw3* in the regulation of wg

The segment polarity mutant *zw3* has a reciprocal cuticular phenotype to that of the *wg* class of mutants. In *zw3* mutants, virtually all ventral denticles are replaced with naked cuticle (Perrimon and Smouse, 1989), similar to embryos in which Wg has been expressed uniformly from a heat-shock promotor (Noordermeer et al., 1992). In the absence of *zw3* activity, *en* expression expands posteriorly, away from the *wg*-expressing cells, in each segment during gastrulation (Siegfried et al., 1992). After this expansion of *en*, ectopic *wg* stripes appear posterior to the expanded *en* expression domain, resulting in embryos with twice the normal number of *wg* stripes (Siegfried et al., 1992; Fig. 3A). The expansion of *en* stripes also occurs in *zw3; wg* double mutants. The loss of *zw3* activity therefore uncouples *en*-expressing cells from their requirement for *wg*. Notably however, in *zw3; wg* double mutant embryos, all *wg* stripes fade by late stage 11, even though *en* stripes remain broadened (Hooper, 1994; A. S. M. and K. B. Y., unpublished observations). This indicates that, although *wg* activity is no longer required for its paracrine function in *zw3* embryos, *wg* activity is still required for the maintenance of *wg* stripes. Thus *zw3* does not appear to mediate direct *wg* autoregulation.

We examined the expression of *wg* RNA in double mutants for *zw3* and either *dsh*, *arm* or *porc*. Unlike *zw3; wg* double mutants, *zw3 dsh* double mutants display stable wild-type and ectopic *wg* stripes (in addition to broadened *en* stripes), just as in *zw3* single mutants (Fig. 3B; Siegfried et al., 1994). Thus, although *wg* activity is required for maintenance of *wg* transcription in *zw3* embryos, *dsh* is not. In contrast to *zw3 dsh*, all *wg* stripes are lost in *arm zw3* double mutants (Fig. 3C). Since this could be a secondary effect of the complete loss of *en* expression in these double mutants (Siegfried et al., 1994; Peifer et al., 1994), we cannot assess from this experiment alone whether *arm* is required for direct *wg* autoregulation.

In *zw3 porc* embryos, *en* expression is expanded as in *zw3* mutants (Siegfried et al., 1994). Thus if direct *wg* autoregulation requires *porc*, it follows that *wg* transcription would fade in *zw3 porc* even in the presence of this ectopic *en* expression. Indeed, *wg* transcription ceases in *zw3 porc* double mutant embryos (Fig. 3D), just as in *zw3; wg* double mutant embryos. Therefore both *wg* and *porc* are indispensible for the maintenance of *wg* expression, even in the case of the 'constitutive' paracrine *wg* signaling observed in *zw3* mutant embryos (Siegfried et al., 1992).

porc is required for autoregulation by exogenous Wg

In *wg*, *porc*, *dsh* and *arm* embryos, *wg* expression fades during stage 9 (Figs 1 and 2). In order to directly test the requirements of *arm*, *dsh* and *porc* in *wg* autoregulation, we have used the

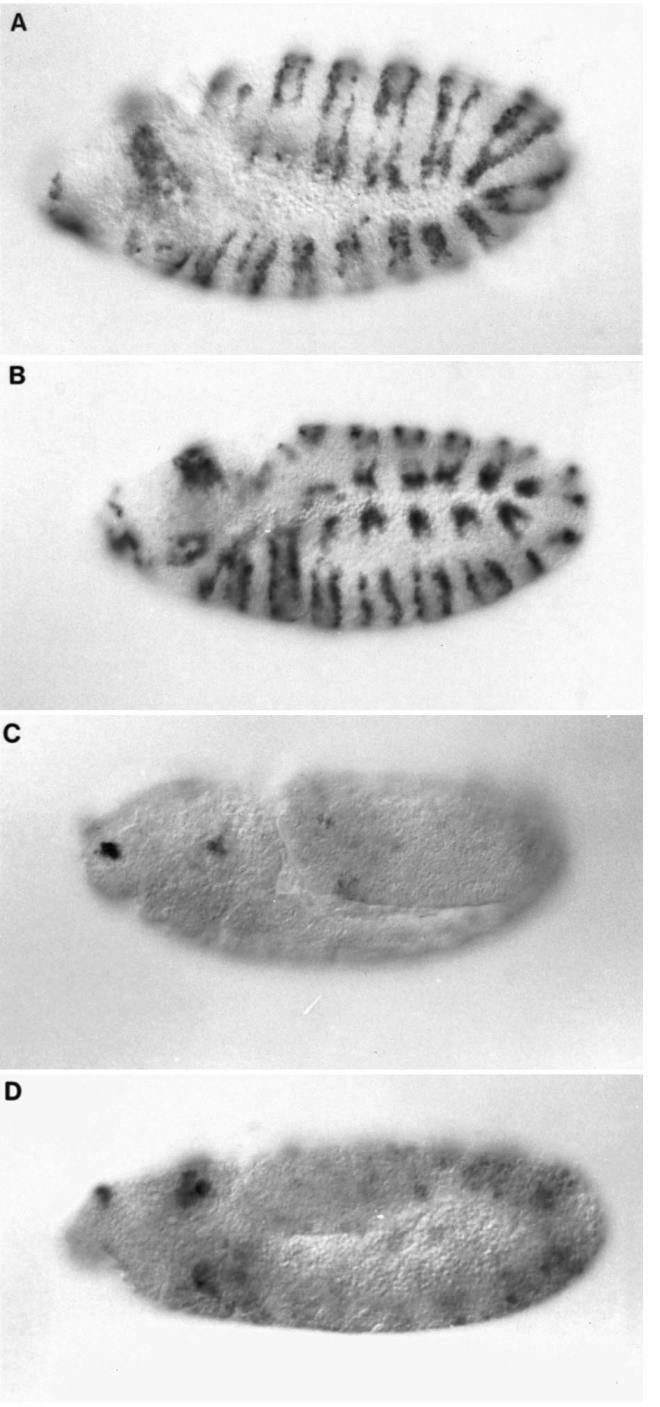

Fig. 3. The epistatic relationship of *dsh*, *arm* and *porc* to *zw3*. Transcription of *wg* is shown in stage 11 embryos lacking maternal and zygotic $zw3^{M11}$ (A), $zw3^{M11}$ dsh^{75} (B), $zw3^{M11}$ arm^{XM19} (C) and $zw3^{M11}$ $porc^{PB16}$ (D) activity. In *zw3* mutant embryos (A), *wg* is expressed in a 28-stripe pattern rather than the wild-type 14-stripe pattern. These ectopic stripes are thought to be induced by the ectopic *en* expression which is apparent in *zw3* embryos (Siegfried et al., 1992). This phenotype is still observed in *zw3 dsh* double mutants (B; Siegfried et al., 1994), but in *zw3 arm* embryos (C) *wg* fades (Siegfried et al., 1994). Since in *zw3 arm* embryos *en* expression fades, the lack of *wg* expression in these double mutants could be secondary to this loss of *en*. In *zw3 porc* embryos (D), *wg* transcripts fade even though *en* stripes persist and are expanded (not shown; Siegfried et al., 1994). Ectopic *wg* stripes initially appear in these embryos with all the *wg* stripes fading by stage 11. This result would appear to be at odds with previous work showing that wild-type and ectopic Wg protein stripes persist in *zw3 porc* double mutants (Siegfried et al., 1994). However, this discrepancy can be explained by the fact that Wg protein is abnormally stable in the absence of *porc* activity (van den Heuvel et al., 1993; Siegfried et al., 1994) and thus is still present in *zw3 porc* embryos even when *wg* transcripts are gone.

GAL4 system (Brand and Perrimon, 1993) to misexpress Wg in the absence of each of these gene products. By utilizing a *hairy (h) GAL4; UASwg* recombinant chromosome (Wilder and Perrimon, 1995), which we will refer to as *h-wg*, we are able to drive exogenous Wg in the spatial pattern of the *h* pair rule gene. This exogenous Wg misexpression persists in the epidermis, overlapping alternate endogenous *wg* and *en* stripes, from stages 8-10 (Yoffe et al., 1995). In *h-wg* embryos, ectopic endogenous *wg* stripes are induced, resulting later in the transformation of alternate denticle belts to naked cuticle in the larva (Yoffe et al., 1995; Wilder and Perrimon, 1995). In *wg*; *h-wg* embryos, alternate endogenous *wg* stripes are often restored while the others fade, leading to 'seven striped' embryos (Yoffe et al., 1995). This incomplete *wg* expression pattern allows us to unambiguously identify *wg* mutant embryos with exogenous Wg. Using *h-wg*, we have previously shown that exogenous Wg can activate endogenous *wg* in *en* mutants, demonstrating an *en*-independent autoregulatory mechanism (Yoffe et al., 1995).

The *dsh* and *arm* genes are both required for paracrine *wg* signaling in the positive regulation of *en* and the specification of naked cuticle (Siegfried et al., 1994; Noordermeer et al., 1994). Consistent with this, we found that in *dsh; h-wg* or *arm; h-wg* embryos *en* expression fades (not shown), and the 'lawn' phenotypes of *dsh* and *arm* are not affected (Fig. 5A,B). However, *h-wg* can activate endogenous *wg* in the absence of either *dsh* or *arm*, based on the persistence of *wg* transcription in *dsh; h-wg* and *arm; h-wg* embryos (Fig. 4B,C). This rescue of alternate endogenous *wg* stripes is reproducible but fairly weak (see Materials and Methods). This inefficiency of *wg* maintenance in the absence of *dsh* or *arm* could be due to the weak activity of *hGAL4* in our experiments (Yoffe et al., 1995), or to the absence of *en* in these embryos (see Discussion). Nonetheless, we conclude that these two components of the *wg* paracrine feedback loop are not absolutely required for Wg to autoregulate in this assay.

In contrast, *h-wg* cannot activate endogenous *wg* in *porc* mutants (Fig. 4D), indicating that *porc* is absolutely required for *wg* autoregulation. We examined Wg protein in *porc; h-wg* embryos and found that exogenous Wg persists at high levels in seven broad *h*-like stripes through stage 12 (Fig. 4E,F), long past the time when *hGAL4* expression ceases in the epidermis (stage 10; Yoffe et al., 1995). Endogenous and exogenous Wg in *porc; h-wg* embryos appears to be restricted within the *wg*-transcribing cells instead of secreted as in wild type (van den Heuvel, 1989, 1993a; Siegfried et al., 1994; Fig. 4E,F). Although expressed with increased stability, this intracellular Wg in *porc* mutant embryos is nonetheless unable to activate the endogenous *wg* gene. However, the absence of *porc* does not completely abolish *wg* activity, since exogenous Wg is capable of restoring *en* expression and naked cuticle in *porc* mutants (Noordermeer et al., 1994; Fig. 5C).

DISCUSSION

Autoregulation and paracrine signaling are distinct activities of *wg*

It has been established that *wg* has a crucial positive role in the maintenance of *en* expression and specification of diverse cell types in the developing embryonic segment (reviewed in Peifer and Bejsovec, 1992; Siegfried and Perrimon, 1994), a role referred to as paracrine signaling. Since *en* is in turn required for *wg* maintenance, then paracrine signaling represents a positive feedback loop that could be the primary mechanism through which *wg* regulates its own transcription. However, *wg* appears to have an autoregulatory function distinct from this paracrine feedback loop, which we have referred to as direct autoregulation. Recently it has been shown that (1) direct autoregulation differs temporally from paracrine *wg* signaling and that (2) epidermal cells appear to require direct exposure to Wg protein in order to express the *wg* gene (Yoffe et al., 1995).

The initial suggestion for direct *wg* autoregulation, however, came from analyses of *ptc* mutant embryos (Bejsovec and Wieschaus, 1993). In *ptc* embryos, epidermal *wg* stripes expand from their normal width of about one cell to a width of approximately half the segment. It has been postulated that, in the absence of *ptc* function, *wg* maintenance no longer requires signaling from the *en*-expressing cells, this signal perhaps being encoded by the secreted *hedgehog (hh)* product (Ingham et al., 1991; Mohler and Vani, 1992; Lee et al., 1992; Tabata et al., 1992). Notably, although *hh* activity is not required for efficient *wg* transcription in *ptc* embryos (Ingham et al., 1991), *wg* activity is still required: in *wg ptc* double mutants, *wg* expression is weak and inconsistent (Ingham and Hidalgo, 1993; Bejsovec and Wieschaus, 1993; Hooper, 1994). Hooper (1994) has used *ptc* mutants to examine potential components of the direct (or autocrine) *wg* autoregulatory pathway. In this work, we have analyzed *wg* autoregulation using three additional approaches. We suggest that *porc*, but not *arm*, *zw3* or *dsh*, is a crucial component of direct *wg* autoregulation.

The role of *porc* in *wg* function

We have shown a crucial role for *porc* in *wg* autoregulation. In the absence of Porc, *wg* expression fades prior to the disappearance of En (Fig. 2D) indicating that, like *wg*, *porc* is required prior to *en* for *wg* maintenance (Yoffe et al., 1995; Fig. 1). We find that exogenous Wg is unable to rescue the lost *wg* expression in *porc* embryos (Fig. 4F). Since exogenous Wg is capable of maintaining *en* expression and specifying naked cuticle in *porc* mutant embryos (Noordermeer et al., 1994; Fig. 5C), we suggest that *porc* is required for direct *wg* autoregulation but not paracrine *wg* signaling (Fig. 6). This possibility is further supported by our observation that, although *zw3* is epistatic to *porc* in the maintenance of *en* and the specification of naked cuticle (Siegfried et al., 1994), *porc* is epistatic to *zw3* in the regulation of *wg* (Fig. 3D).

It has been noticed that, in *porc* mutant embryos, Wg protein appears to be confined to the cells in which it is transcribed (van den Heuvel et al., 1993; Siegfried et al., 1994). While the significance of this confinement and increased stability of Wg in *porc* mutant embryos is unknown, this particular role of *porc* is not crucial for *wg* paracrine signaling (Noordermeer et al., 1994; Fig. 5C). It is possible that proper secretion or processing may be mandatory for Wg to regulate its own transcription. Molecular characterization of the *porc* gene product might give insights into these roles in *wg* function.

dsh, *zw3* and *arm* may not be components of direct *wg* autoregulation

In both *dsh* and *arm* mutant embryos, *wg* RNA expression

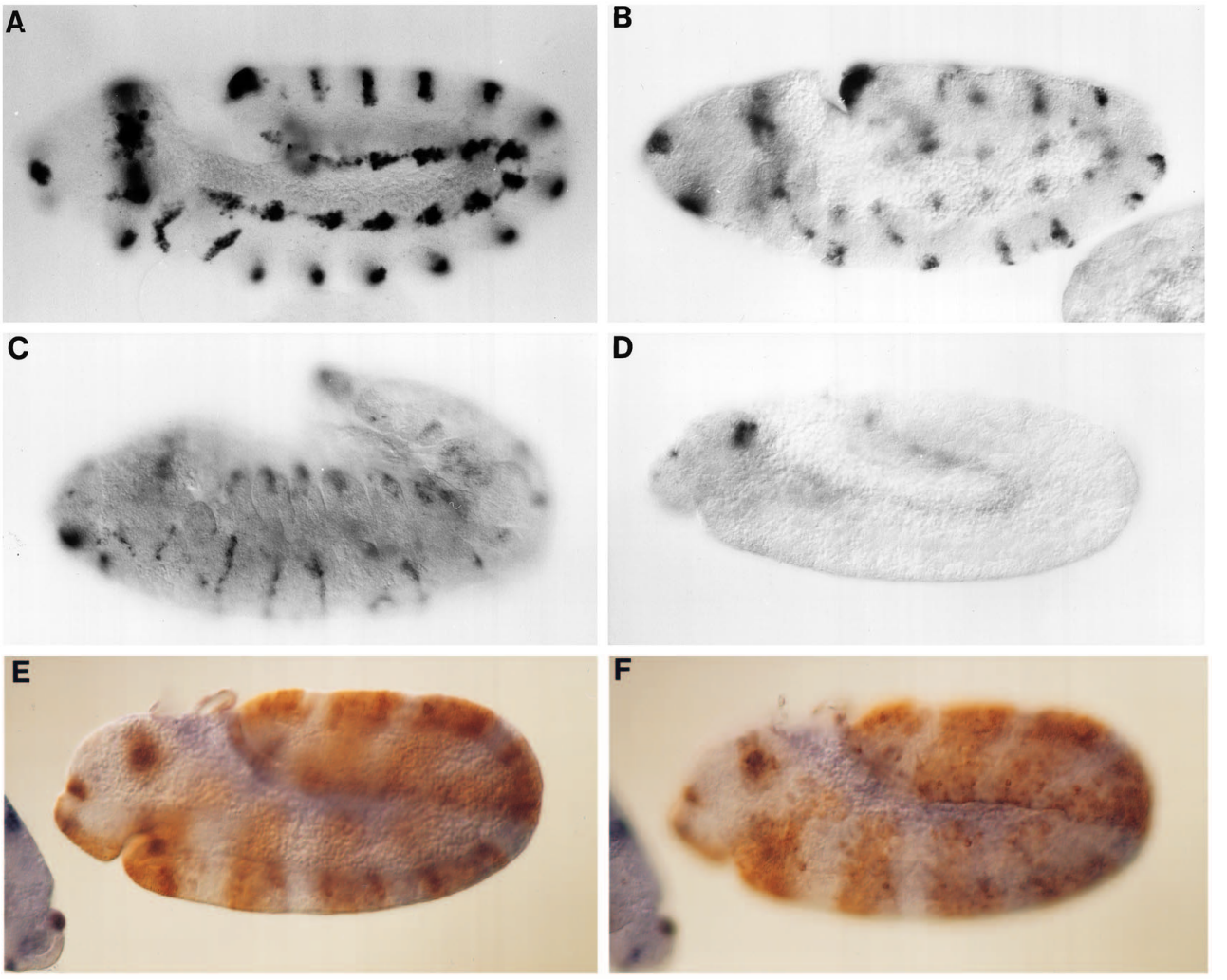

Fig. 4. The autoregulatory effects of exogenous Wg in mutant embryos. Transcription of endogenous *wg* is shown in stage 11-12 wild-type (A), dsh^{75}; *h-wg* (B), arm^{XM19}; *h-wg* (C) and $porc^{PB16}$; *h-wg* (D) embryos. (E,F) $porc^{PB16}$; *h-wg* embryos double-labeled for *wg* transcripts (blue) and Wg (brown). *h-wg* can rescue endogenous *wg* transcription (in alternate segments) in the absence of *dsh* and *arm* activity (B,C), resulting in 'seven-stripe' embryos. The efficiency of this rescue is fairly low; thus restored *wg* stripes are rarely as robust as in wild type and not all mutant embryos expressing *h-wg* display *wg* expression in all seven segments. Further, most mutant embryos expressing *h-wg* show rescue of *wg* stripes in adjacent segments, due to the broad expression of *hGAL4*, for example, the 10-striped embryo in B and the 8-striped embryo in C (Yoffe et al., 1995). We have also tested dsh^{v26} with our h-wg assay and it behaved identically to dsh^{75} (not shown). In the absence of *porc*, *h-wg* cannot rescue *wg* transcription (D). (E,F) A *porc*; *h-wg* embryo double labeled for *wg* protein (Wg) (brown) and *wg* transcripts (blue). In these embryos, *wg* transcription is lost from the trunk and the seven Wg stripes are evident. The seven broad exogenous Wg protein stripes are still present and stable until the end of stage 12, due to the increased stability of Wg in the absence of *porc* (van den Heuvel et al., 1993; Siegfried et al., 1994).

fades following the disappearance of En (Fig. 2B). Since *en* activity is required for *wg* maintenance (Martinez-Arias et al., 1988; Bejsovec and Martinez-Arias, 1991), these results imply that the loss of *wg* in *dsh* and *arm* mutants is a secondary consequence of the loss of paracrine *wg* signaling, and not directly due to a block in direct *wg* autoregulation. Hh, which is co-expressed with *en* in the epidermis, is postulated to encode a secreted factor involved in the regulation of *wg* transcription. We have not followed the expression of Hh in these embryos. It is possible that *hh* is differentially regulated in *porc* versus *dsh* or *arm* embryos - whether this difference is detectable at the level of Hh antibody staining is unclear. It is therefore possible that the difference between *porc* and *dsh* or *arm* in our experiments could be due to the differential regulation of *hh* transcription or activity in these mutants. As Hh protein enters the *wg*-transcribing cells, we cannot exclude the possibility that *hh* functions in both paracrine and direct *wg* autoregulation. Hooper (1994) has suggested that autocrine (or direct) *wg* autoregulation is *hh*-independent. In any case, we have demonstrated a relevant difference in the regulation of *wg* transcription in *porc* versus *dsh* or *arm* embryos (Figs 2, 3 and 4). We suggest that *dsh* and *arm*, two crucial positive mediators of *wg* paracrine signaling (Noordermeer et al., 1994; Siegfried et al., 1994), do not appear to be crucial for direct *wg* autoregulation (Fig. 6).

zw3 acts between *dsh* and *arm* in the *wg* paracrine signaling

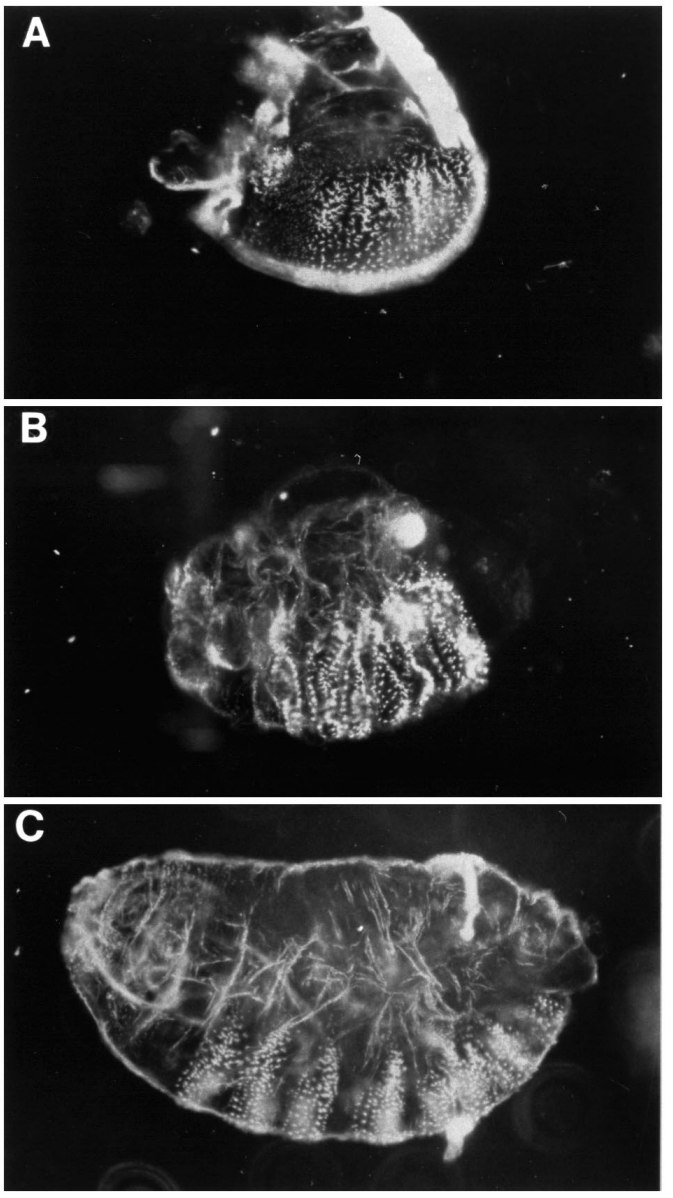

Fig. 5. The effects of exogenous Wg on *dsh*, *arm* and *porc* mutant cuticles. The cuticle phenotypes of dsh^{75}; *h-wg* (A), arm^{XM19}; *h-wg* (B) and $porc^{PB16}$; *h-wg* (C) are shown. Consistent with previous work (Noordermeer et al., 1994), the presence of exogenous Wg has no effect on the cuticle phenotype of *dsh* or *arm* mutants (compare Fig. 5A to Fig. 2A), while naked cuticle can be restored by exogenous Wg in *porc* mutants (compare Fig. 5C to Fig. 2C).

pathway (Siegfried et al., 1994). In the absence of *wg* signal, the *zw3* kinase directly or indirectly inactivates Arm by phosphorylation, changing Arm's concentration and intracellular distribution (Siegfried et al., 1994; Peifer et al., 1994). In *zw3* mutants, Arm (and thus *wg* signaling) is thought to be active ubiquitously, leading to ectopic *en* expression and naked cuticle (Peifer et al., 1994). Thus *zw3*, in a negative manner, mediates the paracrine *wg* functions. In contrast, *zw3* does not mediate direct *wg* autoregulation, since *wg* is still required for its own maintenance in *zw3* mutants (Hooper, 1994; A. S. M. and K. B. Y., unpublished observations). As *zw3* functions

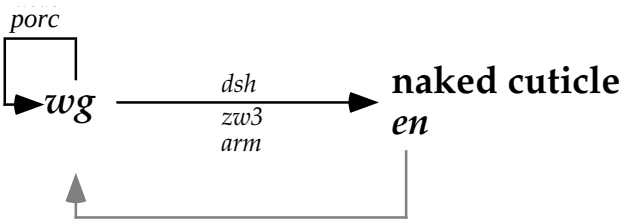

Fig. 6. The two pathways through which *wg* regulates its own transcription. One pathway involves *porc* and is required for 'direct' *wg* autoregulation. The second pathway involves the regulation of *en* in neighboring cells. *en* is in turn required for the maintenance of *wg* transcription, thus completing a 'paracrine feedback loop'. The latter pathway includes the *dsh*, *zw3* and *arm* genes and is also required for the specification of naked cuticle and the generation of cell type diversity.

downstream of *dsh* and upstream of *arm* in the paracrine *wg* signaling pathway (Noordermeer et al., 1994; Siegfried et al., 1994; Peifer et al., 1994), the exclusion of *zw3* from direct *wg* autoregulation provides further evidence for the exclusion of *dsh* and *arm* in this pathway. We can therefore only propose the inclusion of *porc* in the direct *wg* autoregulatory pathway.

As Wg is a secreted molecule, the existence of transcription factor(s) mediating *wg* autoregulation must be postulated. The *gooseberry* protein (Gsb) has been shown to be involved in an autoregulatory loop with *wg*, perhaps functioning as a transcription factor (Li and Noll, 1993). Therefore, it is possible that the *dsh*- or *arm*-independent autoregulation of *wg* in our *h-wg* experiments may be mediated by *gsb*. As *gsb* expression is a target of *wg* signaling as is *wg* expression (Li and Noll, 1993), this would again indicate a *dsh*- or *arm*-independent function of Wg.

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