Signaling Activities of the Drosophila *wingless* Gene Are Separately Mutable and Appear to be Transduced at the Cell Surface

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ABSTRACT

The *Drosophila* segment polarity gene *wingless* encodes an intercellular signaling molecule that transmits positional information during development of the embryonic epidermis. We have explored the mechanism of *wg* signal transduction by perturbing cellular processes genetically and by performing structure/function analysis of the Wg protein. We present evidence that Wingless protein may transduce signal at the cell surface and that Wg may bind to its cell surface receptor without necessarily activating it. We demonstrate that two specific signaling activities of the Wg molecule can be disrupted independently by mutation. Sequence analysis indicates that these different signaling activities are not promoted by discrete functional domains, but rather that the overall conformation of the molecule may control distinct signaling functions. We conclude that *wg* signaling may involve complex interactions between the Wg ligand and its cell surface receptor molecule(s) and that some of this complexity resides within the Wg ligand itself.

THE Drosophila *wingless* (*wg*) gene, and its vertebrate homologue, *Wnt-1*, play crucial roles in establishing cell identities during embryogenesis, but the mechanism of *wg/Wnt-1* signal transduction is poorly understood (reviewed in PEIFER and BEJSOVEC 1992; MCMAHON 1992; NUSSE and VARMUS 1992). Both *wg* and *Wnt-1* encode proteins with features typical of secreted growth factors; they are rich in cysteine residues and have a signal sequence followed by a consensus signal peptidase cleavage site at their amino-termini (VAN OOYEN and NUSSE 1984; RIJSEWIJK *et al.* 1987). However, the Wingless and Wnt-1 proteins are larger than other known growth factors: *e.g.*, Wg consists of 468 amino acids compared with epidermal growth factor, which consists of 53 amino acids (DEUEL 1987; RIJSEWIJK *et al.* 1987).

Wg and Wnt-1 proteins are known to enter the secretory pathway and appear to be exported from cells that express the genes (PAPKOFF *et al.* 1987; VAN DEN HEUVEL *et al.* 1989; PAPKOFF and SCHRYVER 1990; GONZALEZ *et al.* 1991). In the segmented Drosophila embryonic epidermis, *wg* transcription begins at ~3 hr of development (stages 7 and 8), in one row of cells per segment under the control of pair-rule gene transcription factors (reviewed in AKAM 1987). Wg passes through the endoplasmic reticulum and Golgi apparatus of *wg*-expressing cells and can be detected in intracellular vesicles and multivesicular bodies in those cells and in adjacent non-*wg*-expressing epidermal cells (VAN DEN HEUVEL *et al.* 1989; GONZALEZ *et al.* 1991). Such intracellular locations are typical of secreted growth factors (reviewed in ULLRICH and SCHLESSINGER, 1990), which are internalized in vesicles upon binding and activating cell surface receptors. These vesicles are transported to multivesicular bodies where ligand and receptor are dissociated and receptor is recycled to the plasma membrane or sorted to the lysosome for degradation. However no receptor molecule has yet been identified for Wingless or Wnt-1, and it is not clear what relevance particular subcellular locations have to *wg/Wnt-1* function.

Both Wg and Wnt-1 act as intercellular signals, producing detectable effects on neighboring populations of cells. For example, in the Drosophila embryonic epidermis, *wg* and *engrailed* expression are activated by the pair-rule gene transcription factors, in adjacent rows of cells in each segment (reviewed in AKAM 1987). In the absence of *wg* function, *en* expression in the neighboring row of cells decays between 4 and 5 hr of development (stages 8 and 9) (DINARDO *et al.* 1988; MARTINEZ ARIAS *et al.* 1988). *wg* activity during this time is required to stabilize *engrailed* transcription (BEJSOVEC and MARTINEZ ARIAS 1991; HEEMSKERK *et al.* 1991). Intercellular *wg* signaling that results in stabilized *en* expression has also been demonstrated in a Drosophila cell culture system (CUMBERLEDGE and KRASNOW 1993). A similar interaction has been documented in mice: *Wnt-1* gene activity in the developing mouse brain stabilizes the expression of the vertebrate *engrailed* homologues, *En-1* and *En-2*, in an adjacent population of cells (MCMAHON *et al.* 1992).

In Drosophila, evidence for *wg* intercellular signaling

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can also be seen in epidermal cell populations other than the *en*-expressing cells: *wg* activity influences cell fate decisions across many rows of cells. Epidermal cells secrete a stereotyped pattern of cuticular protrusions, called denticles, in the anterior of each segment and an expanse of smooth or naked cuticle in the posterior (LOHS-SCHARDIN *et al.* 1979; CAMPOS-ORTEGA and HARTENSTEIN 1985). These features provide easily scored markers for the positional identities of the cells that secrete them. Drosophila embryos that lack *wingless* activity secrete no naked cuticle and instead of the wild-type pattern of diverse denticle types, they show reiteration of a single denticle type across the entire epidermis (NÜSSLEIN-VOLHARD and WIESCHAUS 1980; BAKER 1988). Thus *wg* activity is required for correct cell fate specification many cell diameters away from the cells that express the *wg* gene. Experiments with a temperature-sensitive allele of *wingless*, wg^{IL114}, have revealed that *wg* acts to specify these different components of the wild-type pattern at different times during development (BEJSOVEC and MARTINEZ ARIAS 1991). *wg* function before 6 hr of development (early stage 11) generates the diversity of cell fates that give rise to the wild-type denticle belt pattern and *wg* function after 6 hr instructs cells in the posterior of the segment to secrete smooth or naked cuticle.

Thus *wg* signaling produces a complex array of cellular responses: *wg* activity can instruct cells to produce one of two completely different cuticular properties, denticle diversity *vs.* naked cuticle, and it can have a direct effect on *engrailed* transcription in neighboring cells. In addition, *wg* signaling activity has an effect on its own transcription. In the *wg*-expressing cells, *wg* expression is lost during stages 10 and 11 in *wg* mutant embryos (BEJSOVEC and MARTINEZ ARIAS 1991; INGHAM and HIDALGO 1993) and therefore *wg* activity is required to stabilize its own expression. These very different cellular consequences of *wg* signaling suggest that the *wg* signaling mechanism is complex.

We have investigated the basis for the complexity of this process using two approaches. First, we have assessed the importance of the subcellular localization of Wg protein. We have determined that the *wg* signaling mechanism does not depend on endocytosis of the Wg ligand into responding cells, suggesting that Wg acts through a cell surface receptor. Second, we have characterized a collection of Wg mutant alleles to relate structural features of the protein to its signaling function. We find that although Wg can transduce signal without being endocytosed, a truncated mutant Wg molecule can be endocytosed without transducing signal, indicating that Wg can interact with its cellular receptor without activating it. Furthermore, we find that the distinct signaling activities that give rise to different components of the wild-type cuticle pattern (BEJSOVEC and MARTINEZ ARIAS 1991) can be disrupted independently in mutant *wg* alleles. Sequence analysis of these mutant alleles suggests that these activities are not promoted by discrete functional domains but rather that regions of the Wg protein may contribute differentially to the overall activity of the molecule. We conclude that at least part of the complexity of the Wg signaling mechanism resides in the Wg ligand and not in the as yet unidentified receptor molecule(s).

MATERIALS AND METHODS

Drosophila stocks and culture conditions: Fly strains were cultured at 25° on standard cornmeal/yeast medium or on apple juice agar plates for egg collection. Lethal *wg* mutations were isolated based on a noncomplementation F1 screen using the adult viable wg^{1} allele, as described by BABU (1977), and were balanced over *CyO*. EMS mutageneses were conducted according to GRIGLIATTI (1986) using a *pr cn* Adh^{UF3} parental chromosome. The suppressor mutation, $wg^{PE1\ CE7}$, was obtained in an F1 screen for suppressors of wg^{CE7} lethality in combination with the pupal lethal wg^{CX3} allele (BAKER 1987). Animals heteroallelic for wg^{CX3}/wg^{CE7} normally die as pupae; suppression was initially assayed by absence of the *CyO* balancer chromosome. $wg^{PE1\ CE7}$ was recovered as a viable non-*Cy* adult fly among 166,592 *Cy* adult flies screened.

Cuticle preparations were performed according to WIESCHAUS and NÜSSLEIN-VOLHARD (1986).

Analysis of *shibire* mutant embryos: A homozygous stock of $shibire^{ts}$ flies was maintained at 18°. Embryos were collected at 1-hr intervals and aged to gastrulation, when *wg* expression begins (roughly 6 hr of development at 18°). Collections were examined under a dissecting scope to verify stage and shifted to a temperature block at high temperature. To ascertain that *shi* function is eliminated, we repeated these experiments at 30, 32 and 35°. Similar results were obtained at all three temperatures; all experimental data presented in this paper are derived from the 35° trials. Embryos were incubated for 1.5 hr at the restrictive temperature and fixed for antibody staining with anti-Wg, anti-Arm or anti-En antibodies. It was not possible to assess the cuticle pattern produced by shifted *shi* mutants because they die before secreting cuticle. Embryos collected from a heterozygous stock carrying the wg^{IL114} temperature-sensitive mutation were processed in parallel with the *shi* mutant embryos to determine whether disrupted *wg* activity could be detected under the conditions of the experiment. As expected, one quarter of the embryos from this stock failed to redistribute Arm protein and showed decay of Engrailed expression; and three quarters appeared completely wild-type for Arm and En distribution at both 18 and 35°. At 35°, only $\frac{1}{4}$ of the embryos were completely wild-type for Wg protein distribution since wg^{IL114} heterozygotes produce some mutant protein that shows retention in the secretory apparatus.

Analysis of gene expression patterns: RNA *in situ* hybridizations were performed according to TAUTZ and PFEIFLE (1989). Wingless antibody used in this work is described in VAN DEN HEUVEL *et al.* (1989), Engrailed in DINARDO *et al.* (1985), Armadillo in PEIFER *et al.* (1991), and Neurotactin in HORTSCH *et al.* (1990). Anti-Engrailed antibody staining was performed according to PATEL *et al.* (1989) except that bovine serum albumin was used instead of normal goat serum and embryos were cleared in xylene and mounted in DPX (BDH Chemicals). Anti-Armadillo antibody staining was performed using the modified BBS fixation protocol described for anti-Wingless staining (GONZALEZ *et al.* 1991). Double-labeling

with Wingless and Neurotactin antibodies was performed with mixed primary antibodies (anti-Wg polyclonal diluted at 1:500, anti-Neurotactin monoclonal at 1:4) and mixed secondary antibodies from Boehringer Mannheim (rhodamine-conjugated anti-rabbit, fluorescein-conjugated anti-mouse). Embryos were mounted in Aquapolymount (Polysciences) and viewed with laser scanning confocal microscopy (Krypton-Argon Laser, Biorad MRC600).

Sequence analysis of mutant alleles: PCR primers based on cDNA sequences (RIJSEWIJK *et al.* 1987) were designed to prime from within exons across intervening sequences in genomic DNA. Single-stranded templates were generated using asymmetric PCR (McCabe 1990) and sequenced using the method of SANGER *et al.* (1977). For analysis of wg^{CE7} and its suppressor, cDNA sequences were obtained from embryos of four genotypes: homozygotes for the parental *pr cn* Adh^{UF3} chromosome, homozygotes for the $wg^{PE1\ CE7}$ suppressor chromosome, heterozygotes for $wg^{CE7}/wg^{PE1\ CE7}$ and heterozygotes for wg^{CE7}/*pr cn* Adh^{UF3}. Poly-A+ RNA was extracted from 3–6-hr embryo collections and reverse transcription was performed using AMV reverse transcriptase and an oligo-dT primer (both from Promega). These first strand reactions were purified over a Microcon 100 column (Amicon) and subjected to PCR amplification using primers from within exons 4 and 5 that prime across intron 4. PCR products representing the different splice variants were separated on an agarose gel, excised and eluted from the gel using disposable spin columns (Fisher). Gel-pure products were then subjected to a second round of PCR amplification using the same primers and sequenced directly as above.

RESULTS

***wingless* transduces signal at the cell surface:** In wild-type embryos, Wg antibody staining appears punctate due to the accumulation of Wingless protein in intracellular vesicles; these vesicles can be detected in a graded distribution over several cell diameters on either side of the single row of cells that express *wg* RNA (VAN DEN HEUVEL *et al.* 1989; GONZALEZ *et al.* 1991). To test whether Wg protein must be endocytosed into these vesicles in order to transduce signal, we assessed Wg protein distribution and activity in *shibire*^{ts} mutant embryos. *shibire* (*shi*) encodes a dynamin homologue (CHEN *et al.* 1991; VAN DER BLIECK and MEYEROWITZ 1991) that is essential for endocytosis (POODRY and EDGAR 1979). In *shibire*^{ts} mutants at permissive temperature, Wingless protein distribution is wild type (Figure 1A) and the embryos develop normally. *shi* embryos shifted to restrictive temperature at 3 hr of development, when *wg* expression begins, and cultured for 1.5 hr at high temperature show a Wg protein distribution different from the wild-type distribution seen in control embryos treated under the same conditions. In *shi* embryos at restrictive temperature, Wg protein is not detected in intracellular vesicles and instead appears at high levels around the *wg*-expressing cells, presumably accumulating in the extracellular space (Figure 1B). Thus the Wg-containing vesicles observed in wild-type embryos depend on the endocytotic process and probably represent Wg being endocytosed from the cell surface. In *shi* mutants, Wg protein is restricted to the area immediately surrounding the *wg*-expressing cells. This row of *wg*-expressing cells is mostly one cell wide and never more than two cells wide. This contrasts with the wild-type Wg distribution: at this stage of development, Wg can be detected over three to six cell diameters (GONZALEZ *et al.* 1991). Thus *shi* activity is required for the broad distribution of Wg protein observed in the wild-type situation. *shi* mutation does not appear to affect export of Wg protein: the distribution of Wg protein in *shi* mutants is clearly different from the distribution observed in the temperature-sensitive $wg^{IL.114}$ mutant (Figure 1C), which produces a protein that is not secreted at the restrictive temperature (GONZALEZ *et al.* 1991).

We measured the activity of *wg* in *shi* mutants by examining the distribution of Armadillo protein, which serves as an early molecular marker of *wg* signaling. Arm protein is present at cell membranes in all epidermal cells but accumulates in the cytoplasm of cells responding to *wg* signal (PEIFER *et al.* 1994). In wild-type embryos, three- to six-cell-wide stripes of cytoplasmic Arm staining are centered over the *wg*-expressing row of cells, a distribution that correlates with the broad distribution of Wingless protein; these stripes are absent in mutants lacking *wg* activity (RIGGLEMAN *et al.* 1990; PEIFER *et al.* 1991). *shi* mutants shifted to restrictive temperature (Figure 1E) show stripes of cytoplasmic Arm staining similar to wild type (Figure 1D) in intensity but narrower in distribution. The width of the Arm stripes ranges from two to four cell diameters and never exceeds four cell diameters. This correlates with the restricted distribution of Wingless protein in *shi* mutants and indicates that only *wg*-expressing cells and their immediate neighbors are responding to *wg* signaling. The observation that the Arm stripes are 1 cell diameter wider than the Wg stripes suggests that non-*wg*-expressing cells are responding to the Wg signal and therefore that extracellular Wg is capable of transducing signal into adjacent cells. The intensity of cytoplasmic Arm staining observed in the *shi* mutants is comparable to the wild-type response to *wg* signal; it is markedly higher than Arm levels observed in partial function mutants of *wg* (described below). $wg^{IL.114}$ embryos shifted at the same time and treated under the same conditions show no cytoplasmic striping of Armadillo (Figure 1F). We also assayed the expression of *engrailed* in the cells adjacent to the *wg*-expressing cells. In *shi* mutants shifted to restrictive temperature, *en* expression is maintained properly as it is in wild-type controls (Figure 1, G and H), whereas it is disrupted in $wg^{IL.114}$ mutants shifted at the same time and treated under the same conditions (Figure 1I). This provides further evidence that Wg signal can be transduced in a non-*wg*-expressing population of cells in the absence of endocytosis.

Thus in *shi* mutants, Wg trapped at the cell surface

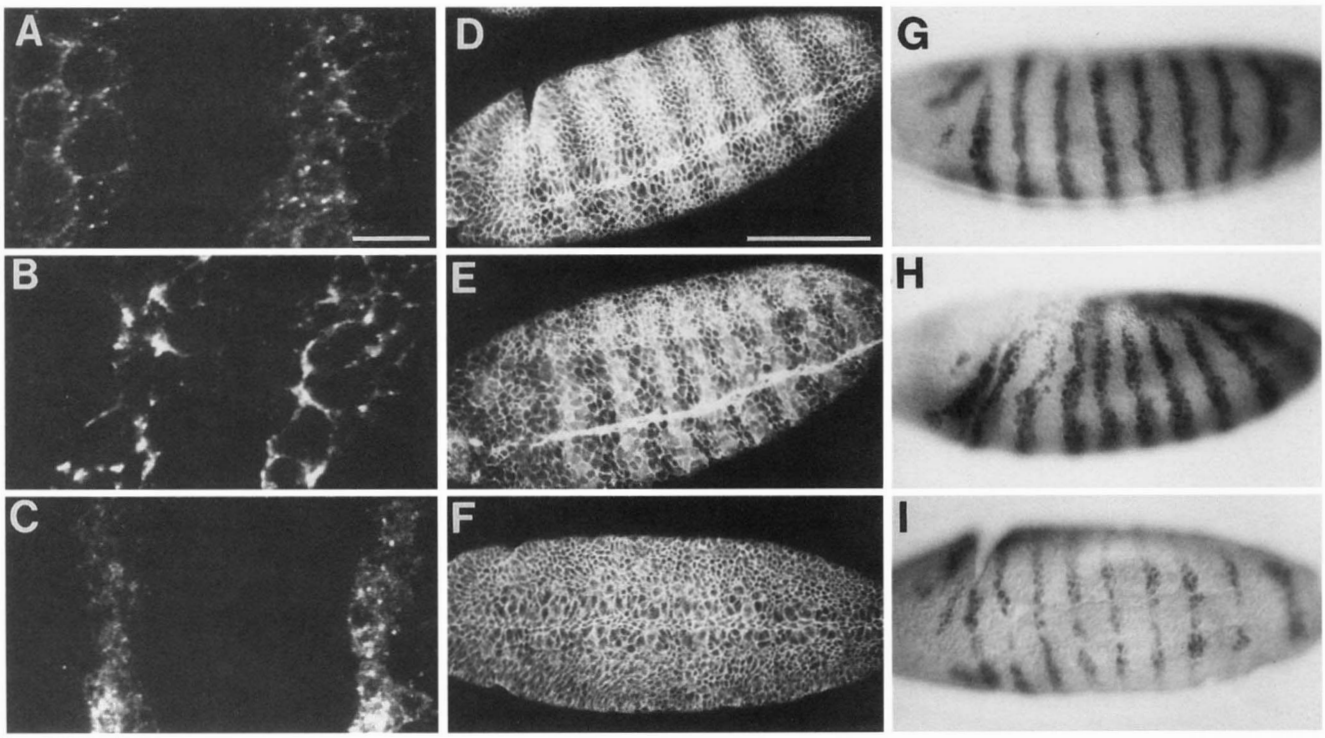

FIGURE 1.—Wingless and Armadillo protein distribution in *shibire*^{ts} mutant embryos. (A) Wingless antibody staining in *shi* mutant embryo at 18° is wild type in appearance. This single confocal image was taken midway through the epidermal cell layer, just below the cell nuclei and roughly 5 μm below the apical surface. Intracellular vesicles containing Wingless protein are ~1 μm in diameter. Scale bar, 10 μm. (B) Wingless antibody staining in *shi* mutant embryo shifted to 35° at 3 hr of development and cultured at that temperature for 1.5 hr. Wingless protein accumulates around cells, presumably in the extracellular space. Note that intracellular vesicles are reduced in number and none are detected in non-*wg*-expressing cells when endocytosis is blocked. Wild-type embryos treated under the same conditions show wild-type Wingless protein distribution as in A. (C) Wg staining in wg^{IL114} mutants shows the mutant protein retained in the *wg*-expressing cells at restrictive temperature. (D) Armadillo antibody staining in *shi* mutant embryo at 18° shows wild-type pattern of cytoplasmic stripes centered over the row of *wg*-expressing cells. Wild-type embryos treated under the experimental conditions used in E also show this wild-type pattern. Scale bar, 100 μm. (E) Armadillo antibody staining in *shi* mutant embryo shifted to 35° and cultured for 1.5 hr. Cytoplasmic stripes indicate normal response to *wg* signal in the *wg*-expressing cells and their immediate neighbors. Note that these stripes are narrower than wild type, ranging between two and four cell diameters as opposed to three to six cell diameters in wild type; these widths correlate with the restriction in Wg protein distribution when endocytosis is blocked. Ventral cells are larger than dorsal cells because *shi* mutation arrests cell division: dorsal cells in domain 11 (FOE 1989) have divided shortly after gastrulation, just after the embryos are shifted, while the later cell divisions in the ventral region are blocked. These embryos die before secreting cuticle and therefore we cannot assess effects on the cuticle pattern. (F) Armadillo staining in wg^{IL114} mutant shifted to 35° at the same stage as the *shi* mutants and cultured under the same conditions. No cytoplasmic Arm stripes are observed in the absence of *wg* activity. Engrailed antibody staining in wild-type embryo (G) and *shi* mutant embryo (H), both shifted to 35° at the same stage. *en* expression is stabilized by *wg* signaling activity in these embryos, whereas it decays in wg^{IL114} mutant (I) shifted to 35° and cultured under the same conditions. All embryos were fixed at 4.5 hr of development, but because germ band extension is retarded in *shi* mutant embryos, the morphology of this embryo differs from the control embryos.

appears to transduce a wild-type level of signaling in *wg*-expressing cells and in the immediately adjacent non-*wg*-expressing cells. Blocking endocytosis does not block response to *wg* signaling but it does restrict the range over which *wg* signaling can be detected. Narrower stripes of cytoplasmic Arm correlate with a restricted distribution of Wg protein and indicate that endocytosis is required for producing both the broad distribution of Wg protein and the broad domain of response to *wg* signal observed in wild-type embryos.

Wingless can be internalized and transported properly without transducing signal: We have identified a mutant *wg* allele, wg^{CE7}, which makes protein that is internalized and distributed in a manner indistinguishable from the wild-type protein, but that fails to transduce signal. The cuticle pattern shown by wg^{CE7} embryos is typical of null mutants (Figure 2, A and B) and all known cellular responses to *wg* signaling are absent. wg^{CE7} mutants, like *wg* null mutants (RIGGLEMAN *et al.* 1990), show no intracellular rearrangement of Armadillo protein (Figure 2, C and D) Wild-type *wg* signaling activity during stages 8 and 9 stabilizes expression of the segment polarity gene *engrailed* in the neighboring row of cells (BEJSOVEC and MARTINEZ ARIAS 1991; HEEMSKERK *et al.* 1991). wg^{CE7} mu-

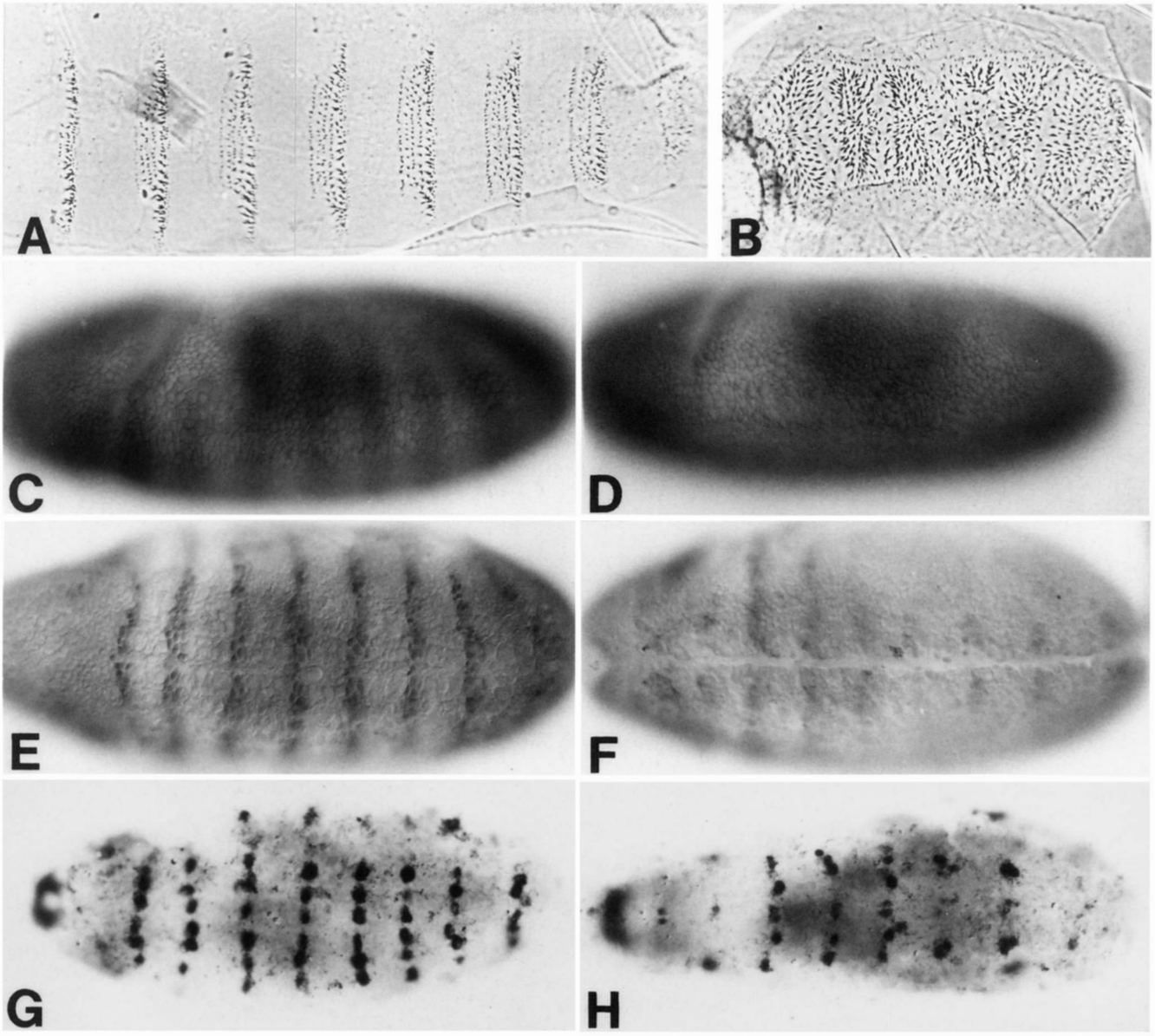

FIGURE 2.—Markers of *wg* signaling activity in wild-type and wg^{CE7} mutants. (A) Wild-type cuticle pattern shows diverse denticle types in the anterior portion of each abdominal segment and naked cuticle in the posterior portion. This embryo is actually homozygous for the $wg^{PE1\ CE7}$ allele, which is completely wild type for *wg* signaling activity (see text). (B) wg^{CE7} mutants produce a cuticle pattern indistinguishable from that of homozygotes for wg^{CX4}, a known molecular null mutation (BAKER 1987). (C) Armadillo antibody staining in an early stage 9 wild-type embryo shows strong segmental striping. This striping of Arm protein is not observed in wg^{CE7} mutants (D) at the same stage. (E) Engrailed antibody staining in a wild-type stage 9 embryo shows Engrailed protein in one-cell-wide segmental stripes. (F) A wg^{CE7} mutant at the same stage shows loss of *en* expression from the epidermis; *wg*-independent staining in segmentally repeating neuroblasts can be seen below this plane of focus. (G) *wg* RNA *in situ* hybridization in a late stage 10 wild-type embryo shows that *wg* expression is restricted to a single row of cells in each segment. (H) Expression begins to decay during stage 10 in wg^{CE7} mutants as it would in a *wg* null mutant.

tants, like *wg* null mutants, show decay of *engrailed* expression in these cells by early stage 9 (Figure 2, E and F). Finally, stable *wg* RNA expression depends on *wg* activity (BEJSOVEC and MARTINEZ ARIAS 1991; INGHAM and HIDALGO 1993). wg^{CE7} mutants, like *wg* null mutants, show decay of *wg* RNA expression during stages 10 and 11 (Figure 2, G and H).

During the developmental stages at which these *wg* signaling events occur, wg^{CE7} protein distribution is identical to the wild-type distribution. To determine whether mutant protein is internalized into adjacent non-*wg*-expressing cells, double labeling was performed using anti-Wg antibody and an antibody against Neurotactin, which outlines epidermal cell membranes (HORTSCH *et al.* 1990). Mutant Wg protein, like wild-type Wg protein, accumulates inside cells over several

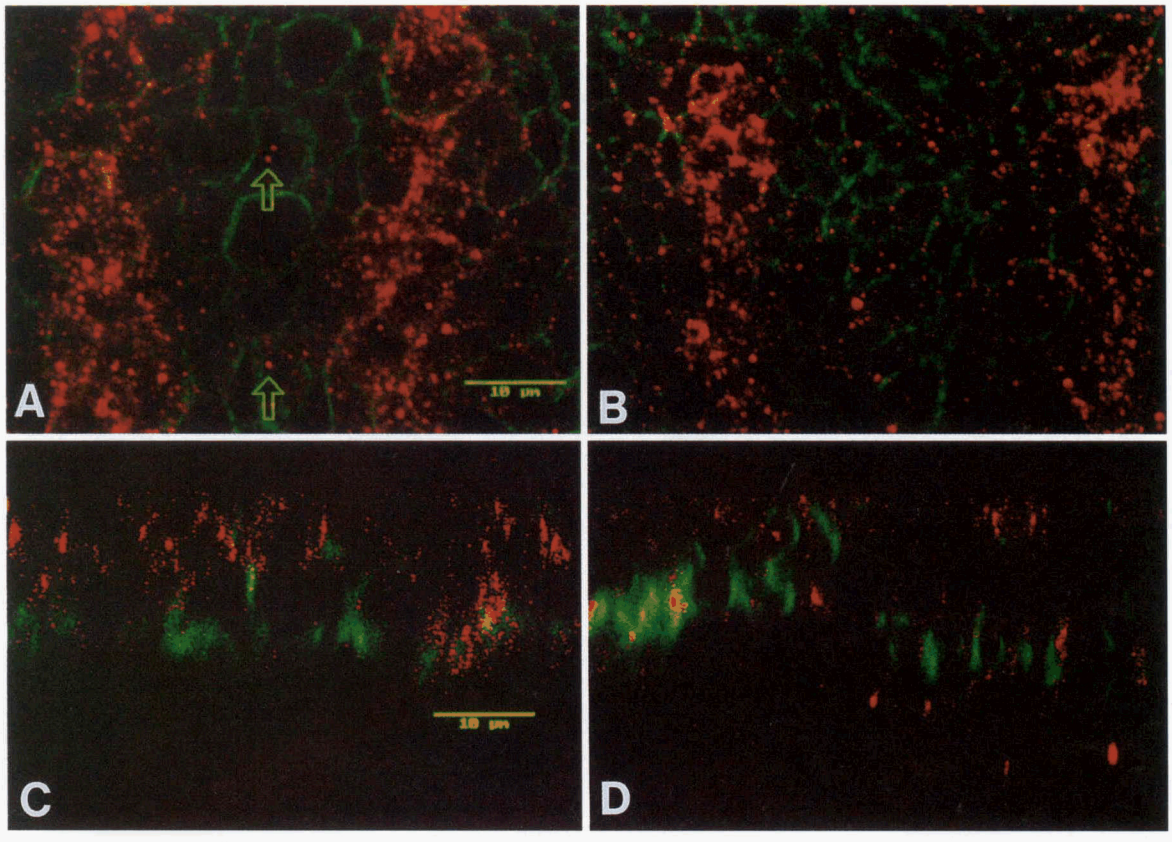

FIGURE 3.—Wingless protein distribution in wg^{CE7} mutants. All panels show Wingless antibody staining in red and cell membrane-specific Neurotactin antibody staining in green. (A) Projection of five focal planes at 1-μm intervals from just above the basal surface up to roughly midway through the epidermal cell layer. The distribution of intracellular vesicles is broader in basal planes of focus (GONZALEZ *et al.* 1991) than at higher levels in the cell, such as those shown in Figure 1, A–C. This stage 9 embryo was obtained from a mating of wg^{CE7} heterozygotes that produces one-quarter homozygous wg^{CE7} mutants. All stage 9 embryos in this collection (46/46) show the same wild-type distribution of Wg. Since wg^{CE7} heterozygotes also synthesize mutant protein, we would expect 3/4 of the embryos to show an abnormal protein distribution if one were detectable at this stage. Arrows indicate Wg-containing vesicles that reside entirely within a non-*wg*-expressing cell. Such internalized vesicles were documented for all 46 stage 9 embryos scored. Scale bar, 10 μm. (B) Late stage 10 wg^{CE7} mutant embryo. At this stage, homozygous *wg* mutants can be distinguished due to morphological effects of *wg* mutation. Notice that mutant protein appears in large clumps and is more broadly distributed across the segment. This abnormal distribution is detected in 68/93 of the late stage 10 and stage 11 embryos, close to the expected 3/4 (see above). (C) Transverse cross section through a wild-type stage 10 embryo using confocal images compiled at 0.13-μm intervals. Neurotactin antibody (green) stains ectodermal layer: epidermal cells on top and neural cells below. Wild-type Wg antibody staining (red) is restricted mostly to epidermal layer, except when neuroblasts delaminate: one is shown just above the 10-μm scale bar. (D) Cross section through homozygous wg^{CE7} mutant embryo. Mutant Wg protein can be detected below the lower Neurotactin-staining ectodermal layer. These vesicles appear to reside in mesodermal tissue.

cell diameters on either side of the *wg*-expressing row of cells, in what appear to be intracellular vesicles (Figure 3A). The neurotactin marker allows us to ascertain that some Wg-containing vesicles reside entirely within non-*wg*-expressing cells in all embryos examined. We find that wg^{CE7} mutant protein is not only internalized into neighboring cells, it is also transported to and internalized into cells that lie at a distance from the cells expressing the RNA. This implies a very specific protein-handling process and therefore it seems likely that the mutant protein traverses the same cellular pathway as does the wild-type Wg protein.

In late stage 10 and stage 11 embryos, the Wg protein distribution begins to appear abnormal in wg^{CE7} homozygotes and heterozygotes. Mutant protein accumulates in large clumps and can be detected inside cells in regions of the segment where wild-type Wg is never detected (Figure 3B). Cross sections through doubly-labeled embryos show that the mutant protein is also detected in cell layers beneath the epidermis (Figure 3D), while wild-type protein is restricted mostly to the epidermal cell layer (Figure 3C). This wider mutant protein distribution appears due at least in part to greater stability of the mutant protein. In wg^{CE7} mutants, *wg* RNA expression decays during stages 10 and 11 (Figure 2, G and H), but Wg protein continues to be detected at high levels in mutant embryos after stage 13 (not shown). This contrasts with wild-type Wg protein.

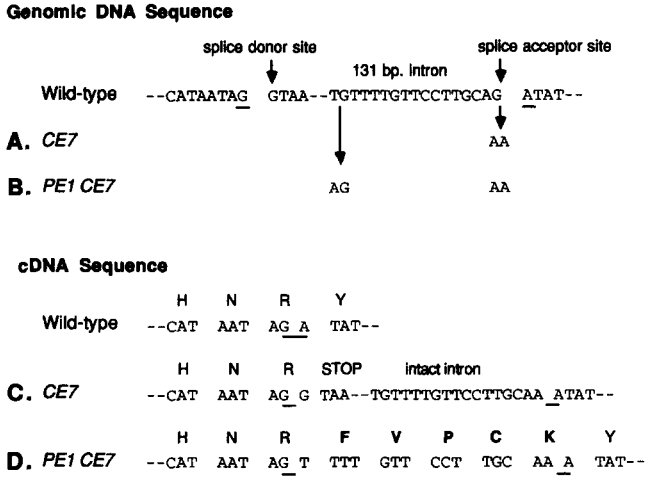

FIGURE 4.—Sequence surrounding intron 4 of wild-type and *wg* mutants. Last nucleotide of exon 4 and first nucleotide of exon 5 are underlined in all sequences shown. (A) Genetic lesion in wg^{CE7} alters the exon 5 splice acceptor site from AG to AA. (B) $wg^{PE1\ CE7}$ mutant DNA contains the original wg^{CE7} lesion and an additional mutation, wg^{PE1}, which changes a TG dinucleotide to an AG dinucleotide. (C) cDNA sequences show that intron 4 is unspliced from transcripts produced by wg^{CE7} and is partially spliced out of $wg^{PE1\ CE7}$ mutant transcripts. (D) New splice acceptor generated by the wg^{PE1} lesion results in transcripts that include 15 nucleotides of intron sequence. Novel amino acids that would be translated from intron sequences are indicated in boldface.

In wild-type stage 12 and 13 embryos, *wg* expression diminishes and Wg protein levels drop concommitantly, suggesting that wild-type Wg protein has a more rapid rate of turnover. Although wg^{CE7} heterozygotes produce mutant protein that accumulates in this abnormal distribution, the mutant protein does not appear to interfere with the function of the wild-type Wg protein as the heterozygotes are phenotypically wild type.

Mutated splice acceptor site results in a truncated Wg molecule: Sequence analysis of wg^{CE7} mutant DNA has revealed a genetic lesion that alters a nucleotide at the 3′ end of the fourth intron, changing the splice acceptor site for exon 5 from AG to AA (Figure 4A). cDNAs from the wg^{CE7} mutant were sequenced and found to contain the fourth intron intact (Figure 4C), thus the wg^{CE7} mutant lesion results in failure to splice out the fourth intron. The first three nucleotides of the intron encode a UAA stop codon. Translation of this transcript will produce a protein truncated at the end of the fourth exon and consisting of 367 amino acids rather than the wild-type 468.

Confirmation that this splicing defect is responsible for the wg^{CE7} mutant phenotype was obtained when an intragenic second site suppressor mutation of wg^{CE7} was sequenced. This suppressor mutation was recovered in a screen for adult viability in heteroallelic combinations of the pupal lethal *wg* allele, wg^{CX3}, with the embryonic lethal wg^{CE7} allele. The suppressed mutant strain $wg^{PE1\ CE7}$ is homozygous viable and shows completely wild-type *wingless* signaling function (Figure 2A). Sequence analysis reveals that in addition to the wg^{CE7} lesion, this strain carries a second mutation within the fourth intron, generating a new splice acceptor site (Figure 4B). cDNAs from $wg^{PE1\ CE7}$ homozygotes were sequenced and the major splice product from the $wg^{PE1\ CE7}$ mutants was found to use the new splice acceptor site. The resulting translation product would have an additional 5 amino acids inserted between exons 4 and 5 (Figure 4D). Apparently these additional residues do not interfere with *wg* function, as the cuticle pattern and the Wg protein distribution observed in $wg^{PE1\ CE7}$ embryos are wild-type.

The wg^{CE7} lesion results in a molecule that, although truncated, must assume a conformation acceptable to the secretory apparatus, as it is exported from the cells expressing the gene. Another mutant allele, wg^{PE3}, is a nonsense mutation that truncates the molecule near the end of the fourth exon (Figure 5) and shows the same phenotype of being secreted and internalized without transducing signal. However, nonsense alleles within the fifth exon (Figure 5) encode molecules that are not secreted from the *wg*-expressing cells, suggesting that these longer Wg molecules do not fold properly and are retained in the endoplasmic reticulum. These results suggest that the carboxy-terminal region may fold independently of the rest of the protein, since it can be cleanly removed without affecting protein export. It may be relevant that the 85 amino acids immediately preceding this region are very hydrophilic, do not include any cysteines and are not conserved between Drosophila and mouse (RIJSEWIJK *et al.* 1987).

***wingless* signaling activities can be mutated independently:** We have isolated mutant alleles of *wg* that appear to disrupt different specific functions of *wg*. Temperature shift experiments with the wg^{IL114} temperature-sensitive mutant have shown distinct phases of *wg* function at different times (BEJSOVEC and MARTINEZ ARIAS 1991). The cuticle patterns produced by wg^{IL114} mutant embryos that have had *wg* function at early stages show denticle diversity without naked cuticle, and those that have had *wg* function only at late stages show naked cuticle with little denticle diversity. These patterns can be specifically phenocopied by point mutations within the *wg* gene.

wg^{PE2} secretes a cuticle pattern that shows denticle diversity but no naked cuticle (Figure 6A). This pattern is identical to wg^{IL114} temperature-sensitive mutant embryos that have been shifted from permissive to restrictive temperature, removing *wg* activity, at 5.5–6 hr (BEJSOVEC and MARTINEZ ARIAS 1991). Unlike null *wg* mutants, wg^{PE2} mutants show little loss of *engrailed* expression. *en* expression is stabilized by *wg* activity between 4 and 6 hr of development (BEJSOVEC and MARTI-

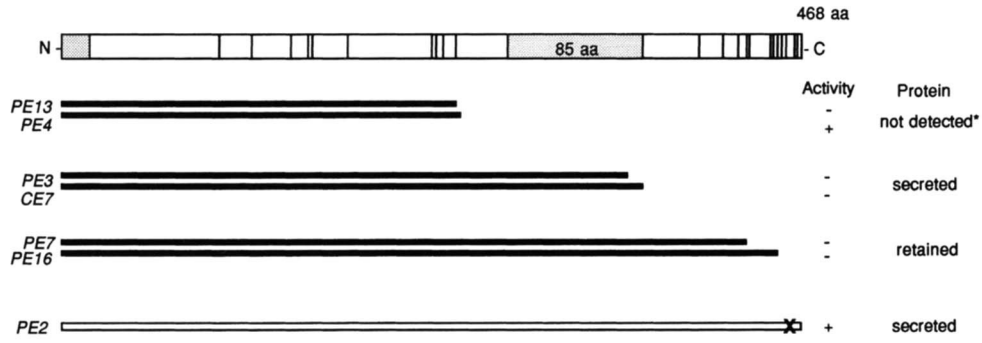

FIGURE 5.—Schematic diagram of truncated Wingless molecules. Top panel shows wild-type Wg protein structure: signal sequence is stippled at N-terminus, and vertical lines represent cysteine residues that are conserved between Wingless and mouse Wnt-1. Stippled box represents 85-amino acid region that is present only in Wingless and in no other *Wnt* gene family member. Nonsense mutant alleles are represented as darkly stippled truncated lines. *Protein distributions for *PE13* and *PE4* are unknown because these mutant proteins are not detected with the rabbit polyclonal Wg antiserum used in this work. We suspect that this is due to the absence of the 85-amino acid nonconserved region of the protein (stippled box). Although the antiserum used is polyclonal, a majority of the antibodies in the antiserum may be directed against the 85-amino acid region that is not present in any vertebrate Wnt protein.

NEZ ARIAS 1991; HEEMSKERK *et al.* 1991). Both wg^{PE2} and shifted wg^{IL114} embryos have almost normal *en* expression, with only an occasional gap in the dorsolateral portion of the stripe. Furthermore, expression of *wg* is normal in these mutants and wg^{PE2} protein appears in a wild-type distribution at all stages of development. The wg^{PE2} mutant phenotype cannot be explained as a general reduction in overall *wg* activity. wg^{IL114} mutant embryos cultured at intermediate temperatures show a more widespread loss of *en* expression (PEIFER *et al.* 1990) and they secrete defective cuticle patterns that display both denticle diversity and naked cuticle (not shown). Similarly, weak alleles of *wg* due to genetic rearrangements affecting the promoter region of the gene result in cuticle patterns that also show both denticle diversity and naked cuticle (A. BEJSOVEC, unpublished data). Thus the wg^{PE2} mutation phenocopies the wg^{IL114} temperature-shift embryos exactly, suggesting a specific disruption of the late *wg* signaling function.

A second mutant allele, wg^{PE4}, secretes a cuticle pattern that suggests specific disruption of early *wg* signaling function. The mutant cuticle pattern shows considerable naked cuticle at 18°, with reduced denticle diversity (Figure 6B). This pattern is identical to wg^{IL114} mutant embryos that have been shifted from restrictive to permissive temperature, restoring *wg* activity, at 5–5.5 hr (BEJSOVEC and MARTINEZ ARIAS 1991). These shifted temperature-sensitive mutant embryos produce considerable naked cuticle, but show reduced denticle diversity. Again, wg^{PE4} mutant embryos and the wg^{IL114}-shifted embryos show similar *engrailed* expression patterns. Restoring *wg* activity late in development rescues stabilization of *en* expression only in cells across the ventral midline region (BEJSOVEC and MARTINEZ ARIAS 1991); an identical pattern of *en* expression is observed in wg^{PE4} mutant embryos (not shown). This pattern is again distinct from the *en* expression pattern observed in conditions of generally reduced *wg* activity. Thus wg^{PE4} produces specific effects similar to those produced by late *wg* signaling activity. Unfortunately, the wg^{PE4} mutant protein is not detectable with our anti-Wg antisera (see legend of Figure 5 for possible explanation) so it is not possible to assess the mutant protein distribution.

Neither the wg^{PE2} nor the wg^{PE4} mutant shows the intense cytoplasmic Armadillo staining that is typical of wild-type *wg* signaling. Instead, a slightly higher level of membrane-bound Armadillo is detected in stripes (not shown), suggesting that partial *wg* activities produce only a subtle effect on Armadillo distribution and that the cuticle pattern provides a more sensitive measure of *wg* signaling activity.

The early *vs.* late signaling activities revealed in the wg^{IL114} temperature-shift experiments appear to be separately mutable within the Wg molecule. Sequence analysis of wg^{PE2} reveals a missense change in a highly conserved region at the carboxy-terminal end of the protein (Figure 6D). A conserved valine is replaced by a glutamic acid at position 453, within a cluster of six cysteines that are conserved between the Drosophila Wg and mouse Wnt-1 proteins. The mutant effect on cuticle pattern suggests that this mutant molecule is competent to perform the early signaling activity, but is not able to promote the late function of specifying naked cuticle. Surprisingly, the reciprocal phenotype observed in wg^{PE4} mutants is not due to a missense mutation but rather to a nonsense mutation roughly halfway through the coding region, at position 250. wg^{PE4} truncates the molecule at the end of a highly conserved region that includes five conserved cysteine residues (Figure 6E). Another nonsense mutation, wg^{PE13}, was found to truncate the molecule two amino acids earlier,

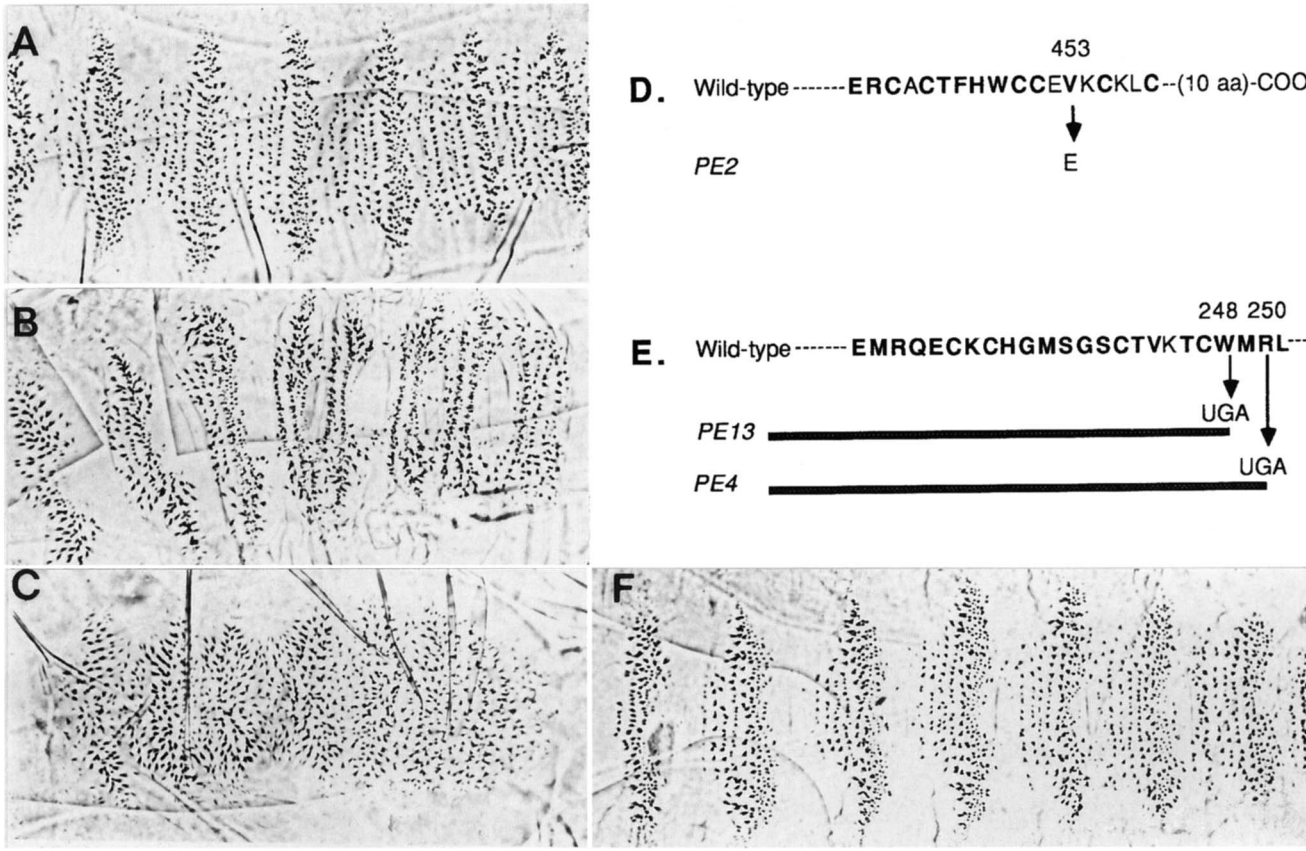

FIGURE 6.—Mutant *wg* alleles that reveal distinct signaling activities. (A) Cuticle pattern produced by wg^{PE2} mutant shows denticle diversity with no naked cuticle. A similar phenotype is observed in embryos that have been exposed to wild-type *wg* signaling activity before 6 hr of development (see Figure 8 in BEJSOVEC and MARTINEZ ARIAS, 1991). (B) Cuticle pattern produced by wg^{PE4} mutant shows naked cuticle with little denticle diversity. A similar phenotype is observed in embryos that have been exposed to wild-type *wg* signaling only after 5.5 hr of development (see Figure 8 in BEJSOVEC and MARTINEZ ARIAS, 1991). wg^{PE4} is temperature sensitive; embryo shown was raised at 18°, slightly less naked cuticle is observed in embryos raised at 25°. (C) Cuticle pattern of wg^{PE13} mutant is typical of null mutants. wg^{PE2} and wg^{PE13} mutant patterns (A and C) are not temperature sensitive; the same cuticle pattern is produced at 18° and 25°. (D) The wg^{PE2} mutant lesion changes valine 453 to glutamic acid. In surrounding sequence, boldface residues are identical between Drosophila Wg and mouse Wnt-1. Mutant protein produced by wg^{PE2} shows wild-type distribution at all stages of development. (E) Nonsense alleles that truncate Wg molecule at nearby positions: wg^{PE4} mutants show late *wg* signaling activity (see panel *B*), while wg^{PE13} mutants show no activity (see panel *C*). These mutations truncate the molecule in a region of strong conservation with mouse Wnt-1. Identical residues are shown in boldface. Protein distributions for wg^{PE4} and wg^{PE13} are not obtainable (see legend for Figure 5). (F) Cuticle pattern produced by embryo trans-heterozygous for wg^{PE2} and wg^{PE4}; embryo shown was raised at 18°, slightly less naked cuticle is observed in embryos raised at 25°.

at position 248, and this mutant shows no evidence of *wg* signaling activity (Figure 6C). Thus the two amino acids between positions 248 and 250 appear to be critical to the activity observed in wg^{PE4} mutants. This activity may be due directly to the tryptophan and methionine residues at these positions, or it may result from changing the context for the cysteine residue at position 247. This cysteine is the last conserved cysteine before the 85 amino acid nonconserved region and so may be crucial for the structural conformation of an amino-terminal domain.

The cuticle pattern produced by wg^{PE4} mutants indicates that this truncated molecule promotes preferentially the late function of *wg*, because it specifically phenocopies temperature-sensitive mutant embryos that have had *wg* function only at late stages of development. The reciprocal pattern seen in wg^{PE2} mutants suggests that the wg^{PE2} lesion disrupts late function without affecting early function. This presents a paradox. The wg^{PE2} lesion alters a residue at the very carboxy-terminus of the protein. This portion of the molecule is deleted in the truncated wg^{PE4} protein, yet the cuticle pattern of the wg^{PE4} mutant indicates that the mutant protein is able to direct late signaling activity. It therefore seems likely that the overall conformation of the Wg protein is critical for its ability to promote early *vs.* late signaling activity.

The heteroallelic combination of wg^{PE2} and wg^{PE4} in

trans shows partial complementation. The trans-heterozygous embryos produce a cuticle pattern with denticle diversity and some naked cuticle (Figure 6F). This pattern is closer to wild type than that produced by either homozygous mutant, but is not completely wild type: it resembles the pattern produced by mutants with slightly lower levels of *wg* expression due to rearrangements in the promoter region (A. BEJSOVEC, unpublished data). This partial complementation stongly suggests that the phenotypes of wg^{PE2} and wg^{PE4} are produced by partially nonoverlapping subsets of the wild-type array of *wg* signaling activities.

DISCUSSION

Our results indicate that the *wingless* signaling mechanism has novel properties that distinguish it from other growth factor signal transduction pathways. Although Wg appears to transduce signal through a cell surface receptor, ligand binding alone is not sufficient to activate this receptor. Endocytosis is dispensable for wild-type levels of signaling activity in cells that are immediately adjacent to the *wg*-expressing cells, but appears to be required for normal Wg transport and signaling in cells at a distance from the *wg*-expressing cells in the segment. Finally, different portions of the Wg molecule appear to promote distinct signaling activities and strongly suggest a complex interaction between Wg and its cellular receptor molecule(s).

Wg protein trapped at the cell surface is able to promote known responses to *wg* signaling activity in the *wg*-expressing cells and in their immediate neighbors. Thus it is likely that Wg transduces signal through a cell surface receptor and that endocytosis of Wg into intracellular vesicles is secondary to signal transduction at the cell surface. This is consistent with the observation that the vertebrate Wnt-1 protein transmits signal in cell culture assays when it is tethered to the expressing cell by a heterologous transmembrane domain (PARKIN *et al.* 1993). The subcellular localizations of wild-type Wg appear to be part of a receptor-mediated endocytotic process similar to that of other secreted peptide growth factors.

Blocking endocytosis also provides insight into the mechanism of Wg protein distribution. In wild-type Drosophila embryos, detection of Wg protein in cells that lie more than one cell diameter away from *wg*-expressing cells could result from its diffusion through extracellular spaces (VAN DEN HEUVEL *et al.* 1989) or from cells actively transcytosing the molecule (GONZALEZ *et al.* 1991). In *shibire* mutants, the distribution of Wg protein is limited to the area immediately surrounding the cells that express *wg*. We conclude that the broad Wg protein distribution observed in wild-type embryos is due to active cellular processes related to the endocytotic pathway, rather than to passive diffusion through extracellular spaces. This is consistent with the observation that both Wg (VAN DEN HEUVEL *et al.* 1989; GONZALEZ *et al.* 1991) and Wnt-1 (PAPKOFF *et al.* 1987; BRADLEY and BROWN 1990; PAPKOFF and SHRYVER 1990) proteins are found to tightly associate with membrane and extracellular matrix and therefore it seems unlikely that they would diffuse freely through extracellular spaces. In addition, blocking endocytosis limits the domain of cells that show Arm relocalization in response to *wg* signal. The concommitant restriction of Wg protein and *wg* response suggests that Wg protein that has been transported to cells that lie several cell diameters away from the *wg*-expressing row of cells in the wild-type segment may act directly to trigger response in those cells.

Properties of a mutant Wg molecule, encoded by wg^{CE7}, suggest that Wg ligand binding alone may not be sufficient to activate its cell surface receptor. wg^{CE7} mutant protein is internalized properly without transducing signal. The mutant Wg protein is detected in cells more than one cell diameter away from the *wg*-expressing cells, strongly suggesting that it interacts with the cellular components that normally handle Wg protein in its transport within and between cells. Therefore this truncated Wg ligand may be able to bind to its receptor without necessarily activating it, suggesting complexity in the interaction between the Wg ligand and its receptor. Known growth factor receptors can be internalized in the absence of signal transduction: a mutant epidermal growth factor (EGF) receptor that cannot be activated because of mutation in the kinase domain is still internalized upon binding ligand (FELDER *et al.* 1990; HONEGGER *et al.* 1990)

Late in development, wg^{CE7} mutants begin to show an abnormal Wg protein distribution apparently due to enhanced stability of the mutant protein. We postulate that the wild-type Wg may be degraded as a consequence of having transduced signal and that mutant Wg that fails to signal may not be properly targetted to the lysosome. Intracellular vesicular trafficking is important in modulating the activity of other known growth factors and can be altered by the status of the receptor molecule. For example, EGF receptor is normally targetted to the lysosome after transducing signal and thereby is down-regulated, but a kinase-defective mutant receptor escapes degradation and is recycled to the cell surface instead (FELDER *et al.* 1990). Such intracellular trafficking may also relate to transcytosis of Wg protein. The mutant Wg is eventually detected in cells far from the *wg*-expressing stripe, both in epidermal cells and in cells beneath the epidermis, where wild-type Wg is not normally detected. Failure to signal may favor a transcytotic pathway, or transcytosis may represent a default state for Wg protein handling. This wider distribution of Wg protein also indicates that cel-

lular receptors able to handle Wg protein are widely distributed within the embryo.

Molecular analysis of mutant *wg* alleles has revealed that the structure of the Wg ligand cannot be explained in terms of simple functional domains. The wg^{CE7} mutant molecule is missing the carboxy-terminal 101 amino acids and fails to trigger any detectable signaling activity. The wg^{PE4} mutant molecule is missing an even larger portion of the carboxy-terminus than is the wg^{CE7} mutant molecule, but it does promote some signaling activity. Thus the carboxy-terminal 101 amino acids missing in wg^{CE7} are not exclusively responsible for activating the receptor. Rather, activity may reside in several portions of the molecule and some intervening sequence in the longer wg^{CE7} mutant molecule may interfere with the activity observed in the shorter wg^{PE4} molecule.

Structural complexity of the Wg ligand is further suggested by properties of the wg^{PE2} and wg^{PE4} mutations. These alterations in the Wg molecule mimic the effects of distinct temporal phases of *wg* function, defined by *wg* temperature shift experiments (BEJSOVEC and MARTINEZ ARIAS 1991). Since the region affected in the wg^{PE2} mutant, disrupting late function, is deleted in the wg^{PE4} mutation, which retains late signaling function, we conclude that discrete functional domains are not responsible for each activity separately. Rather, various portions of the Wg molecule may contribute differentially to the conformation of the protein, accounting for the ability to mutate the early *vs.* late signaling activities independently. The conformation of the Wg molecule might promote these distinct functions in one of two ways. First, Wg may have two different receptors, perhaps expressed at different times, one for transducing the early signaling function and one for the late. Altered conformation of the mutant Wg molecules then may favor binding to one receptor preferentially, so that only one specific signaling event is transduced. Alternatively, Wg may act through a single cell surface receptor but the conformation of Wg protein bound to the receptor may alter the receptor conformation and change the constellation of cellular proteins with which it interacts on the cytoplasmic face of the membrane. In this case the interacting cytoplasmic molecules would modulate *wg* signaling to produce different cellular consequences. Two molecules have been identified that appear to modulate a specific cellular function of *wg* signaling activity. The segment polarity genes *patched* and *naked* (JÜRGENS *et al.* 1984; NÜSSLEIN-VOLHARD *et al.* 1984; HOOPER and SCOTT 1992) have been found to play a role in antagonizing the autoregulatory function of *wg* signaling (BEJSOVEC and WIESCHAUS 1993). In the absence of one or both of these gene activities, *wg* activates its own expression in neighboring cells. Thus the wild-type functions of these gene products restrict the ability of *wg* signaling to influence *wg* transcription, without restricting the ability of *wg* to specify patterning information across the segment or to influence expression of *en* in posterior neighboring cells. It therefore seems likely that other molecules may interact with the *wg* signaling pathway to modulate its range of specific cellular consequences.

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