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# Mutations in the segment polarity genes *wingless* and *porcupine* impair secretion of the wingless protein

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**We have characterized the molecular nature of mutations in *wingless* (*wg*), a segment polarity gene acting during various stages of *Drosophila* development. Embryo-lethal alleles have undergone mutations in the protein-encoding domain of the gene, including deletions and point mutations of conserved residues. In a temperature sensitive mutation, a conserved cysteine residue is replaced by a serine. In embryo-viable alleles, the *wg* transcriptional unit is not affected. Immunostaining of mutant embryos shows that the embryo-lethal alleles produce either no wg antigen or a form of the protein that is retained within cells. Interestingly, embryos mutant for the segment polarity gene *porcupine* show a similar retention of the wg antigen. We have also transfected wild type *wg* alleles into *Drosophila* tissue culture cells, which then display wg protein on the cell surface and in the extracellular matrix. In similar experiments with mutant alleles, the proteins are retained in intracellular compartments and appear not to be secreted. These data provide further evidence that wg acts as a secreted factor and suggest that porcupine provides an accessory function for wg protein secretion or transport.**

*Key words: Drosophila/porcupine*/segment polarity genes/*wingless*/*Wnt* gene

## Introduction

The *wingless* (*wg*) gene of *Drosophila* has various functions during development, ranging from early embryonic patterning to imaginal disc cell specification (Nüsslein-Volhard and Wieschaus, 1980; Baker, 1987; Bejsovec and Martínez-Arias, 1991; Couso *et al.*, 1993; Struhl and Basler, 1993; reviewed in Peifer and Bejsovec, 1992; Cohen and DiNardo, 1993). In all of these processes, it appears that *wg* is involved in intercellular communication. In the embryo, wg regulates the expression of the *engrailed* gene, but is itself expressed immediately anterior to the cells making engrailed protein (DiNardo *et al.*, 1988; Martínez-Arias *et al.*, 1988; van den Heuvel *et al.*, 1989). *wg* encodes a cysteine-rich protein with a signal sequence (Rijsewijk *et al.*, 1987a) that by whole mount immunostaining can be detected between cells (van den Heuvel *et al.*, 1989; González *et al.*, 1991). Consistent with a function as an intercellular signal is the non-autonomous behaviour of *wg* mutant cells (Morata and Lawrence, 1977; Baker, 1988).

*wg*, a member of the rapidly growing *Wnt* gene family (Nusse and Varmus, 1992) is genetically well characterized, but less is known of the biochemistry of the wg protein than of its mammalian ortholog, Wnt-1. *Wnt*-1-transfected tissue culture cells secrete Wnt-1 protein, but with low efficiency (Papkoff, 1989). Wnt-1 protein can be detected on the surface of cells (Papkoff and Schryver, 1990) and in extracellular matrix fractions (Bradley and Brown, 1990). The *Wnt*-1 gene also functions in paracrine assays (Jue *et al.*, 1992). In such assays, mammary epithelial cells, which in direct transfections become morphologically transformed by *Wnt*-1 (Brown *et al.*, 1986; Rijsewijk *et al.*, 1987b), show a transformed phenotype when cocultivated with Wnt-1-expressing cells. Further evidence for secretion of the Wnt-1 protein has been provided by mutational analysis. Wnt-1 mutants lacking the signal sequence are non-functional in both autocrine and paracrine assays (McMahon and Moon, 1989; Mason *et al.*, 1992). Site-directed mutations in some of the cysteine residues that are conserved in the protein products of most members of the large *Wnt* gene family impair activity of Wnt-1 in several assays (McMahon and Moon, 1989; Mason *et al.*, 1992).

Although most available evidence indicates that Wnt proteins are secreted, there are many unresolved aspects on the mechanism by which this is achieved. Free Wnt protein has not been seen in the tissue culture medium of cells transfected with Wnt expression constructs. Extracellular forms of the protein can only be found when the cells are incubated in the presence of charged polymeric molecules such as suramin or heparin (Papkoff, 1989; Bradley and Brown, 1990) which are thought to release proteins from the extracellular matrix and/or cell surface. Most of the Wnt protein in transfected cells accumulates in the endoplasmic reticulum, has been incompletely processed (Brown *et al.*, 1987; Papkoff *et al.*, 1987) and is associated with the resident ER protein, BiP (Kitajewski *et al.*, 1992). These observations suggest that correct folding and secretion of Wnt protein is dependent on an accessory protein, and that the limited presence of such an accessory protein in many cultured cells would impair efficient secretion. There is indeed variability in the efficiency with which mammalian cell lines secrete Wnt protein after transfection (Papkoff, 1989). With respect to the product of the *wg* gene, some reports have even claimed that the protein is not secreted. Chakrabarti *et al.* (1992) expressed the wg protein in *Xenopus* oocytes by injection of *wg* mRNA and could detect wg protein in the medium only in the presence of suramin. The effect of suramin was, however, explained by release from intracellular storage and not by release from the cell surface. In addition, wg protein could not be detected outside *Xenopus* embryo cells by immunohistochemistry. In a study of *Drosophila* embryos, González *et al.* (1991) concluded

that the wg protein is not released from cells but was actively transported to target cells in a membrane bound form.

In this paper, we present a biochemical analysis of synthesis of the wg protein. We have found that, in tissue culture cells, wild type wg protein is present on the cell surface, in the culture medium and in the extracellular matrix. We have also analyzed the molecular nature of a number of existing *wg* mutations and studied the gene products of mutant alleles. It appears that mutant wg proteins are retained within cells. Embryos mutant for another segment polarity gene, *porcupine* (*porc*), show a similar secretion defect, suggesting that the product of *porc* provides an accessory function for wg secretion.

## Results

### *Mapping of wg alleles*

To establish the sites of mutation in *wg* alleles, we isolated several additional *wg* genomic clones from a wild type *Drosophila* genomic library. The region obtained in clone extends from 11 kb 5′ from the approximate transcription start to 12.4 kb 3′ from the transcription end. Restriction maps of the clones with the positions of the exons of the *wg* gene as established previously (Rijsewijk *et al.*, 1987a) are shown in Figure 1A.

We then determined the molecular lesions in the homozygous viable allele, $wg^1$ (Sharma and Shekaran, 1983), and the pupa-lethal alleles, $wg^{CX3}$ and $wg^P$, by restriction enzyme analysis. Previously, Baker (1987) mapped these lesions on the *wg* locus, but since the structure of the gene was only partially established, the results were not accurate relative to the transcription unit. In $wg^1$ homozygous flies, a 2.5 kb deletion located 9 kb downstream of the end of transcription was detected. DNA from $wg^{CX3}$ flies contained an insert of a large piece (≥17 kb) of unknown DNA between 2 and 4 kb from the transcriptional endpoint of the gene. $In(2L)wg^P$ is described cytologically as a large inversion with breakpoints at 28A1-3 and 32E-F (see Baker, 1988). We mapped the distal breakpoint to 9−11

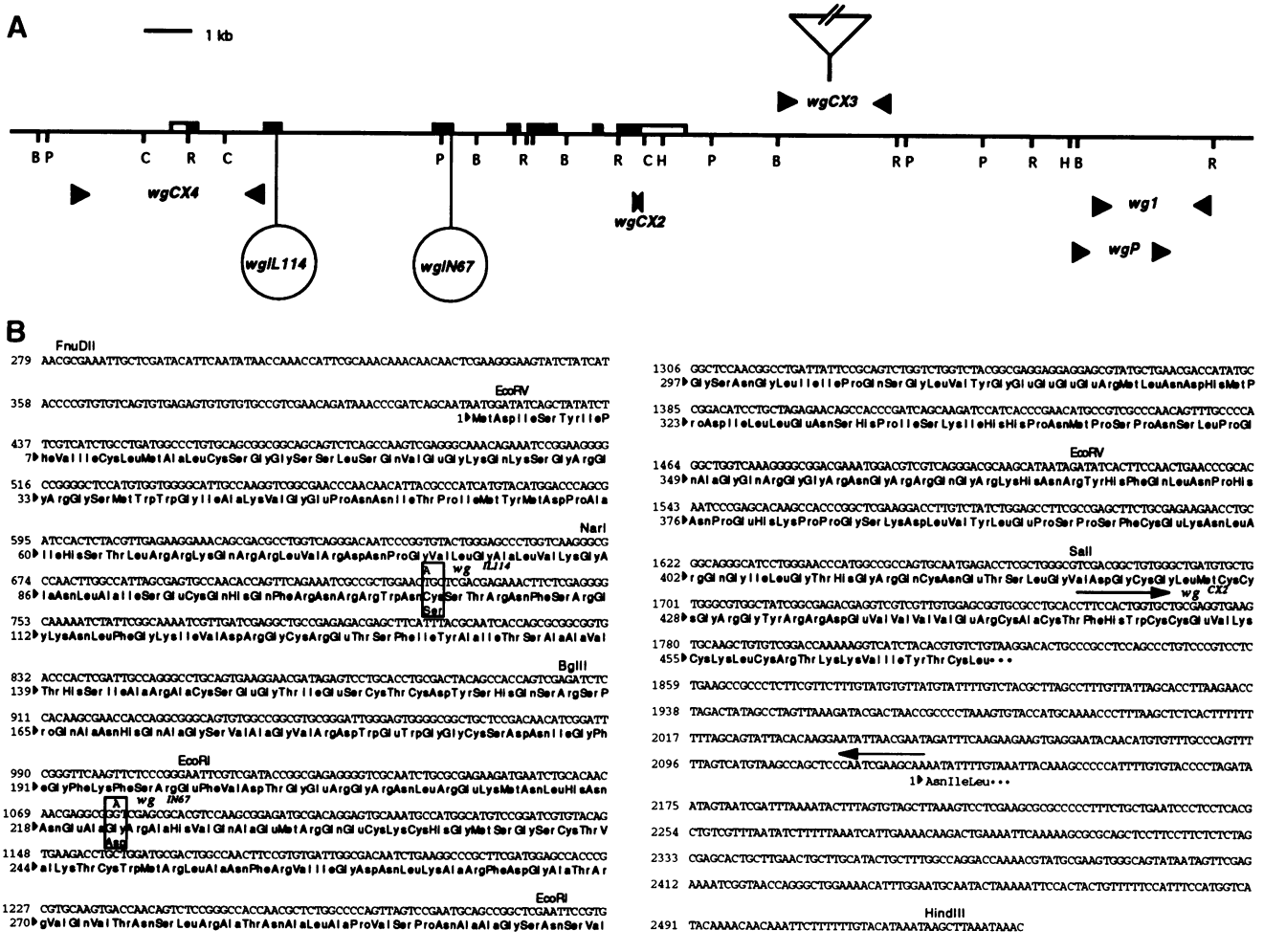


**Fig. 1.** (**A**) Schematic representation of the *wg* locus and mutations. A genomic map of the *wg* locus relative to its transcription unit is shown (5′ at left). The open boxes represent untranslated *wg* sequences while the closed boxes represent translated. The open triangle represents the insertion (at least 17 kb) in the $wg^{CX3}$ allele. In $wg^{CX4}$, $wg^{CX2}$ and $wg^1$ sequences are deleted (the limits of the deletions are indicated by the arrowheads). In the $In(2L)wg^P$ inversion mutant, the distal breakpoint occurs between the two proximally pointing arrowheads. The locations of the amino acid changes in wgIL114 (amino acid position 104) and wgIN67 (amino acid position 221) are indicated. R: *Eco*RV, C: *Cla*I, B: *Bam*HI, P: *Pst*I, H: *Hind*III, X: *Xba*I. (**B**) Sequence of *wg* cDNA, showing the positions of the mutations. Numbering is according to Rijsewijk *et al.* (1987a). The open boxes indicate the base pair and amino acid changes in the alleles $wg^{IL114}$ (nucleotide 727; amino acid 104) and $wg^{IN67}$ (nucleotide 1079; amino acid 221). The deletion in $wg^{CX2}$ is indicated by arrows. At the site of deletion an extra G is inserted; the last codon before the deletion is therefore still intact. Above the sequence, restriction enzyme sites are indicated that were used to clone the fragments containing the mutations back into the cDNA. The *Fnu*DII site at the 5′ end was used to clone the cDNA into shuttle vectors.

kb 3′ from the transcriptional endpoint of the gene (Figure 1A). Therefore, in all of the embryo-viable alleles analyzed here, alterations occur 3′ of the transcribed region of the gene and do not disrupt the transcriptional unit. It is known that *wg* is required during imaginal development (Baker, 1988), and our results suggest that regions 3′ of the transcription unit contain sequences essential for correct expression of *wg* during this stage of development. The small deletion in $wg^{1}$ identifies a region that is not required for adult viability but its absence can lead to transformations of distal structures in the wing into more proximal ones. In the two pupa-lethal alleles, similar defects in the wing are seen along with many other axial patterning defects; in both of these alleles the same region is disrupted as in $wg^{1}$.

Next, we analyzed embryo-lethal alleles of *wg* generated by chemical (Nüsslein-Volhard *et al.*, 1984) and X-ray (Baker, 1987) mutagenesis (Table I). The phenotypes of these alleles are similar: cuticle patterns are largely identical and expression of *engrailed*, which is dependent on *wg*, decays in a similar manner. At the end of stage 9 of development, hardly any engrailed antigen is detected in the germband of homozygous mutant embryos (not shown). One of the chemically induced alleles is temperature sensitive ($wg^{IL114}$). At 16−18°C the expression of *engrailed* in the embryo and the larval cuticle phenotype are the same as wild type (data not shown). At the non-permissive temperature (25−29°C), this allele displays the embryo-lethal phenotype (Nüsslein-Volhard *et al.*, 1984; Baker, 1988; Bejsovec and Martínez-Arias, 1991).

Embryos homozygous for the $wg^{CX4}$ allele have no *wg* mRNA (data not shown) and carry a 2.7 kb deletion (Figure 1A), resulting in removal of the first exon. Embryos mutant for $wg^{CX2}$, $wg^{IN67}$ and $wg^{IL114}$ display wg protein. RNA was isolated from single embryos or from heterozygous pupae of these strains and converted into cDNA which was then used as a template for the polymerase chain reaction (PCR). The products of the reactions were either used directly for sequencing or first cloned. We confirmed the changes by analyzing several PCR-generated clones.

In $wg^{CX2}$, a deletion of 374 bp was detected within the fifth exon, with an extra base pair (G) inserted at the site of the deletion (Figure 1A and B). This results in a mutant protein shorter by 20 amino acids: the deletion removes 23 amino acids from the carboxy-terminus and adds three amino acids from codons of the normally untranslated trailer (Figure 1B). In the $wg^{IN67}$ allele, a single base pair change (G to A; nucleotide 1079; Figure 1A and B) was found. In the encoded protein, a glycine at amino acid 221 is changed to an aspartic acid (Figure 1B). In the temperature sensitive mutation $wg^{IL114}$, a single base pair change (T to A) was detected, mapping to nucleotide 727 (Figure 1A and B). This leads to a serine replacing a cysteine at amino acid 104 (Figure 1B).

### *Patterns of wingless RNA and protein in mutant embryos*

In all embryo-lethal alleles, except for $wg^{CX4}$ (see above), *wg* mRNA was found in a striped pattern initially indistinguishable from wild type embryos. During stage 10, however, expression in mutants started to deviate from that in wild type embryos. The mRNA disappeared first from the dorsal side, while ventrally a few cells still expressed the gene. During stage 10 and early stage 11, *wg* expression had completely disappeared from the germband region (Table I).

To examine the wg protein produced by the embryo-lethal alleles, we stained embryos with an anti-wg antibody. Many mutant strains showed either no or very weak wg antigen staining (Figure 2C and Table I). Three embryo-lethal alleles, $wg^{CX2}$, $wg^{IN67}$ and $wg^{IL114}$, expressed wg antigen

**Table I.** Summary of the results of the characterization of the various *wingless* alleles

| Allele | Phenotype | Embryonic expression | | Molecular defect |
|---|---|---|---|---|
| | | mRNA | Protein | |
| $wg^{1}$ | viable | + | + | ~2.5 kb deletion 3′ of end transcript |
| $wg^{CX3}$ | pupa-lethal | + | + | ~17 kb insertion 3′ of end transcript |
| $In(2L)wg^{P}$ | pupa-lethal | +[a] | +[a] | breakpoint ~9−11 kb 3′ of end transcript |
| $wg^{CX4}$ | embryo-lethal | − | − | deletion of 2.7 kb (deletes first exon) |
| $wg^{CX2}$ | embryo-lethal | + | + | deletion of 374 bp (deletes carboxy-terminal 23 amino acids) |
| $wg^{IG22}$ | embryo-lethal | + | −[b] | N.D. |
| $wg^{IJ69}$ | embryo-lethal | + | −[b] | N.D. |
| $wg^{IID23}$ | embryo-lethal | + | −[b] | N.D. |
| $wg^{7L74}$ | embryo-lethal | + | −[b] | N.D. |
| $wg^{IIS34}$ | embryo-lethal | + | −[b] | N.D. |
| $wg^{IN67}$ | embryo-lethal | + | + | Gly221→Asp |
| $wg^{IL114}$ | embryo-lethal (conditional) | + | + | Cys104→Ser |

Some of the mapping of the embryo-viable alleles has been described before (Baker, 1987). N.D.: not determined.
[a]In homozygous $wg^{P}$ embryos only seven *wg* stripes are detected, in a pair-rule pattern. No abnormal pattern is seen in $wg^{P}/wg^{lethal}$ embryos. The defect in $wg^{P}$ homozygous embryos could arise because the inversion uncovers an unknown pair-rule locus (see also Materials and methods).
[b]Faint staining for wg antigen can be detected only at early germband to elongated stages.

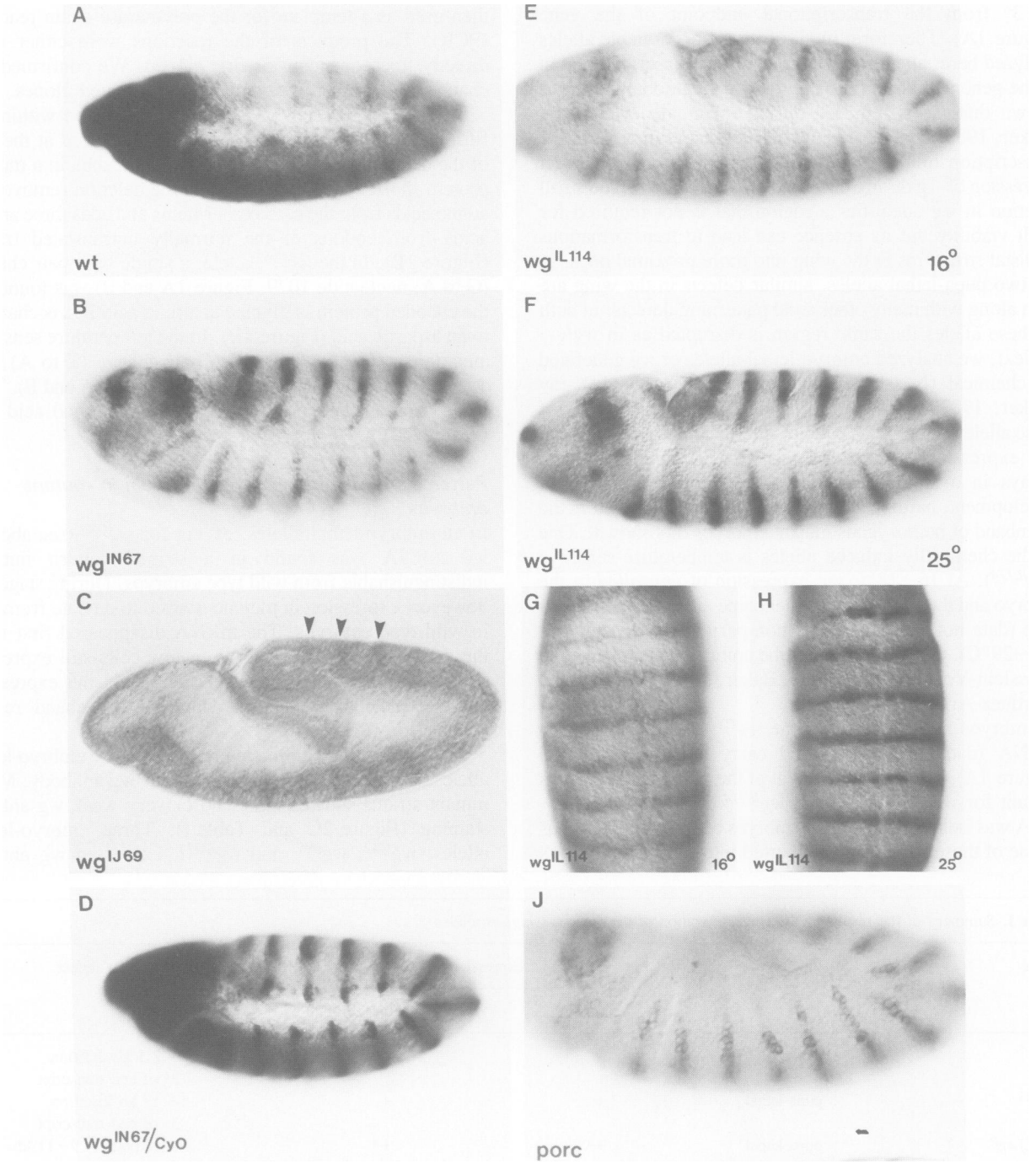


**Fig. 2.** Localization of wg protein and β-galactosidase in wild type, heterozygous mutant and homozygous mutant embryos. Stocks contained balancer chromosomes with a *hunchback−LacZ* construct; the genotypes of embryos could be determined by the extent of β-galactosidase staining. Whole mount embryos photographed with differential interference contrast microscopy. (**A**) Wild type embryo (*CyO hb-β*-gal/*CyO hb-β*-gal) (stage 10). The staining for β-gal extends over the curve of the germband. Note the diffuse appearance of the wg staining. (**B**) Homozygous mutant (stage 11) ($wg^{IN67}$) embryo. wg staining is in sharp bands, more intense than in wild type embryos. (**C**) Mutant ($wg^{IJ69}$) embryo (stage 9). Arrowheads indicate faint stripes of wg staining. (**D**) Heterozygous embryo ($wg^{IN67}$/*CyO hb-β*-gal) (stage 10). The β-gal staining is now limited to an area including only the first abdominal segments. Note the strong, limited staining of wg. (**E**) Homozygous mutant ($wg^{IL114}$ at 16°C) embryo, stage 10. No β-gal staining is present. wg staining is similar to wild type. (**F**) Homozygous mutant ($wg^{IL114}$ at 25°C) embryo, stage 10. wg staining is seen in sharp bands. (**G**) Homozygous mutant ($wg^{IL114}$ at 16°C) embryo, ventral view. (**H**) Homozygous mutant ($wg^{IL114}$ at 25°C) embryo, ventral view. (**J**) Embryo (stage 10) derived from $porc^{PB16}$ germline clone and stained with an anti-wg antibody. Note the sharp appearance of the stripes, similar to those in the *wg* mutant embryos.

(Figure 2, Table I). Antigen was detected to stage 12/13 mostly in non-epidermal cells (at this stage many epidermal cells have died in *wg* mutant embryos (Klingensmith *et al.*, 1989). Since *wg* mRNA has decayed in these mutant embryos by stage 11, the mutant wg proteins appear to be stable for at least 2 h.

Although the global pattern of wg antigen in $wg^{CX2}$, $wg^{IN67}$ and $wg^{IL114}$ embryos is similar to wild type, we

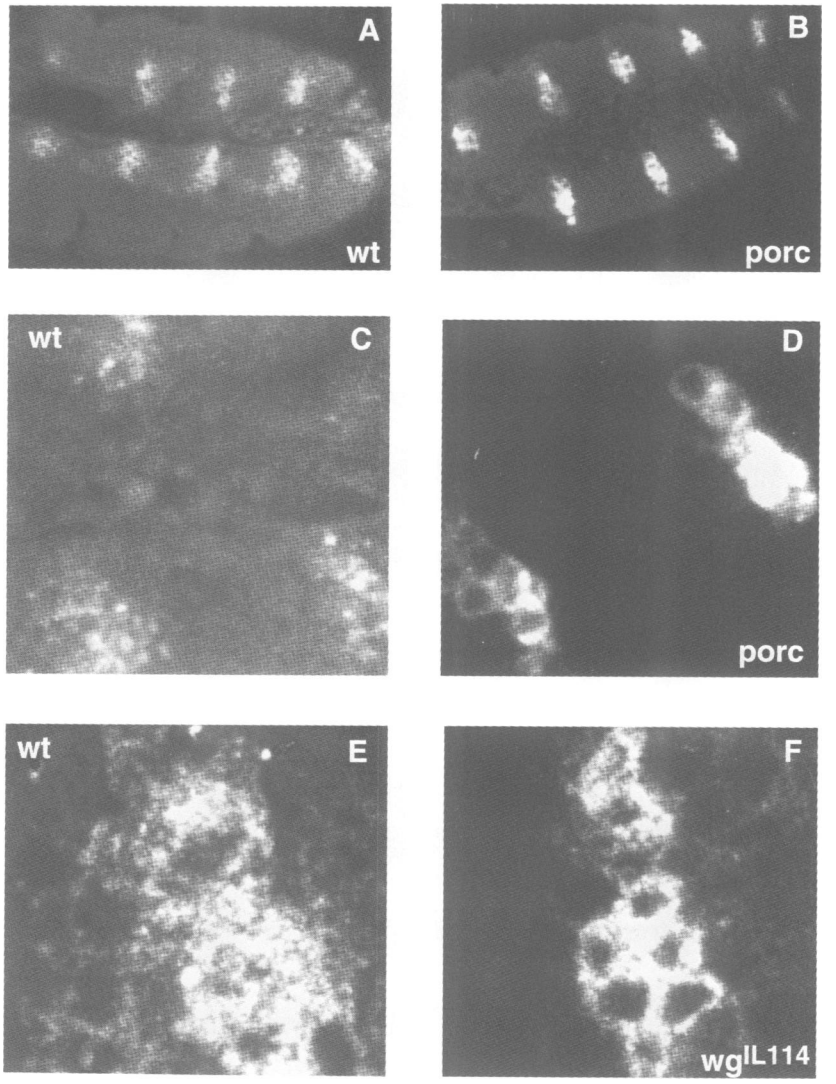


**Fig. 3.** Localization of wg protein in wild type and mutant embryos by confocal laser scanning microscopy. (**A**) Wild type embryo (stage 10/11). The scan visualizes the dorsal-lateral patches of wg expression along the germband. Circular spaces devoid of staining correspond to the cell nuclei. Note the diffuse appearance of the staining. (**B**) Mutant embryo (stage 10/11) derived from *porc* germline clone. The scan shows approximately the same area of an embryo as shown in (A). Note the confined localization of the wg antigen. (**C** and **E**) Magnification of stained areas of wild type embryos. The diffuse distribution of antigen includes many strong dots of staining. (**D**) Magnification of two stained areas of the *porc* embryo shown in (C). The antigen seems to be confined to the cytoplasmic area of wg-expressing cells. (**F**). Part of a stripe of a *wg* mutant embryo ($wg^{IL114}$ at 29°C). Note the non-diffuse appearance of wg staining and the absence of dots.

noted a change in the subcellular localization of the antigen. In contrast to the diffuse stripes of staining seen in wild type embryos (Figure 2A), the mutant embryos displayed sharply demarcated bands. The staining was always very strong (Figure 2B) relative to wild type embryos in the same batch (Figure 2A). In embryos homozygous for $wg^{IL114}$, staining was more diffuse at the permissive temperature (16°C, Figure 2E and G) than at the non-permissive temperature (Figure 2F and H). A similar observation was reported by González *et al.* (1991).

As an additional means to study the location of the wild type and mutant proteins, we used the confocal laser scanning microscope. Wild type embryos showed wg antigen in stripes, in a diffuse pattern and in intensely labeled dots (Figure 3A, C and E) possibly corresponding to similar dots seen in the light microscope (van den Heuvel *et al.*, 1989; González, 1991). In $wg^{IL114}$ mutant embryos at the non-permissive temperature, wg staining appeared confined to an area around the nuclei of the cells expressing the protein. No antigen was concentrated in the dots characteristic of wild type wg staining (Figure 3F).

### *wg protein distribution in wg mutants is similar to that in porc mutants*

We have performed a survey of the distribution of wg protein in embryos mutant for other segment polarity genes. Most of these mutations do not affect the cellular localization of the wg protein (not shown), except for the segment polarity gene *porcupine* (*porc*). The gene product of *porc* is contributed maternally as well as zygotically and its phenotype can be detected in embryos derived from female germline clones. The cuticle phenotype of *porc* is very similar to that of *wg*. Like *wg* mutants, *porc* mutant embryos show stable wg protein throughout most of embryogenesis in an altered subcellular pattern. In the light microscope, the *porc* mutant embryos show the same intense and confined

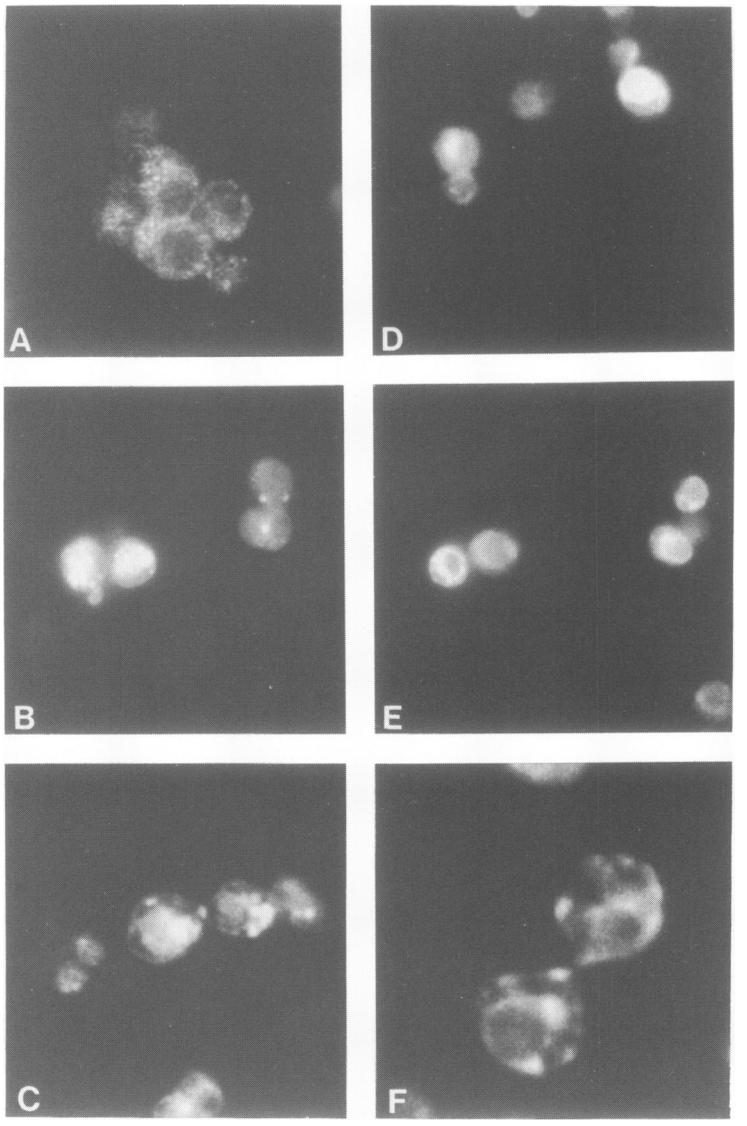


**Fig. 4.** Localization of wg protein in transfected cells. Confocal laser scanning microscope images (A, C and F) and fluorescence microscope photographs (B, D and E) of permeabilized Schneider cells. (**A**) Cells expressing wild type *wg*, fixed with formaldehyde. Note the appearance of *wg* antigen in small dots. (**B**) Cells expressing wild type *wg*, fixed with formaldehyde. (**C**) Cells expressing wild type *wg*, fixed with methanol. (**D**) Cells expressing $wg^{IL114}$, at 25°C, fixed with formaldehyde. Note the perinuclear location of staining. (**E**) Cells expressing $wg^{IL114}$ at 18°C, fixed with formaldehyde. Note the presence of both perinuclear staining and small dots. (**F**) Cells expressing $wg^{IN67}$, fixed with methanol.

staining pattern of wg antigen (Figure 2J). As seen in the confocal laser scanning microscope, the protein appears confined to the cell (Figure 3B and D) when compared with the diffuse nature of the staining in wild type embryos (Figure 3A, C and E). In *porc* mutant embryos we found that the *wg* mRNA disappears as it does in *wg* embryos (data not shown), while the wg protein persists.

The distribution of the wg protein in wild type and mutant embryos suggests that mutant proteins are not secreted but accumulate within cells. Furthermore, embryos mutant for *porc* show the same defect, suggesting that the porc protein functions to promote wg secretion. It should be noted, nevertheless, that the subcellular locations of the mutant proteins appear dominant over the wild type protein. Heterozygous embryos (which develop normally) display the confined staining pattern quite prominently (Figure 2D). Possibly, the intense staining of the intracellular mutant wg protein obscures the weaker staining of the secreted protein produced by the wild type allele. In addition, embryos derived from *porc* mutant germlines can be paternally rescued; these rescued embryos also show prominent retention of the wg protein (E.Siegfried, E.Wilder and N.Perrimon, submitted).

### *Expression of wild type and mutant wg in cultured cells*

We further investigated the wg protein in a tissue culture system. Wild type *wg* cDNA, an antisense *wg* cDNA control and *wg* cDNAs carrying *wg* mutations were cloned into an expression vector under the control of the *Drosophila* metallothionein promoter (Koelle *et al.*, 1991) and transfected into *Drosophila* Schneider 2 (S2) cell lines.

We first located the wg antigen in these cells by immunostaining. In permeabilized cells expressing wild type protein, staining for wg was localized in dots (Figure 4A, B and C). In similarly treated cells expressing mutant forms ($wg^{CX2}$ and $wg^{IN67}$) of the protein, staining was restricted to the perinuclear space, suggestive of the endoplasmic

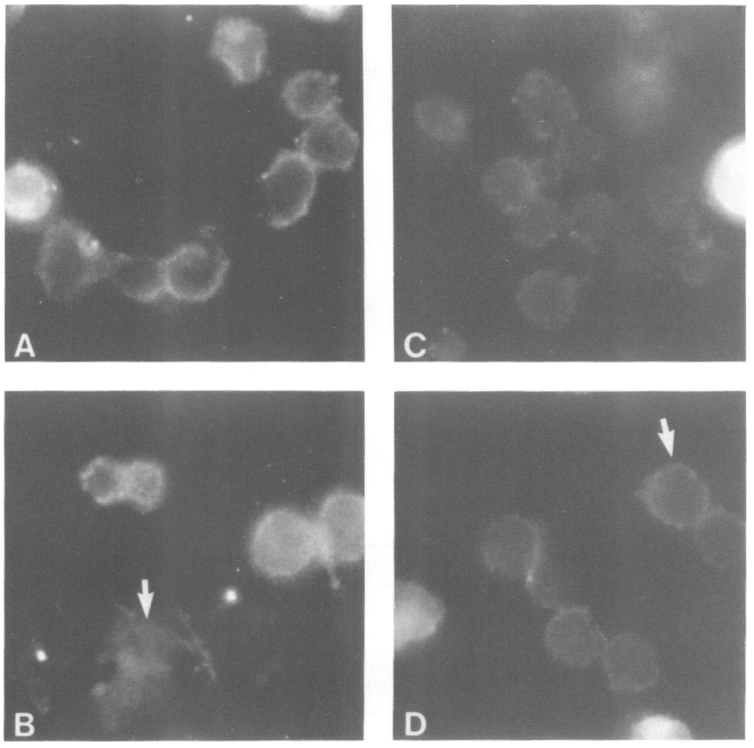


**Fig. 5.** Localization of wg protein in transfected cells. Fluorescence microscopy of non-permeabilized Schneider cells. **(A)** Cells expressing wild type *wg*. Note the cell surface staining. **(B)** Cells expressing wild type *wg*. Arrow points to non-cellular material that is stained and most likely is extracellular matrix material. **(C)** Cells expressing $wg^{IN67}$ at 18°C. **(D)** Cells expressing $wg^{IL114}$ at 18°C. Note the weak cell surface staining (arrow).

(Figure 4F). The location of the $wg^{IL114}$-encoded protein was to some extent dependent on the temperature. At the non-permissive temperature (25°C), the pattern of staining was very similar to the non-conditional mutants: mostly localized to the perinuclear space (Figure 4D). At the permissive temperature (16−18°C), wg antigen was also localized to smaller vesicles as seen in cells transfected with wild type cDNA, although the perinuclear staining was still prominent (Figure 4E). The fixation conditions changed the appearance of wild type staining in cells producing wg protein. Small dots were seen in formaldehyde-fixed cells, and larger ones in cells fixed with methanol (compare Figure 4A and C).

When wild type cDNA transfected cells were not permeabilized, wg antigen was observed at the cell surface (Figure 5A). Additionally, material deposited by the cells on to the plastic of the tissue culture dish, which most likely represents an extracellular matrix fraction, stained for wg antigen (Figure 5B). In non-permeabilized cells expressing mutant proteins ($wg^{CX2}$, $wg^{IN67}$ and $wg^{IL114}$ at 25°C), no antigen was detected at the cell surface or on the tissue culture dish (Figure 5C). However, in cells expressing the $wg^{IL114}$ protein, wg antigen was detected on the cell surface at the permissive temperature (18°C, Figure 5D), although the surface staining was less intense than that of cells expressing wild type wg (compare Figure 5D with A).

### *Immunoprecipitation and immunoblotting of wg protein*

The staining results in embryos and in transfected cells indicate that wild type wg protein is secreted from the cell, but that the mutant forms are not. To substantiate these results, we immunoprecipitated wg protein from transfected and metabolically labeled cells. A protein species of 52 kDa, corresponding to the expected size of the wg protein, was found in the medium of cells expressing wild type wg (Figure 6A). When cells were grown in the presence of suramin, the secreted 52 kDa protein was more prominent (Figure 6A). A protein of 65 kDa was detected in the medium fractions shown in Figure 6A, but this protein species was not consistently seen (compare Figure 6A with B). We do not know what this protein represents and have not investigated it further.

We did not detect wg protein in the medium of the cells expressing mutant ($wg^{IN67}$ and $wg^{IL114}$) proteins, regardless of the addition of suramin or of the temperature (Figure 6B), whereas the protein was detected in cell lysates. The carboxy-terminal deletion ($wg^{CX2}$) led to the intracellular production of a slightly smaller, 40 kDa protein species which was not detected in the medium (data not shown).

By immunoblotting analysis, we found wg protein of a similar size in lysates of cells expressing wild type or mutant genes (Figure 6C). A larger protein, possibly a processing intermediate, was found in cells expressing the mutants. Smaller protein species (33−37 kDa) were seen in the cells producing wild type wg. These most likely represent breakdown products of wg protein that are prominent here because of the abundant protein production in these cells.

To determine if wg protein was present in the extracellular matrix, we collected matrix material from plates after removal of the cells (see Materials and methods). In matrix fractions produced by cells expressing wild type wg, a 52 kDa protein species was detected on a Western blot using antibodies against wg (Figure 6C). Most of this material was sensitive to trypsin treatment, indicating that few residual intact cells were present (Figure 6D). In the matrix fractions

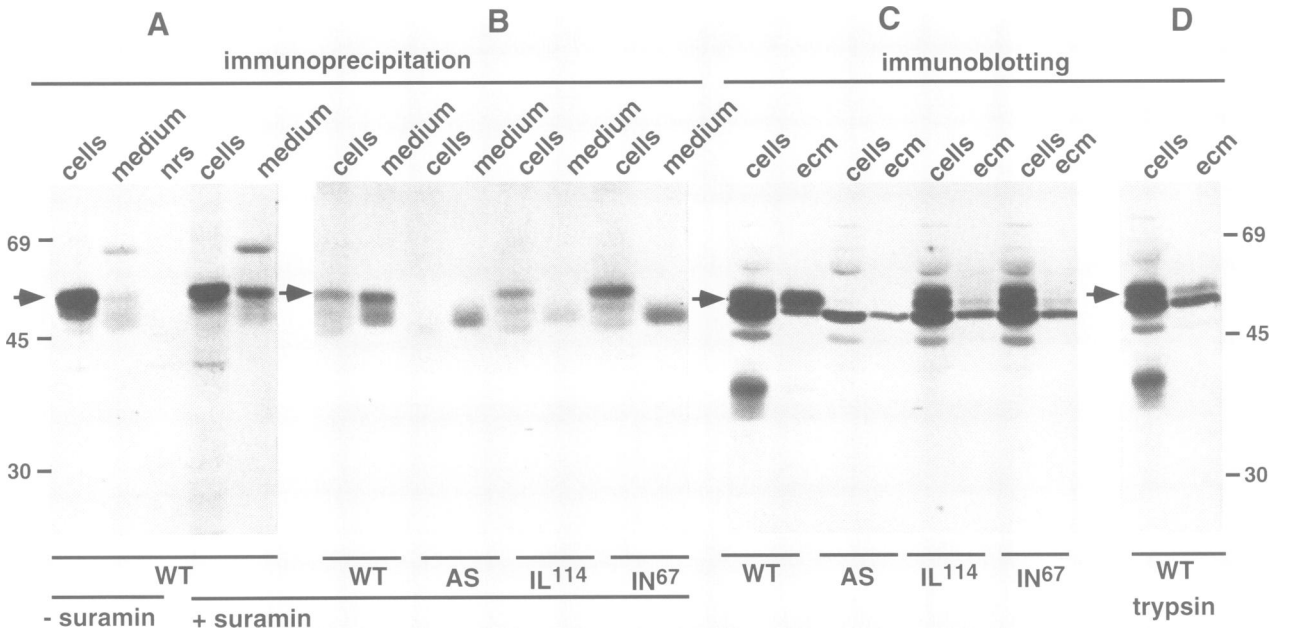


**Fig. 6.** Immunoprecipitation and immunoblot analysis of the wg protein. Arrows indicate the wg protein which has an expected and observed size of 52 kDa. A protein of ~50 kDa reacts non-specifically with the anti-wg antibody. (**A**) Immunoprecipitation of wild type wg from cell lysates (cells) and medium in the absence and presence of suramin (−/+ sur). nrs: preimmune serum control of cell lysates. (**B**) Immunoprecipitation of wild type and mutant wg proteins from cell lysates and medium in the presence of suramin; cells grown and labeled at 25°C. WT: wild type; AS: antisense construct; IL114: $wg^{IL114}$ construct; IN67: $wg^{IN67}$ construct. (**C**) Detection of wg protein by immunoblotting in cell lysates and extracellular matrix fractions (ecm) from transfected Schneider cells. Wild type and mutant wg in cell lysates (cells) and extracellular matrix (ecm), at 25°C. (**D**) Extracellular matrix (ecm) and cell fractions collected in the presence of trypsin. Most of the 52 kDa wg protein species in the ecm is sensitive to trypsin.

of cells expressing the mutant ($wg^{IL114}$ and $wg^{IN67}$) wg protein, low amounts of wg protein were found, in quantities below the trypsin-resistant fraction. This was independent of the temperature at which the cells were cultured (Figure 6C and not shown).

## Discussion

In this paper, we have characterized the molecular defects of several *wg* alleles and the resulting effects on the *wg* products. The main conclusions are that wild type wg protein is secreted from cells, that mutant proteins appear not to be secreted and that mutations in another segment polarity gene, *porc*, lead to a similar secretion defect. These conclusions are based on examining the wg protein in embryos and in a cell culture system.

### *The nature of wg mutations*

For three lethal *wg* mutants, we mapped the mutations in detail. In genetic analysis, these mutants appear as lack of function alleles although the wg protein accumulates. In the product of $wg^{IN67}$, a glycine in a region conserved among Wnt proteins is replaced by an aspartic acid. A second allele ($wg^{CX2}$) encodes a protein which is truncated at the carboxy-terminus, a region also highly conserved among the Wnt family of proteins. A carboxy-terminal truncated form of Wnt-1 has been reported to be inactive after injection into *Xenopus* oocytes; no bifurcation of the embryonic axis was seen (McMahon and Moon, 1989). The third allele mapped, $wg^{IL114}$, is temperature sensitive; at the permissive temperature it suffices for completely normal embryonic development, while at the restrictive temperature it results in patterning defects similar to those in protein nulls. In this allele the amino-terminal conserved cysteine is changed into a serine. Cysteine residues at specific intervals are highly conserved throughout the Wnt family (Nusse and Varmus, 1992). Mutational analysis of Wnt-1 *in vitro* has identified two temperature sensitive proteins (Mason *et al.*, 1992). In the first, a glycosylation site not conserved in the wg protein was eliminated. The second, like the $wg^{IL114}$ mutant, has a serine substituted for a cysteine, but this cysteine is not at an equivalent position.

The immunostaining experiments, particularly the expression studies in cultured cells, showed that the mutant wg proteins are not secreted. This is not unusual for mutant forms of normally secreted proteins; they become folded incorrectly and are retained in the endoplasmic reticulum (Lodish, 1988), consistent with the perinuclear staining we observed. At the permissive temperature, the temperature sensitive *wg* allele behaves as an intermediate form. In stained cells, the protein was found in the perinuclear space, in small vesicles and on the cell surface. At the non-permissive temperature, the protein was solely seen in the perinuclear space and no cell surface staining was detected. By immunoprecipitation and immunoblotting experiments, we were unable to detect any wg protein from the medium or extracellular matrix from cells grown at the permissive temperature. It appears that the $wg^{IL114}$ protein is secreted at the permissive temperature but at low levels relative to wild type.

The accumulation of mutant wg protein inside the cell is also seen in embryos heterozygous for mutant alleles. We interpret these results as evidence that the product of one mutant *wg* allele accumulates inside cells without affecting the function of the wild type gene product. The prominent staining for the mutant gene product most likely results from

its stability relative to the wild type protein. For some growth/differentiation factors that are made as dimers, it has been described that inactive forms of the protein can act as dominant negative inhibitors of function. Mutations that lead to the formation of non-functional dimers titrate out the wild type monomers (Mercola *et al.*, 1990; Lopez *et al.*, 1992). The formation of multimers of Wnt-1 protein has been described (Papkoff, 1989) but their functional significance is not clear. The fact that the heterozygous *wg* mutant embryos develop normally suggests, however, that wg protein does not function as a multimer, although it could also be that mutant forms are incapable of dimerizing.

### *porc may provide an accessory function for wg secretion or transport*

The finding that embryos mutant for another segment polarity gene, *porc*, display a staining pattern for wg protein that is similar to *wg* mutants may be relevant to the observations that Wnt proteins in general are inefficiently secreted when expressed in tissue culture cells. The poor secretion of Wnt proteins and their retention in the ER could be explained by a requirement for an accessory protein that facilitates correct folding. Much of the Wnt-1 protein produced in various cells is indeed associated with the molecular chaperone, BiP, which binds incorrectly folded proteins and prevents their release from cells (Kitajewski *et al.*, 1992). It is possible that Wnt proteins, including wg, are normally secreted with the aid of an accessory protein, which could be the product of the *porc* gene or a gene regulated by *porc*.

The molecular structure of the *porc* gene product is not known. *porc* mutant cells are, like *wg* mutant cells, non-autonomous in mosaic animals, indicating that the porc protein works in presenting rather than in interpreting or receiving the wg signal (J.Klingensmith and N.Perrimon, in preparation). An indication for an accessory function of porc in wg secretion also comes from observations made on expression of the *armadillo* (*arm*) gene, one of the downstream effectors of *wg* in the *Drosophila* embryo. wg normally regulates the local accumulation of the arm protein, both within wg-producing and in adjacent cells. In *porc* embryos, the arm protein accumulates only within cells expressing wg (Riggleman *et al.*, 1990). This may imply that wg has both autocrine and paracrine effects and that porc is required for the paracrine functions by facilitating wg secretion or transport.

## Materials and methods

### *Stocks*

We have used the following *wg* alleles: $wg^{CX3}$, $wg^{CX2}$, $wg^{CX4}$ (Baker, 1987), *In(2L)*$wg^{P}$ (unknown origin, P.A.Lawrence), $wg^{1}$ (Sharma and Chopra, 1976), $wg^{IL114}$, $wg^{IG22}$, $wg^{IJ69}$, $wg^{IID23}$, $wg^{IN67}$, $wg^{7L74}$ and $wg^{IIS34}$ (Nüsslein-Volhard *et al.*, 1984). $wg^{1}$ is homozygous viable and flies display a variable phenotype of loss of one or two wings (Sharma and Chopra, 1976; Morata and Lawrence, 1977). $wg^{P}$ and $wg^{CX3}$ are both pupa-lethal in combination with a $wg^{lethal}$ allele (Baker, 1988). The pharate adults show a wing phenotype similar to that of $wg^{1}$ flies, but many other parts of the imago are also affected. $wg^{P}$ is an inversion associated with breakpoints at 28A1-3 and 32E-F (see Baker, 1988). Homozygous $wg^{P}$ embryos show a pair-rule phenotype (M.van den Heuvel, unpublished results). *In trans* over a deficiency that includes the pair-rule gene *paired* (at 33B6,7-33E2,3 (Kilchherr *et al.*, 1986), *Df(2L)prd D293*) this phenotype is lost (M.van den Heuvel, unpublished results). An unknown locus leading to a pair-rule phenotype might be uncovered by this inversion (see Vavra and Carroll, 1989). All fly stocks were reared at 25°C under standard conditions. The temperature sensitive allele ($wg^{IL114}$) was used for egg collections at 25°C (non-permissive temperature) and 16–18°C (permissive temperature). The embryo-lethal alleles were balanced over a *CyO* chromosome carrying a *hunchback–LacZ* transposable element (Struhl *et al.*, 1992). The homo/heterozygosity of the embryos can be unambiguously determined using immunological detection of β-galactosidase (β-gal). Embryos were staged according to Campos-Ortega and Hartenstein (1985) and Wieschaus and Nüsslein-Volhard (1986).

Germline clones of *porc* (the alleles $porc^{PB16}$ and $porc^{i8}$ gave the same results) were obtained using the dominant female sterile technique as previously described (Perrimon *et al.*, 1984). Irradiation was performed at the end of the first larval instar stage at a dose of 1000 rads (Torrex 120D X-ray machine).

### *Analysis and sequencing of mutant alleles*

Several genomic clones covering the *wg* locus were isolated from a genomic library in the lambda phage Charon 40 (Russell *et al.*, 1992). Restriction maps of the two furthest extending clones were established. Chromosomal DNA was isolated from $wg^{CX4}$, $wg^{CX3}$ and $wg^{P}$ heterozygous flies and from $wg^{1}$ homozygous flies. The parental chromosomes of $wg^{1}$ and $wg^{P}$ were not available. DNA was digested with various restriction enzymes and hybridized to several probes from the *wg* area. Details and probes are available on request.

Total RNA was isolated from heterozygous mutant animals (mostly from pupal stage). cDNA was made using *wg*-specific, oligo(dT) or random primers (USB) according to the manufacturer's protocol. *wg* cDNA was amplified with the polymerase chain reaction using two pairs of primers: (i) nucleotides 404–422 and 1313–1329 and (ii) nucleotides 1140–1157 and 2200–2217. This generates two fragments, covering the whole of the translated sequence. For the allele $wg^{IL114}$, these fragments were reamplified asymmetrically and sequenced directly after purification over a Centricon column (Amicon) using internal primers. Only one base pair (T to A) alteration was detected. The fragments generated after the first round of amplification were also cloned into pGEM (Promega) and the fragment that contained the T to A (base pair 727) change was resequenced in seven independent clones; three of these clones contained the single base pair change. For the allele $wg^{IN67}$, five independent clones were sequenced and in two a single G to A (basepair 1079) change was found. This base pair change generated a *Sau*3AI site which was used to screen 24 more clones. Nine out of these 24 contained the new site. For the allele $wg^{CX2}$, RNA was isolated from single embryos. On DNA blots of the amplified fragments, the smaller homozygous mutant fragments could be distinguished after hybridization with a *wg* cDNA probe. The fragments containing the deletion were subcloned and sequenced.

### *RNA and protein localization*

Whole mount *in situ* hybridizations were performed as described by Tautz and Pfeifle (1989), using a full-length, digoxigenin-labeled *wg* cDNA (Figure 1B).

Antibody stainings with a polyclonal rabbit anti-wg antibody were performed as described by van den Heuvel *et al.* (1989). Embryos were either mounted in 80% glycerol or dehydrated and mounted in Araldite. For fluorescence and confocal laser scanning microscopy, embryos were labeled as described above. The bound secondary biotinylated antibodies were detected using avidin-FITC (Vector Laboratories, DCS grade at 2 mg/ml). The embryos were mounted in 4% propyl gallate/80% glycerol (Giloh and Sedat, 1982) and investigated using a Zeiss Axioscop attached to a Phoibos 1000 Confocal Laser Scanning Microscope. The images were digitalized using Phoibos/4D software and were photographed from the monitor.

### *In vitro expression*

Cloned and sequenced fragments of the alleles $wg^{IN67}$, $wg^{IL114}$ and $wg^{CX2}$ were subcloned into a *wg* cDNA construct. For the $wg^{IL114}$ allele, a *NarI–BglII* fragment containing the point mutation was directly cloned into a *wg* cDNA missing this fragment (Figure 1B). The $wg^{IN67}$ point mutation was isolated on a small *Eco*RI–*Eco*RI fragment. This was cloned into the *wg* cDNA in a two step ligation. First the whole cDNA was digested with *Eco*RI and *Hin*dIII, generating vector plus 5′ sequences of the gene, a large 3′ *Eco*RI–*Hin*dIII fragment, a small *Eco*RI–*Eco*RI fragment from the middle of the gene and some additional non-translated sequences. Following this, the large 3′ *Eco*RI–*Hin*dIII fragment was ligated back into the vector and the missing *Eco*RI–*Eco*RI fragment containing the mutation was cloned in (Figure 1B). The small deletion in $wg^{CX2}$ was cloned into a partial digest with *Sal*I and *Hin*dIII of the *wg* cDNA vector, as a *Sal*I–*Hin*dIII fragment (Figure 1B). All of the above *wg* cDNAs in pBlueScript (Stratagene) comprise the complete translated sequence of the *wg* gene. The constructs start at a *Fnu*DII site in the 5′ untranslated sequence to avoid the presence of any initiation codons before the initiating methionine of the wg protein

(Figure 1B). The cDNAs including a wild type and an antisense construct, were isolated as a whole from these vectors as a *XbaI*–blunt end fragment (using sites in the vector); *XbaI* linkers were ligated on. The *XbaI*-digested fragments were then cloned into a *SpeI*-digested pMK vector (Koelle *et al.*, 1991), generating a *wg* cDNA driven by the metallothionein promoter. The presence of the molecular lesions was confirmed by sequencing the fragments as cloned in the pMK vectors.

The expression plasmids were transfected into Schneider S2 cells using calcium phosphate (Krasnow *et al.*, 1989). The expression vectors contain a hygromycin resistance gene and stable mixed clones were established under hygromycin selection (200 μg/ml). wg protein in transfected cells could be detected in cell lysates on Western blots without induction of the promoter. In all experiments therefore the cells were not induced with copper sulfate.

Immunodetection of proteins in cells was done using both formaldehyde (4% in PBS) and methanol (100%, −20°C) fixed cells. For detection of wg protein on the cell surface and in the extracellular matrix, no detergents were added to any of the buffers, on formaldehyde-fixed cells. As a control for the integrity of the cells, cells expressing ecdysone receptor (EcR) (Koelle *et al.*, 1991) were cultured together with the cells expressing wg. In immunostainings with antibodies against both wg and the EcR, no staining for the nuclear EcR protein was seen. As secondary antibodies, a FITC-conjugated goat anti-rabbit antibody or a rhodamine-conjugated horse anti-mouse antibody was used (Vector Laboratories).

Confluent cultures were labeled in 0.750 ml of regular *Drosophila* Schneider's medium (Gibco) with 250 μCi [$^{35}$S]cysteine in six-well dishes for 4 h. Suramin was added to a final concentration of 1 mM. In temperature dependent experiments, the cells were grown 14 h at the appropiate temperature before labeling. Cells were lysed in CSK buffer (Wollner *et al.*, 1992) and the resulting lysates were spun at 15 000 *g*. The pellet fraction was boiled in the presence of 1% SDS. Soluble and insoluble fractions were combined, and this total cell lysate and the medium of the cells were used for immunoprecipitation with antibodies against wg [standard protocol using buffers as described in Wollner *et al.* (1992)]. Samples were run on 9% polyacrylamide–SDS gels; the gels were processed for fluorography (Amplify, Amersham).

For preparation of extracellular matrix fractions, cells were cultured in the wells of a six-well dish for 18 h. Preparation of extracellular matrix fractions was as described in Bradley and Brown (1990). Schneider cells were washed with PBS and incubated with PBS, 2 M urea. About 30% of the cells detached from the plate in 10 min. The rest of the cells were removed by squirting PBS, 2 M urea over the cells. The cell fraction was removed, washed with PBS and lysed in sample buffer (4% SDS; 0.125 M Tris–HCl pH 6.8; 6 M urea; 1.4 mM β-mercaptoethanol). The plates were scraped five times with a cell scraper in PBS, 2 M urea. After three more washes with PBS, sample buffer was added to the plate; these fractions were defined as extracellular matrix. Cells were also removed using trypsin (0.1% in PBS). 10% of the cell lysate fractions and 50% of the extracellular matrix fractions were analyzed on 9% polyacrylamide–SDS gels and blotted on to nitrocellulose. The nitrocellulose filters were incubated with antibodies against wg; bound rabbit antibodies were detected with a horseradish peroxidase-conjugated anti-rabbit antibody.

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