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The consequences of ubiquitous expression of the *wingless* gene in the *Drosophila* embryo

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Summary

The segment polarity gene *wingless* has an essential function in cell-to-cell communication during various stages of *Drosophila* development. The *wingless* gene encodes a secreted protein that affects gene expression in surrounding cells but does not spread far from the cells where it is made. In larvae, *wingless* is necessary to generate naked cuticle in a restricted part of each segment.

To test whether the local accumulation of *wingless* is essential for its function, we made transgenic flies that express *wingless* under the control of a *hsp70* promoter (HS-*wg* flies). Uniform *wingless* expression results in a complete naked cuticle, uniform *armadillo* accumulation and broadening of the *engrailed* domain. The expression patterns of *patched, cubitus interruptus Dominant* and *Ultrabithorax* follow the change in *engrailed*.

The phenotype of heatshocked HS-*wg* embryos resembles the segment polarity mutant *naked,* suggesting that embryos that overexpress *wingless* or lack the *naked* gene enter similar developmental pathways.

The ubiquitous effects of ectopic *wingless* expression may indicate that most cells in the embryo can receive and interpret the *wingless* signal. For the development of the wild-type pattern, it is required that *wingless* is expressed in a subset of these cells.

Key words: *Drosophila melanogaster*, *wingless*, ectopic expression, segmentation.

Introduction

Five hours after fertilization, the *Drosophila* embryo exhibits the first morphological signs of repeated units that will form the segments of the larva and adult fruit fly (reviewed by Lawrence, 1992). A gene essential for correct formation of this pattern is *wingless*; in its absence, the precisely banded pattern in the larval cuticle is replaced by a continuous field of denticles (Babu, 1977; Nüsslein-Volhard and Wieschaus, 1980). The *wingless* gene is also needed to form the wing and a variety of adult structures; in *wingless*[1] mutants the group of cells that should make the wing sometimes become misdirected to duplicate a part of the thorax (Sharma and Chopra, 1976; Morata and Lawrence, 1977).

In the *Drosophila* embryo, *wingless* is expressed in a precise pattern, initially in narrow stripes of cells that line the parasegment borders on their anterior sides (Baker, 1987; van den Heuvel et al., 1989). *wingless* is a member of the class of segment polarity genes. Mutations in these genes affect the cuticle pattern of every segment. Some sixteen segment polarity genes have been identified and several have been cloned: *armadillo* (Riggleman et al., 1989), *cubitus interruptus Dominant* (Orenic et al., 1990), *fused* (Préat et al., 1990), *gooseberry* (Baumgartner et al., 1987), *engrailed* (Fjose et al., 1985; Kornberg et al., 1985), *patched* (Hooper and Scott, 1989; Nakano et al., 1989), *shaggy/zeste-white3* (Bourouis et al., 1990; Siegfried et al., 1990) and *wingless* (Baker, 1987; Rijsewijk et al., 1987). Zygotic transcripts from these genes can be first detected towards the end of the blastoderm stage as the cells form; from then on cell interactions are likely to be important in maintaining and elaborating pattern.

The predicted molecular structures of several segment polarity gene products are consistent with roles in intercellular signaling: *fused* and *shaggy/zeste-white3* are putative serine-threonine kinases, *patched* encodes a putative transmembrane protein and *armadillo* is related to β-*catenin* (McCrea et al., 1991) and *plakoglobin* (Peifer and Wieschaus, 1990), proteins associated with cell-cell junctions. An indication that *wingless* might play a role in these interactions is that the gene is necessary to maintain *engrailed* expression in adjacent cells, shown by the disappearance of *engrailed* in *wingless*$^-$ embryos, just after gastrulation (DiNardo et al., 1988; Bejsovec and Martinez-Arias, 1991; Heemskerk et al., 1991). Moreover, the two

genes are interdependent; in *engrailed*$^{-}$ embryos *wingless* expression decays prematurely (Martinez-Arias et al., 1988).

wingless is also necessary for local distribution of *armadillo* protein, most likely by some form of post-transcriptional control (Riggleman et al., 1990). *wingless* encodes a cysteine-rich protein with a signal peptide (Rijsewijk et al., 1987). The protein is secreted, becomes associated with the extracellular matrix (van den Heuvel et al., 1989) and can apparently spread from cells in which it is made into nearby cells (van den Heuvel et al., 1989; Gonzáles et al., 1991).

The *wingless* gene is highly conserved; there are both vertebrate and *Drosophila* homologs (The *Wnt* gene family; reviewed by Nusse and Varmus, 1992) and there are indications that these genes behave similarly to the *wingless* gene product; the mammalian *Wnt-1* protein is also secreted and presumably retained by the cell surface or the extracellular matrix (Papkoff and Schryver, 1990; Bradley and Brown, 1990) and can influence the behaviour of cells in close proximity (Jue et al., 1992). In general, the *wingless* and *Wnt* proteins may all be necessary for regional specification of groups of cells. No receptor for any of these molecules is known.

One way to test whether the local distribution of the *wingless* protein is important in determining cell fate is to alter the *wingless* distribution in embryos. We have therefore made transgenic flies that express the gene under the control of a heatshock promoter. Universal expression of the protein produces a striking phenotype, suggesting that most cells can respond to the *wingless* signal. This phenotype strongly resembles that produced by a mutation in the segment polarity gene *naked*.

Materials and methods

*Construction of pHS-*wingless *plasmid and generation of transgenic flies*

A construct was made which consists of a 231 bp *Xho*I-*Alu*I fragment of the promoter region of the *hsp*70 gene from clone 56H8 (Moran et al., 1979), cloned in front of 2.9 kb *wingless* sequence containing its own polyadenylation signal. The *Xho*I site of the *hsp*70 promoter region is located at −188 bp and the *Alu*I site is located at +43 bp with respect to the transcription start site (Török and Karch, 1980). The *Xho*I site was made blunt ended and to the *Alu*I site a *Bam*HI linker was ligated. The *wingless* sequence consists of a 1.2 kb *Fnu*DII-*Eco*RV cDNA fragment (with a *Bam*HI linker at the *Fnu*DII end) ligated to 1.7 kb *Eco*RV-*Pvu*I genomic sequence. This construct was cloned into the *Hpa*I site of the P-element vector Carnegie 20, containing the *rosy* gene as a marker and injected into *rosy*^{506} flies together with the pII 25.1 WC helper plasmid (Karess and Rubin, 1984). One line was obtained that contained a single P-element insertion at position 66A/B on the third chromosome. This stock was homozygous viable.

Fly stocks

All flies were maintained at 23°C on medium containing agar, yeast and glucose. For the study of the effect of heatshock on cuticle phenotype, homozygous HS-*wg* flies were used, while *rosy*^{506} flies were used as a control. Embryos of the strains HS-*wg*/TM3Sb and *Canton S* were used for antibody stainings and RNA in situs. A stock was made that contain the HS-*wg* construct and a β-*galactosidase*-marker gene: *eve*-β-*gal*; HS-*wg*/TM3Sb which has a P-element inserted on the first chromosome, containing the β-*galactosidase* gene driven by the *even-skipped* promoter (Lawrence et al., 1987).

The *naked* alleles *nkd*^{7E89} and *nkd*^{7H16} were a gift from C. Nüsslein-Volhard.

Heatshock procedure and larval cuticle preparation

The effect of heatshock on cuticle pattern was studied in embryos homozygous for the HS-*wg* construct. Eggs were collected on apple-juice plates for 4 hours, washed in PBS and 0.1% Triton (X-100) transferred to slides and covered with 3 S-oil. Staging of the embryos was done according to Wieschaus and Nüsslein-Volhard (1986). Groups of embryos of a particular stage were picked out, transferred to Eppendorf tubes and submerged in a waterbath at 36°C for 20 minutes. At the end of the heatshock period, the embryos were placed on apple-juice plates and allowed to develop. After two days the embryos were dechorionated, devitellinized by hand and mounted in Hoyer's medium to study the cuticle phenotype (Wieschaus and Nüsslein-Volhard, 1986). *rosy*^{506} embryos were used as a control for the general effects of heatshock on embryonic development. Heatshocked *rosy*^{506} embryos mostly hatched with normal cuticle pattern. In some cases, larvae were found with fused denticle belts.

For antibody stainings and RNA in situs, embryos were collected from HS-*wg*/TM3Sb flies. Embryos were collected for 5 hours, aged for 1 hour and heatshocked for 20 minutes at 36°C as described. After the first heatshock, embryos were transferred to apple-juice plates and maintained at room temperature. Second and third heatshocks were carried out 2 and 4 hours after the start of the first heatshock. The embryos were transferred to apple-juice plates and maintained for different time periods until they were dechorionated and fixed in 4% formaldehyde. Heatshocked *CantonS* and non-heatshocked *CantonS* and HS-*wg* embryos were used as a control. A slight increase in background was observed as a general effect of heatshock.

Whole-mount antibody staining

Embryos were fixed in 4% formaldehyde and prepared for single or double antibody staining (van den Heuvel et al., 1989).

Antibodies were kindly provided by E. Wieschaus (anti-*armadillo*), T. Kornberg (anti-*engrailed)*, M. Wilcox (anti-*Ubx*), M. van den Heuvel (anti-*wingless*) and C. Goodman (anti-β-*galactosidase*).

Whole-mount RNA in situ

RNA in situ hybridization on whole-mount embryos using digoxigenin-labeled DNA probes was performed according to a modification of the protocols of Tautz and Pfeiffle (1989) and D. Andrew (personal communication). Embryos were collected, heatshocked, fixed in formaldehyde and prepared for hybridization.

Probes were made of the following purified inserts:

2.9 kb *Fnu*DII - *Pvu*II *wingless* sequences used to make the HS-*wg* construct (Rijsewijk et al., 1987);

240 bp *Eco*RI-*Sph*I most 5′ fragment of *wingless* cDNA, specific for the endogenous transcript (Rijsewijk et al., 1987);

3.5 kb *Eco*RI fragment of *patched* DNA (Hooper and Scott, 1989);

2.4 kb *Eco*RI fragment of *armadillo* DNA (Riggleman et al., 1989);

1.0 kb *Eco*RI fragment of *cubitus interruptus Dominant* DNA (Orenic et al., 1990);

1-3 μg of purified insert and 2 mg/ml hexanucleotide primers (Pharmacia) were boiled, chilled on ice and added to a mixture of hexanucleotide mix, dNTP labeling mix and Klenow fragment (as described in Genius protocol, Boehringer Mannheim). After

overnight incubation at 14°C, 250 U/ml Klenow was added and the mixture was incubated for another 4 hours at room temperature. The probe was precipitated, washed, dissolved in TE containing 0.2 % SDS and incubated for 2 hours prior to use at 37°C to prevent the probe from sticking to the plastic tube. Hybridization was done in a final volume of 200 µl using 100 µl of pretreated embryos with a probe concentration of approx. 2 ng/µl. After hybridization, the washed embryos were incubated for 1 hour with anti-digoxigenin-alkaline phosphatase antibody (1:1000) that was preabsorbed against embryos for 2 hours at room temperature. Staining was performed using NBT and X-phosphate as substrates. After dehydration, embryos were transferred to methylsalicylate and mounted in Araldite.

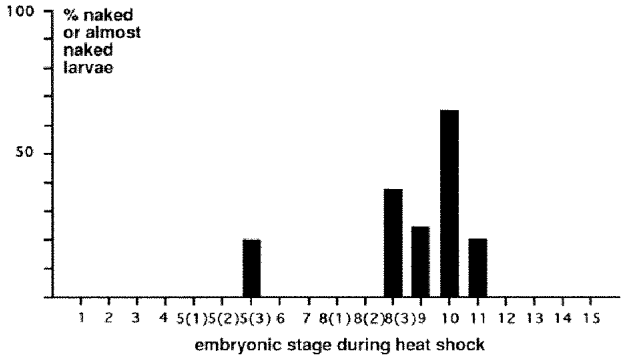

Fig. 1. Percentage of HS-*wg* embryos that show naked or almost naked larval ventral cuticle as a result of uniform *wingless* expression at different developmental stages.
Notes
(1) HS-*wg* embryos of developmental stages 0 to 15 (Wieschaus and Nüsslein-Volhard, 1986) were heatshocked for 20 minutes at 36°C (Materials and methods).
(2) HS-*wg* embryos heatshocked during stages 1 to 5 [2] of development did not survive to generate cuticle. The differences in cuticle phenotype of HS-*wg* embryos heatshocked during stages 5[3] to stage 11 are described in the Results section. Embryos heatshocked during stages 12 to 15 showed a normal cuticle phenotype. HS-*wg* embryos with abnormal cuticle phenotype did not hatch.
(3) We heatshocked different numbers of embryos of each developmental stage: 46 at stage 5[3]; 25 at stage 6; 14 at stage 7; 21 at stage 8[1]; 17 at stage 8[2]; 29 at stage 8[3]; 24 at stage 9; 35 at stage 10; 10 at stage 11; 8 at stage 12; 11 at stage 13; 41 at stage 14/15.
(4) Heatshocked *rosy*^{506} embryos of all described developmental stages were used as a control. In most cases *rosy*^{506} larvae hatched after two days with wild-type cuticle pattern; in some cases larvae were found with fused denticle belts.

Results

Ubiquitous wingless *expression results in naked ventral cuticle pattern*

To study the effect of ectopic *wingless* expression on the cuticle pattern and on the expression of segmentation genes, we made a line of transgenic flies (HS-*wg*) in which the *wingless* gene is controlled by the *hsp*70 promoter. These flies were homozygous viable but weak; the insert maps to cytological location 66A/B. Individually staged embryos were shocked for 20 minutes at 36°C, kept for 2 days and the cuticle prepared. *rosy*^{506} embryos were used as a control (Materials and methods).

In HS-*wg* two heatshock-sensitive periods during embryogenesis could be distinguished (Fig. 1). The first period is during late blastoderm (stage 5 [3], Lawrence and Johnston, 1989; Wieschaus and Nüsslein-Volhard, 1986) when heatshock results in a dramatic but incomplete loss of ventral denticle structures. This is the stage during which, in wild-type embryos, the *wingless* protein is first detected. The second period is later and longer, spanning from when the germ band is fully extended (stage 8 [3]) until germ band shortening (stage 12). The most extreme phenotype found after heatshock in this period was a lack of all abdominal denticle bands (Fig. 2B); we call this the HS-*wg* phenotype. Some denticles (such as the T1 beard) are unaffected, showing that ectopic *wingless* changes the pattern of the cuticle without interfering with the ability to make denticles as such (Fig. 2C).

In wild-type embryos, many interactions between segment polarity genes are believed to take place during this second sensitive period. An indication for this is that expression of *wingless* and *engrailed* is altered at stage 9 to 11 in most segment polarity mutants (van den Heuvel et al., unpublished data).

HS-*wg* embryos heatshocked before stage 5 [3] do not survive to make cuticle, but were not studied in detail, while those shocked during stages 12 to 15 make a cuticle with a normal pattern.

We compared the complete HS-*wg* pattern to wild type. In the wild-type ventral abdomen, naked cuticle is characteristic of the posterior region of the segment, including parts both anterior and posterior to the parasegment border (Lohs-Schardin et al., 1979; Figs 2A and 7B). Each wild-type segment consists of a larger anterior and a smaller posterior compartment and these alternate throughout the 14 parasegmental compartments in the main part of the body (Martinez-Arias and Lawrence, 1985). The compartments have been precisely mapped in the larva using an *engrailed-lacZ* construct (Hama et al., 1990) so that we know which structural elements are anterior and which posterior and where they are located within the compartments. In the HS-*wg* phenotype, parts of the wild-type pattern appear unchanged while other parts are not present; probably the response of any particular element in the pattern is characteristic of the level in the segment from which it derives. For example, the beard, which is formed by the posterior region of the anterior segmental compartment of T1 (called T1a) is intact in the HS-*wg* phenotype (Fig. 2C). Dorsally, the sharply defined hairs characteristic of the most anterior region of the T2a compartment are absent but the similar hairs of the T3a compartment are not (Fig. 2D). In wild-type larvae, these latter hairs are formed further back in T3 than in T2 (Hama et al., 1990) suggesting that the part of the pattern eliminated includes only the anterior half of the anterior compartments - just sufficient to remove the denticle bands ventrally, the T2 anterior dorsal denticles, but not to affect the beard or the T3 anterior dorsal denticles. Ventrally, the first row of abdominal denticles actually belongs to the posterior compartment (Lohs-Schardin et al., 1979; Hama et al., 1990) and these are also eliminated; it

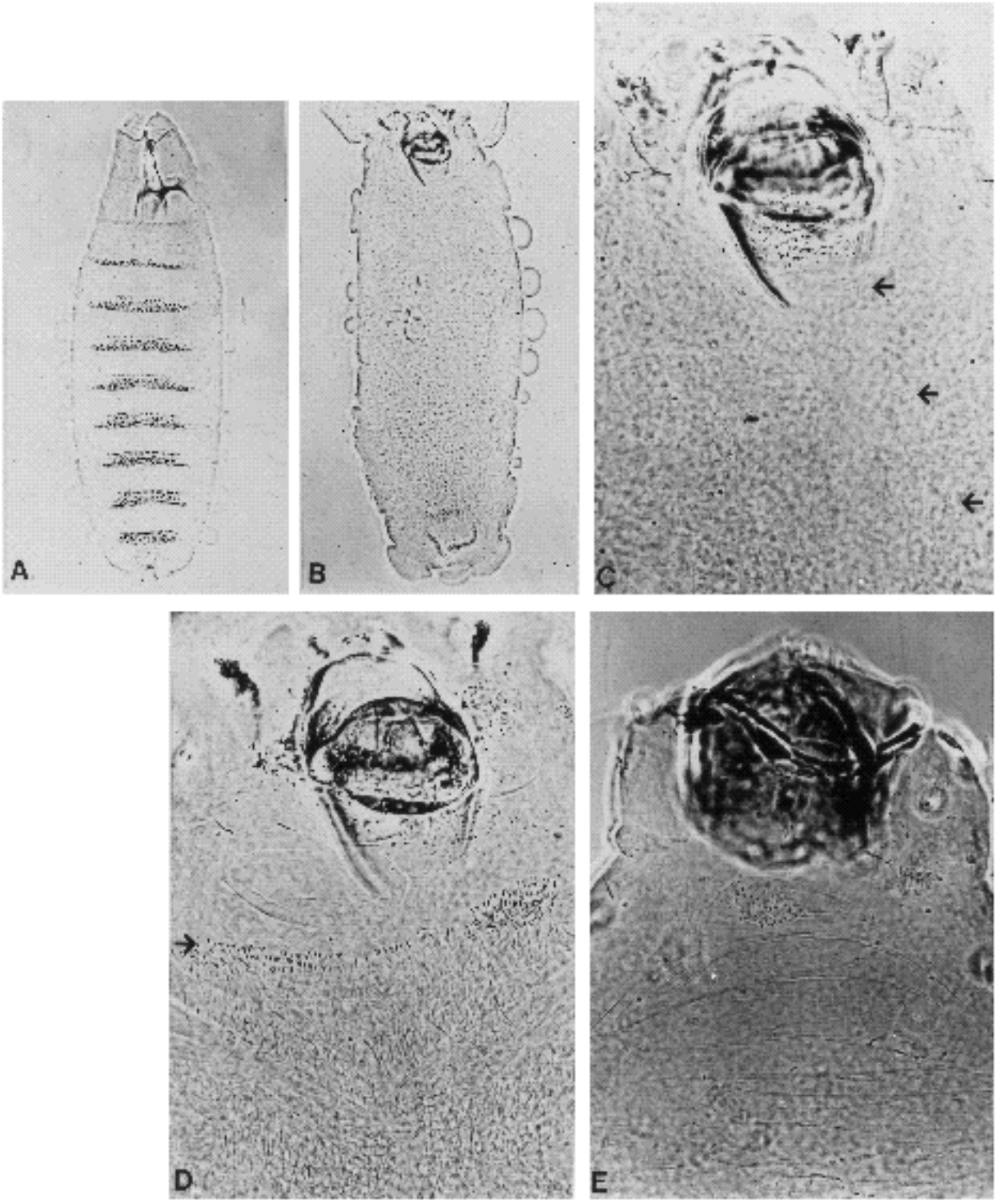

Fig. 2. Comparison of cuticle phenotype of heatshocked *rosy*^{506} (A), heatshocked HS-*wg* (B, C and D) and *naked7E89* (E) larvae. Anterior is up. (A) Ventral view of heatshocked *rosy*^{506} larval cuticle. Note the presence of denticle belts in the anterior portion of each segment. (B) Ventral view of heatshocked HS-*wg* larval cuticle. Uniform *wingless* expression results in a complete naked cuticle, except for the beard (shown in C), the tuft and the Fell. (C) Detail of HS-*wg* larva in B, showing the ventral cuticle with misformed head, the T1 beard and the Keilin's organs indicated by the arrows. (D) Detail of HS-*wg* larva in B, showing the dorsal cuticle in the head region. The most anterior row of hairs is located in compartment T3a, indicated by the arrow. (E) Detail of *naked*^{7E89} larvae with misformed head, naked ventral cuticle and beard.

follows that at least part of the posterior compartment is not present in the HS-*wg* phenotype.

Structures near the parasegment border, such as the Keilin's organs (Fig. 2C) and some other sensory structures are not eliminated and this, as well as the normal size of the beard, suggests that those parts of the pattern that remain are normal or nearly so. Outside the parasegmental trunk there are also effects - the head is misformed (Fig. 2B) and so are parts of the tail. The A9 tuft develops as in wild type.

Partial phenotypes, observed after heatshocking during all stages between 5 [3] and 11, suggest a hierarchy - the regions of denticles are removed in a specific order. When the phenotype is weak, only the two most anterior rows of denticles are removed, resulting in a narrower denticle band. As the phenotype becomes more severe, the next most anterior row is removed and those elements characteristic of the posteriormost part of the denticle belt – the last row is finer and smaller and trails out further dorsally – are unaffected. A still further increase in the phenotype removes more of the denticle bands, particularly near the ventral midline, so that the bands become divided into two lateral patches.

Ectopic wingless *protein induces endogenous* wingless *expression in a specific pattern*

A 20 minute heatshock at 36°C produces a strong response in HS-*wg* embryos, resulting in a high uniform level of *wingless* protein (Fig. 3A, B). Until 45 minutes after heatshock there is universally high levels of *wingless* protein but by 1.5 hours most ectopic *wingless* protein has disappeared, leaving a normal pattern of *wingless* expression. However, by 3 hours after heatshock, two new *wingless* domains appear, which are not present in wild-type embryos at this stage. In wild-type stage-10 embryos, *wingless* is expressed in 14 stripes; the thoracic and abdominal stripes being split into ventral stripes and dorsolateral patches (van den Heuvel et al., 1989; Fig. 4A, G). In HS-*wg* embryos, high levels of ectopic *wingless* antigen can be detected in a large dorsal patch and lower levels in a stripe in the ventral region (Figs 4B, H, and 7A). Ventrally, protein can be detected between the ectopic and the normal stripes, but this is probably due to spreading of the secreted protein – the RNA is localized strictly to cells in the two stripes. Using a probe specific for endogenous *wingless* RNA that distinguished it from the product of the HS-*wg* construct, we determined that the newly formed *wingless* comes from the endogenous gene (data not shown).

When embryos are heatshocked for 20 minutes, only a few percent of the embryos show the induction of endogenous *wingless* in the ectopic domain after 3 hours. Since the effect of heatshock on cuticle phenotype is restricted to a certain rather limited period in development, we heatshocked three times for 20 minutes with intervals of 2 hours between the start of each heatshock to increase the chance of hitting the sensitive period (Materials and methods). In this way, we are able to obtain a higher percentage of the embryos showing the described phenotype. The same temporal pattern of *wingless* expression is observed (disappearance of HS-*wg* protein after 1.5 hours and induction of endogenous *wingless* after 3 hours), but now almost all of stage 10 HS-*wg* embryos have the ectopic *wingless* domain 3 hours after the last heatshock. *Canton S* control embryos do not exhibit the change in *wingless* expression after three heatshocks. HS-*wg* embryos treated in this way show a developmental delay; many embryos of a 1-6 hour egg collection, heatshocked three times with 2 hour intervals between the heatshocks and then aged for 3 hours, are still in stages 10 to 11.

Ectopic wingless *expression results in expansion of the* engrailed *expression domain in a posterior direction*

In every parasegment in the embryo, an anterior stripe of cells expresses *engrailed* (Fig. 4D, G). In HS-*wg* embryos, collected for 1 to 6 hours and heatshocked once for 20 minutes at 36°C, a change in *engrailed* expression is observed 1 to 1.5 hours after heatshock, resulting in a broader *engrailed* stripe. 3 hours after heatshock, the *engrailed* stripe has broadened further, spanning maximally about one-third of the segment. This effect is seen in only a few percent of the embryos. When we used the multiple heatshock regime (Materials and methods), virtually all of the HS-*wg* embryos showed a broader *engrailed* domain. In the HS-*wg* embryos, treated in this way, no change in the *engrailed* pattern is detected 30 minutes after the last heatshock (when the level of *wingless* protein is universally high). However, by 1.5 hours after the last shock, the *engrailed* stripe has begun to broaden and this continues progressively. By 3 hours, the *engrailed* domain has broadened further, apparently to a maximum of half the width of the parasegment. Just posterior to the broadened *engrailed* stripe, grooves form and these are much deeper than the normal parasegment grooves - which can still be seen at the anterior limits of the *engrailed* bands (Figs 4E, 7A). Heatshocked *Canton S* control embryos do not show this change in *engrailed* expression or formation of the new deep grooves.

We determined the direction of broadening of the *engrailed* stripe with reference to fixed landmarks. The anterior limits of the *even-skipped* stripes mark the odd-numbered parasegment borders; we therefore used an *eve-lacZ* construct to label them (Lawrence et al., 1987). The results revealed that the original parasegment borders remain unchanged while the *engrailed* stripes broaden posteriorly (Fig. 5).

Double labelling for both *wingless* and *engrailed* showed that the most prominent *wingless* stripe is the normal one found just anterior to the parasegment boundary; the ectopic *wingless* dorsal patch is located just posterior to the *engrailed* domain and the deep groove (Fig. 4E,H).

Expression of several segmentation and homeotic genes changes as a consequence of ectopic wingless *expression*

To understand more about how the HS-*wg* phenotype is established, we studied the changes in expression of several segmentation and selector genes caused by ubiquitous *wingless* protein. We concentrated on a gene thought to function in the *wingless*-signalling pathway (*armadillo*) and three genes (*patched*, *cubitus interruptus Dominant* and *Ubx*) that are regulated by *engrailed* and essential for correct formation of the anterior compartment.

armadillo RNA is normally expressed uniformly but the protein concentration is uneven; it is found at high levels in those cells where *wingless* is expressed (Riggleman et al., 1989; Fig. 6A). In *wingless*$^-$ embryos, the RNA pattern is unchanged, while *armadillo* protein is distributed at uniform low levels (Riggleman et al., 1990). When HS-*wg* embryos are shocked three times and kept for 3 hours (Materials and methods), the *armadillo* RNA shows the normal pattern of uniform distribution. The protein distribution is quite different from that in wild-type embryos, being evenly distributed and showing intense staining throughout the embryo (Fig. 6B).

The *patched* gene encodes a transmembrane protein which, by the end of germ band extension, is expressed in stripes that correspond to the anterior compartment (Hooper and Scott, 1989; Nakano et al., 1989; Fig. 6E). By stage 12, *patched* transcripts disappear from the middle of each stripe so that each original stripe becomes two narrow ones. In HS-*wg* embryos, 3 hours after the last shock, the *patched* domain becomes confined to those cells that do not express *engrailed*. The anterior border of the *patched* stripe coincides with the deep groove that marks the posterior limit of the broadened *engrailed* stripe (Fig. 6F).

The *cubitus interruptus Dominant* gene encodes a protein with a zinc-finger motif and is expressed in the anterior compartments (Orenic et al., 1990; Fig. 6H). In HS-*wg* embryos, after heatshock treatment, *cubitus interruptus Dominant* RNA expression becomes restricted to those cells that are outside the expanded *engrailed* domain and therefore coincides with the *patched* stripe (Fig. 6I).

The *Ubx* gene is a member of the bithorax complex and is expressed in a parasegmental register; the anterior limit of *Ubx* is at the anterior boundary of parasegment 5 (White and Wilcox, 1985; Fig. 6K). In the wild type, expression of Ubx is high in the anterior compartments of parasegments 7-12 and low in the posterior compartment where *engrailed* protein is present and suppresses transcription of *Ubx*. In HS-*wg* embryos, after shock, we observe that the outcome expected in parasegments 7-12 *Ubx* protein is only seen in those cells that do not express *engrailed* (Fig. 6L).

*The phenotype of HS-*wg *embryos and the segment polarity mutant* naked *are similar*

The cuticle phenotype and pattern of gene expression in HS-*wg* embryos strongly resembles the *naked*$^-$ phenotype (Jürgens et al., 1984; Martinez-Arias et al., 1988). *naked* is a segment polarity gene, whose molecular nature is unknown. *naked*$^-$ embryos are shorter than HS-*wg*, but are otherwise similar. As in the HS-*wg* phenotype after heat shock, the *naked*$^-$ embryos have no denticle bands in the ventral cuticle (apart from the beard in T1; Fig. 2E) but do have the Fell and the A9 tuft. The Keilin's organs are present but, unlike those in HS-*wg* embryos, are abnormal. Often they consist of four or more hairs, rather than three, and occasionally there are three rather than two organs per segment (Simcox et al., 1989).

The expression patterns of *wingless* and *engrailed* in HS-*wg* embryos, 3 hours after heat shock, are similar to those in *naked*$^-$ embryos (Figs 4C, F, I). As with HS-*wg*, we used embryos carrying an *eve-lacZ* construct to determine that the *engrailed* stripe broadens in the posterior direction. There is a similar deep groove formed at the posterior limit of the *engrailed* domain, and anterior to the ectopic *wingless* stripe (Fig. 4F). Moreover, the distributions of *patched* and *cubitus interruptus Dominant* RNA as well as *Ubx* protein were the same in the two kinds of embryos (Figs 6G, J, M). For *armadillo*, the RNA pattern is identical but the protein pattern differs. In *naked*$^-$-embryos, there is an equal distribution of protein (as in HS-*wg*) but, in addition, there are two rows of cells that stain more intensely with the *armadillo* antibody (Fig. 6C, D). These cells are located in the *wingless*-expressing half of the parasegment.

Discussion

The striking and defined new phenotype formed when *wingless* protein is made ubiquitously favors the hypothesis that, in wild-type embryos, local accumulation of *wingless* in the segment is essential for correct pattern formation. When *wingless* is expressed throughout the embryo, larvae lose specific pattern elements, resulting in a complete naked ventral cuticle. We have described some of the many correlated changes in HS-*wg* embryos in expression of segment polarity and selector genes.

One simple model for *wingless* function can be considered: the amount of *wingless* present in a cell in the segment could be instructive for its fate. Within the anterior compartment of the wild-type segment, the local concentration of *wingless* protein might determine the local pattern - this active *wingless* protein forming a concentration gradient in the anterior compartment from a high level near the posterior border (where the cuticle is naked) to a low level at the anterior border (where the cuticle forms denticles). Lack of *wingless* might then produce a continuous mass of denticles, as observed. A uniformly high concentration of *wingless* protein would be predicted to produce an even field of naked cuticle, as seen in the HS-*wg* phenotype.

However, the proposed model leaves much unexplained: in the HS-*wg* phenotype, the naked cuticle is not homogeneous, in each metamer there is a posterior domain where there is *engrailed* expression and an anterior region where *wingless* protein is present. In *patched*$^-$, *naked*$^-$ and *shaggy/zeste-white3*$^-$, ectopic expression of *engrailed* and *wingless* is restricted to the same two domains, suggesting that only cells in these domains are capable of expressing either *engrailed* or *wingless* but not both (Martinez-Arias et al., 1988; Perrimon and Smouse, 1989). This predisposition of cell fate could be determined by expression of earlier acting pair-rule genes (Ingham et al., 1991). In the extended germ band HS-*wg* embryo, where the two cell types meet, a new deep groove forms (Fig. 7). The loss of pattern elements that form near the segment border, specifically the denticle bands on both sides of it, could be due to either respecification or to cell death - there probably is cell death in HS-*wg* embryos as they are somewhat smaller than wild type.

The maintenance of the parasegment borders is dependent on *wingless* and *engrailed* and, as we show here, they survive transient ubiquitous expression of *wingless*. The pattern near the parasegment border seems to be unaffected

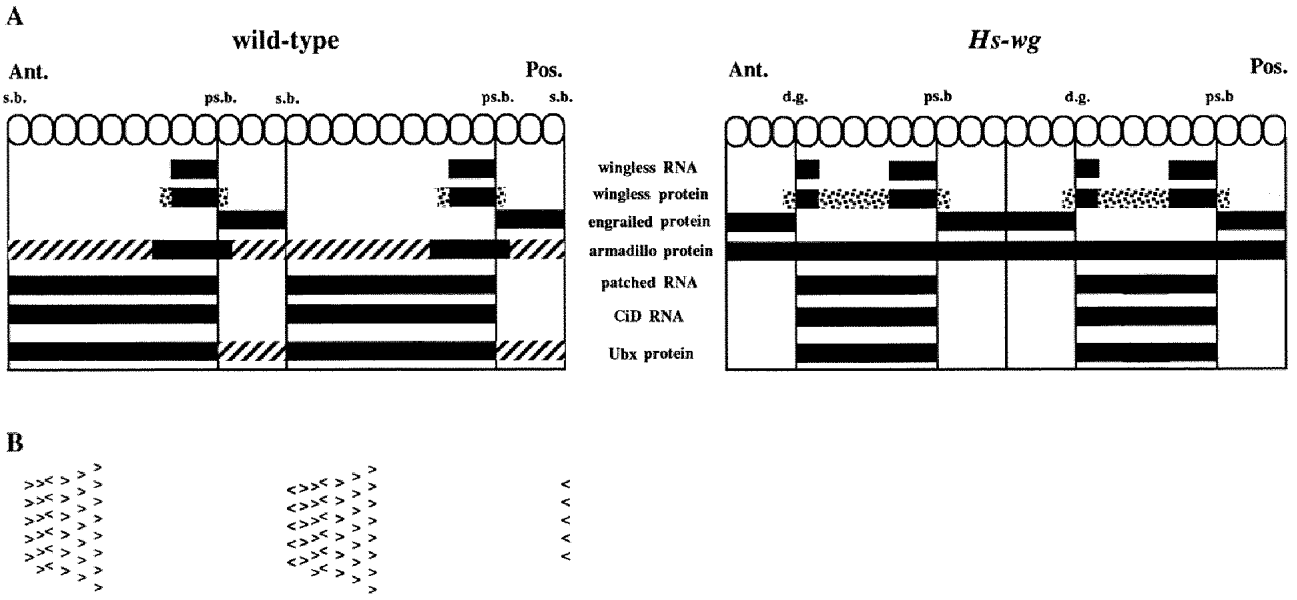

Fig. 7. Schematic representation of the expression pattern of several segmentation genes and cuticle phenotype in wild type and HS-*wg* . (A) Expression of segmentation and selector genes in a stage 10/11 wild-type and HS-*wg* embryo, 3 hours after heatshock (Materials and methods). A schematic drawing of two adjacent segments is shown. At this stage of embryogenesis, each segment is about 12 cells in width. The domain of expression of each gene is depicted by the black boxes. Directly after heatshock in HS-*wg* embryos *wingless* is expressed uniformly throughout the segment. Here, *wingless* expression is shown 3 hours after heatshock; the HS-*wg* RNA has disappeared and an ectopic domain of endogenous *wingless* RNA is observed just posterior to a deep groove. This deep groove is formed in HS-*wg* embryos posterior to the new *engrailed* domain (Fig. 4E). The *wingless* protein is detected at a low level between the ectopic *wingless* domain and the normal *wingless* stripe (Fig. 4B), most likely due to spreading of the protein (spread protein is indicated as dots). In wild-type embryos, the protein can also be detected outside the cells where it is made (shown by the dots; Van den Heuvel et al., 1989). *engrailed* in HS-*wg* embryos has broadened in the posterior direction, spanning half a segmental unit (Fig. 4E), while the high level of *armadillo* protein, normally only found in the *wingless* domain (Fig. 6A), has extended to the whole segment (Fig. 6B). The low basal level of *armadillo* protein in wild-type embryos is shown by stripes. In HS-*wg* embryos *patched* RNA, *cubitus interruptus Dominant* RNA and *Ubx* protein expression seem to correlate with and perhaps follow the altered distribution of *engrailed*. In the wild type, *engrailed* represses *patched* (Hildalgo and Ingham, 1990), *cubitus interruptus Dominant* (Eaton and Kornberg, 1990) and *Ubx* (Martinez-Arias and White, 1988) in a temporally and spatially distinct pattern. In HS-*wg*, where we observe an expansion of *engrailed*, we see a retraction of *patched* (Fig. 6F) and *cubitus interruptus Dominant* (Fig. 6I). *Ubx* is down-regulated in the anterior half of parasegments 7-12, where *engrailed* is expressed at a high level (Fig. 6L). The lower level of *Ubx* in the posterior compartment of the wild-type segment is indicated by the stripes. (Abbreviations: Ant., anterior; Pos., posterior; s.b., segment border; ps.b., parasegment border; d.g., deep groove.) (B) Location of denticle belts and naked cuticle in wild-type and HS-*wg* larvae. In wild-type embryos, abdominal segments are characterized by a belt of denticles in the ventral cuticle of which the first row is in the posterior segmental compartment of the preceding segment, while the other approx. six rows are present in the anterior part of the anterior segmental compartment. The position of the larval belts is shown relative to the embryonic gene expression patterns in A. The more posterior part of the anterior segmental compartment of the larval segment is naked, corresponding to the region where *wingless* is expressed in the embryonic segment. In HS-*wg* embryos the denticle belts are lost, resulting in a complete naked segment.

with normally orientated Keilin's organs and other sensilla formed in their usual positions. Intermediate cuticle phenotypes suggest the pattern is altered progressively out from the segment border since denticle rows are lost, proceeding from the most anterior row in a posterior direction. Changes in gene expression also appear to spread out from the borders - the *engrailed* stripe broadens progressively; 1.5 hours after heatshock when most of the ectopic *wing - less* product has decayed, the *engrailed* stripe begins to broaden from its posterior margin to a maximum of about half the segment width (Fig. 7).

One general explanation for these progressive changes is that the requirements for (or the effects of) *wingless* protein could vary quantitatively across the segment. There is other evidence for this: if the *wingless* gene is inactivated (by temperature shifts using a *wingless*^{ts} mutant) at 5-6 hours of development, the ventral cuticle is almost entirely covered with denticles. After temperature shifts at later and later times during the extended germband stages in the embryo, the cuticle pattern progressively approaches wild type as a naked area first appears near the segment border and then extends towards the anterior (Bejsovec and Martinez-Arias 1991).

The effects of uniform *wingless* expression on the cuticle phenotype seem to be restricted to two periods in development: stage 5(3) and stages 8(3) to 11. During the later period, in particular around stage 9-10, a dramatic change in *engrailed* expression is observed in HS-*wg* embryos. Interestingly, this sensitive period in HS-*wg* embryos coincides with the developmental stage during which *engrailed* expression is dependent on the presence of *wingless* protein (Heemskerk et al., 1991).

Possibly, the action of *wingless* is mediated in part by the *armadillo* protein. It has been shown that the gradient distribution of the *armadillo* protein depends on *wingless* (Riggleman et al., 1990) and, in HS-*wg* embryos intense *armadillo* staining is seen in virtually all the cells of the segment. This ubiquitous response to the *wingless* signal indicates that most cells in the segment can respond by changing the distribution of *armadillo* and generating naked cuticle. This might mean that the receptors for the *wingless* signal are present in many more cells than where *wingless* is expressed in the wild-type segment. Normally, *wingless* could act as a local ligand, triggering a response only in cells where it is expressed or cells to which the protein can spread. The amount of *wingless* and consequently of *armadillo*, could determine what type of cuticle is generated. During various other patterning events in *Drosophila*, regional specification is similarly regulated by locally acting ligands and more widespread receptors (Stevens et al., 1990; Stein et al., 1991; Krämer et al., 1991).

The HS-*wg* phenotype shows a striking similarity with the *naked*$^{-}$ phenotype. The expansion of the *engrailed* domain in a *naked*$^{-}$ background led to the suggestion that *naked* might act as a repressor of *engrailed* in the anterior compartment (Martinez-Arias et al., 1988). Our results suggest that any repression of *engrailed* by *naked* could be overruled by induction of *engrailed* by *wingless*. The mechanism of the interactions between *naked* and *engrailed* is not known, since the molecular structure of the *naked* product has not been established. The segment polarity mutant *shaggy/zeste-white3* has an almost identical phenotype to *naked*$^{-}$ (Perrimon and Smouse, 1989). *shaggy/zeste-white3* encodes a serine-threonine protein kinase, highly homologous to the mammalian protein *glycogen-synthase-kinase 3* (Siegfried et al., 1990; Bourouis et al., 1990). The repressive effect of *shaggy/zeste-white3* on *engrailed* expression could be brought about by phosphorylation and be competed by the activating action of *wingless*. The relative amount of *wingless* present could determine the outcome of this competition.

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Fig. 3. A high uniform level of *wingless* protein is observed in HS-*wg* embryos after heatshock. HS-*wg* (A) and *Canton S* (B) control embryos were heatshocked for 20 minutes at 36°C and incubated for 30 minutes before fixation and antibody staining (Materials and methods).

Fig. 4. *wingless* and *engrailed* protein expression pattern in whole-mount *Canton S*, heatshocked HS-*wg* and *naked*^{7E89} embryos at stage 10 of development (Wieschaus and Nüsslein-Volhard, 1986). Anterior is to the left, dorsal is up. A, B and C show a ventral surface view of the embryo; D, E and F give a sagittal section, while G, H and I are surface views. In the double-labeled embryo, *wingless* protein is black, *engrailed* is red. (A,D,G) *Canton S* embryos stained for *wingless* (A), *engrailed* (D) or both proteins (G). (B,E,H) HS-*wg* embryos were heatshocked three times and maintained for 3 hours after the last heatshock before fixation (Materials and methods) and stained for *wingless* (B), *engrailed* (E) or both (H). The deep groove posterior to the new *engrailed* domain is indicated by the arrow. (C,F,I) *naked*^{7E89} embryos stained for *wingless* (C), *engrailed* (F) or both (I). A similar deep groove as present in HS-wg embryos, can be seen in *naked*^{7E89} (arrow).

Fig. 5. *engrailed* expression in HS-*wg* embryos broadens in the posterior direction. A double-labeled embryo, containing both the HS-*wg* gene and the *even-skipped-lacZ* gene (Lawrence et al., 1987) is shown, stained for *engrailed* protein (blue) and β-*galactosidase* protein (brown). The embryo was heatshocked three times and maintained for 3 hours before fixation (Materials and methods). Anterior is to the left and dorsal is up. The anterior edges of the odd-numbered parasegments are independently marked by the anterior sharp borders of the *even-skipped-lacZ* expression domains (e.g., arrowhead at parasegment 13). Here, as in wild type, this edge coincides with the anterior edge of the *engrailed* stripe. The *engrailed* stripes, which are abnormally broad in this heatshocked embryo, must therefore have broadened away from the parasegmental borders in the posterior direction.

Fig. 6. Comparison of expression patterns of several segmentation genes in whole-mount *Canton S*, heatshocked HS-*wg* and *naked*^{7E89} embryos at stage 10 of development. Anterior is to the left, dorsal is up. A-C and H-J show a surface view of the embryo, while E-G and K-M are sagittal sections. HS-*wg* embryos were heatshocked three times and maintained for 3 hours after the last heatshock before fixation (Materials and methods). (A-C) Distribution of *armadillo* protein in *Canton S* (A), heatshocked HS-*wg* (B) and *naked*^{7E89} embryos (C). (D) Detail of Fig. 6C, showing two rows of cells in the *naked*^{7E89} embryo with higher intensity of *armadillo* staining in the *wingless*-expressing half of each segment. (E-G) *patched* RNA expression pattern in *Canton S* (E), heatshocked HS-*wg* (F) and *naked*^{7E89} (G) embryos. (H-J) *cubitus interruptus Dominant* RNA expression pattern in *Canton S* (H), heatshocked HS-*wg* (I) and *naked*^{7E89} (J) embryos. (K-M) *Ubx* protein distribution in *Canton S* (K) heatshocked HS-*wg* (L) and *naked*^{7E89} (M) embryos. The newly formed deep groove in HS-*wg* and *naked*^{7E89} embryos is indicated by an arrow. Expression is detected in parasegments 5-13.