

A role for *wingless* in the segmental gradient of *Drosophila*?

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SUMMARY

The wild-type functions of the *Wnt* family of genes are still little understood (for review see Nusse and Varmus, *Cell* 69, 1073-1087, 1992). In *Drosophila*, the *wingless* (*D-Wnt-1*) protein is expressed in segmental stripes: its absence leads to a complete failure of segmentation, loss of *engrailed* expression and lack of pattern in the cuticle. A predominating hypothesis is that the spatial distribution of *wingless* is crucial to pattern; it might carry an instructive signal from cells that secrete the protein to cells nearby, or it might form a concentration gradient which acts as a morphogen.

We tested these hypotheses by expressing *wingless* ubiquitously in *wingless*⁻ embryos. The distribution of *wingless* protein in these embryos is uniform. Despite this, *engrailed* expression persists, is confined to the most

anterior third of the parasegment, and delineates the parasegment border. The cuticle shows a segmentally reiterated pattern and, dorsally, the denticles are normally distributed and oriented. Because all these position-specific features cannot have been placed by a local source or a differential distribution of *wingless* protein, we conclude that, in the early embryo, the role of *wingless* is neither to act as a local instructive signal, nor as a morphogen. We propose an alternative hypothesis that the wild-type function of the *wingless* protein is to maintain and 'seal' the parasegment borders; in its absence the borders fail to isolate abutting segmental gradients.

Key words: *wingless*, heat-shock construct, cell junctions, gradients, *Drosophila*

INTRODUCTION

As soon as cells form at the end of the *Drosophila* blastoderm stage, they become allocated to a series of parasegments along the antero-posterior body axis (reviewed in Lawrence, 1992). Parasegments have stable borders, which delimit the realms of action of selector genes (Struhl, 1984; Lawrence et al., 1987). Moreover, parasegments are polar and this is evident in the array of cuticular pattern elements they form at the end of embryogenesis. The first signs of this asymmetry can be seen much earlier in the expression patterns of several genes. For example, the most anterior cells in the parasegment express *engrailed*, the most posterior *wingless*, and this is evident from when these genes first become expressed at the end of the blastoderm stage (DiNardo et al., 1985; Ingham et al., 1985; Baker, 1987).

Parasegmental borders and polarity are established by the pair rule genes. In particular, the borders are defined by the anterior boundaries of expression of *even-skipped* and *fushi tarazu*, two pair rule genes expressed at blastoderm in alternating stripes (Lawrence et al., 1987; Carroll et al., 1988). Since these stripes are asymmetric, it is possible that they also define the polarity of the parasegment, although other pair rule genes could contribute. However, pair rule genes cease to be expressed shortly after the blastoderm stage, but parasegmental borders and polarity have to be maintained for the rest of embryogenesis. How is this achieved?

Genetic and molecular analysis of *wingless* has led to suggestions that it acts as a local and instructive signal

which is directly involved in patterning the parasegment (Cabrera et al., 1987; DiNardo et al., 1988; van den Heuvel et al., 1989; Ingham and Martinez-Arias, 1992; Noordermeer et al., 1992; Vincent and O'Farrell, 1992). Two separate parcels of evidence support this view.

The first parcel concerns the relationship between the *wingless* and *engrailed* genes. The *wingless* gene is required for maintenance of *engrailed* expression. In *wingless*⁻ mutants, *engrailed* transcription begins normally but later ceases (DiNardo et al., 1988; Martinez-Arias et al., 1988). When the *wingless* protein is expressed ectopically by means of a heat-shock promoter-*wingless* hybrid gene (HSwg), more cells than normal express *engrailed* (Noordermeer et al., 1992). The *wingless* gene encodes a secreted protein (Rijsewijk et al., 1987), cells expressing *wingless* are adjacent to those expressing *engrailed* (Baker, 1987; van den Heuvel et al., 1989) and the *wingless* protein can be detected in the *engrailed* expressing cells (van den Heuvel et al., 1989). These observations have led to the hypothesis that only cells in the most anterior part of the parasegment are close enough to the *wingless* transcribing cells, and they receive enough *wingless* protein and are signalled to maintain expression of *engrailed* (DiNardo et al., 1988; Heemskerk et al., 1991; Vincent and O'Farrell, 1992). This hypothesis demands that effective levels of *wingless* protein are confined to part of the parasegment.

The second parcel concerns the cuticle phenotype of *wingless* mutants. By the end of embryonic development, a normal segment has a belt of denticles in the anterior part

where *wingless* is not expressed and naked cuticle in the posterior part where *wingless* is expressed. This suggests that *wingless* might act locally to direct the formation of naked cuticle (Bejsovec and Martinez-Arias, 1991). Indeed it is also found that generalised expression of *wingless* (using HSwg) can produce a naked cuticle with no bands of denticles (Noordermeer et al., 1992), while absence of *wingless* (in *wingless*⁻ embryos) produces a cuticle completely covered in denticles. These facts also support the prevailing idea that it is the **distribution** of *wingless* protein that is crucial to patterning the segment (op cit, above). Further, they have led to suggestions that *wingless* protein (or some active fragment of it) might form a concentration gradient across the segment, a morphogen gradient that would be interpreted to give positional information (Bejsovec and Martinez Arias, 1991; Noordermeer et al., 1992; Peifer and Bejsovec, 1992).

In this paper, we put these ideas to another test: we ask how far generalised *wingless* expression can rescue various mutants including *wingless*⁻. Other experiments (Bejsovec and Martinez-Arias, 1991) and ours subdivide the *wingless* gene in time. In an early and crucial phase, the *wingless* gene is essential for segmentation. We find that, in this phase, evenly distributed *wingless* protein is able to emulate wild-type function, a finding that leads us to a new hypothesis for *wingless*.

MATERIALS AND METHODS

Fly strains

The following alleles have been used:

wingless: *wg*^{CX4} is a null allele; it is a 2 kb deletion in the transcribed region (Baker, 1987) and there is no transcript (Martinez-Arias et al., 1988). *wg*^{LL114} is a temperature-sensitive allele conferring the null phenotype at 25°C and the wild-type one at 18°C (Nüsslein-Volhard et al., 1984; Baker, 1988).

armadillo: *arm*^{XK} (Wieschaus et al., 1984; Klingensmith et al., 1989).

cubitus interruptus-Dominant: *ci*^D (Orenic et al., 1987).

engrailed: *Df(2R)en-E* (generous gift of Z. Ali and T. Kornberg, unpublished).

gooseberry: *gsb*^{lHX62} (Nüsslein-Volhard et al., 1984).

hedgehog: *hh*^{lJ} (Mohler, 1988).

patched: *ptc*^{lN} (Nüsslein-Volhard et al., 1984).

smooth: *smo*^{lHX43} (Nüsslein-Volhard et al., 1984). As other *smooth* alleles, it shows the strongest mutant phenotype at 15°C but has a wild-type pattern at 25°C.

Heat shock-*wingless* (HSwg) (Noordermeer et al., 1992).

CyO, *hb-gal*: generous gift of G. Struhl.

engrailed-lac Z: *ry xho25* (Hama et al., 1990).

even-skipped-lac Z (Lawrence et al., 1987).

A *wg*^{ts} rescuing construct

A lambda genomic library was constructed from *wg*^{LL114}/CyO flies and *wg*^{LL114} phages were recognized by the polymorphic *EcoRI* site marked with an asterisk in Fig. 1. An *EcoRV*-*PstI* 9.2 kb piece from one of such phages (including all *wg*^{ts} coding sequences from the 3rd aminoacid) was fused to a *BamHI*-*EcoRV* 5.7 kb *wg*⁺ piece (including 5.4 kb of *wg* upstream sequences, the transcription start and the first two aminoacids). The resulting 14.9 kb were inserted to the *XbaI* site of C20NX (a modified Carnegie-20 P-

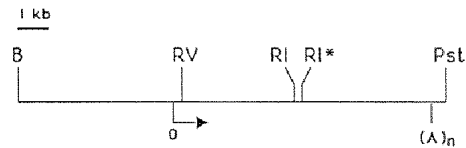


Fig. 1. Schematic map of the *wingless* gene. Only relevant restriction sites are shown. B, *BamHI*; RV, *EcoRV*; RI, *EcoRI*; Pst, *PstI*; O, origin of transcription; (A)_n, poly-adenylation site.

element vector, Struhl, 1989). A single transformant line was obtained (*P[ry⁺, wg^{ts}]*). The insertion (on chromosome 3) in this line partially rescues the *wingless*⁻ phenotype at 18°C (Fig. 2F) but not at 25°C, as expected from the temperature dependence of the *wg*^{LL114} function. During early embryogenesis, *wingless* antigen is expressed in the epidermis in the same pattern as in wild-type embryos, although at a lower level (Fig. 4E). Staining decays during stage 10 and is not detectable at stage 11.

Antibody staining

We have used rabbit anti-*wingless* serum (van den Heuvel et al., 1989) at a dilution of 1:300 and anti-*engrailed* monoclonal antibody obtained from T. Kornberg (Patel et al., 1989). Stainings were performed according to Lawrence and Johnston (1989).

Heat-shock regimes

For heat treatments, embryos were submerged in 0.1% Triton X-100 at 36°C for 20 minutes. This we refer in the text as a *pulse*. When HSwg embryos are heat-shocked for 20 minutes, *wingless* antigen is strongly expressed 30 minutes after the pulse and then starts to fade, the endogenous stripes becoming visible again by 60 minutes. With the *wingless*⁻ embryos, *wingless* staining is uniform at all times after the pulse (Fig. 4C). When several pulses were performed, the embryos were kept in 0.1% Triton X-100 at room temperature between the pulses. After the treatments, they were fixed for antibody staining or transferred to agar plates and left to develop at 25°C until cuticle formation.

The regimes used to generate mirror phenotypes in *wingless*⁻ embryos are described in the Results and in the legend to Fig. 2. The regimes used with other mutants were the following:

gooseberry and *cubitus interruptus-Dominant*: single pulse at 7-10 hours AEL and triple pulse at 5-8, 7-10 and 9-12 hours AEL.

engrailed: triple pulse at 4-7, 6-9 and 8-11 hours AEL.

hedgehog: single pulse at 4-6h AEL and triple pulse at 5-7, 7-9 and 9-11 hours AEL.

patched: single pulse at 6-9 or triple pulse at 4-7, 6-9 and 8-11 hours AEL gave no alteration of the *patched*⁻ phenotype.

wingless engrailed double mutant: single pulse at 4-7 and triple pulse at 4-7, 6-9 and 8-11 hours AEL.

armadillo: triple pulse at 5-8, 7-10 and 9-12 hours AEL.

smooth is cold-sensitive, showing a somewhat variable mutant phenotype at 15°C and a wild-type phenotype at 25°C. For this reason, *smooth*⁻ embryos, with and without the HSwg construct, were treated in parallel in the following way. Eggs were collected for 2.5 hours at 25°C, then aged 13.5 hours at 15°C, given a heat-shock pulse and left at 15°C for 4 days. Although the resulting *smooth* phenotypes are more variable than those of embryos kept at 15°C all the time, we did not observe consistent differences between embryos with or without the HSwg construct.

Classification of embryos

In the antibody staining experiments, we used the CyO, *hb-gal*, balancer to identify the *wingless*⁻ embryos. For the cuticles, all

classifications were based on the phenotypes. Although this is common practise it can be risky, but in the experiments that we describe, the mutant and partially rescued phenotypes were always clearly demarcated from each other and from the wild type. They occurred at the expected frequencies.

X-gal staining of cuticles

For embryos with the mirror phenotype, unhatched larvae of 24–30 hours were heated 10 minutes at 45°C in water, then dechorionated in bleach, softly frozen in liquid nitrogen and cut in half before fixing in 0.1% glutaraldehyde/0.02% Nonidet NP-40/0.01% sodium deoxycholate in Ringer's for 10 minutes. X-gal staining was done by standard procedures (Ashburner, 1989), developing the colour overnight at 37°C. Stained larvae were dehydrated through an isopropanol series and transferred to cedar wood oil. Fat bodies and nervous system were removed, cuticles were stretched by hand and mounted in Araldite. Wild-type larvae could not be properly stretched by this method, particularly in the thorax, where deep folds form at the segment borders. Therefore, newly hatched wild-type larvae were stretched in a two-phase mixture of methanol and heptane for some minutes, then transferred to Ringer's and orientated on a slide in an embedding medium used for frozen sections. The slide was frozen in liquid nitrogen and the larvae cut in half with a razor blade, then fixed in 4% formaldehyde for 20 minutes and stained with X-gal by standard procedures, developing the colour overnight at 37°C. The stained larvae were dehydrated through in isopropanol series, transferred to cedar wood oil and mounted in Araldite.

RESULTS

HSwg restores segmentation to *wingless*⁻ mutants

Ventrally, the cuticle of a normal segment displays an anterior belt of oriented denticles and a posterior naked zone (Fig. 2A). Dorsally, there is a field of denticles with a sharply defined anterior margin that delineates the segment border and a posterior naked area (Fig. 3A). The thoracic pattern has been little studied in previous papers on segment polarity, but here we use the thorax extensively; we examine mutant denticle patterns and the location of *engrailed* expression. As we shall see, the thorax displays both pattern and polarity more clearly, and conclusions obtained from it can be easily extrapolated to the abdomen. *wingless*⁻ embryos are dwarfish and the pattern is depauperate; there are lawns of denticles both ventrally and dorsally (Figs 2B, 3B) and the polarity of these denticles is only slightly patterned.

Our main result is that a single pulse of HSwg soon after gastrulation substantially rescues the cuticle phenotype of *wingless*⁻ embryos and several features of segmentation are recovered. First, the embryos are much larger, reaching nearly the length of wild-type embryos. Second, each segment is clearly demarcated in the ventral abdomen by orderly changes in the width of the denticle band and by the polarity of the denticles (Fig. 2C). Even more remarkably, the dorsal patterns in the thorax and abdomen are very similar to those of the wild type not only in the arrangement and size of the denticles but also in their orientation (compare Fig. 3A,C). The ventral pattern is mirror sym-

metric; in both thorax and abdomen, the denticles of each segment are oriented towards a central mirror plane. The mirror plane runs through the middle of the belt, irrespective of the belt size (compare, for example, T1 with T2 in Fig. 2C). Furthermore, each belt is symmetrical with respect to the stripes of *engrailed* expression (Fig. 4B). This is particularly evident in the thorax, where some naked cuticle remains between the mirrorized belt and the *engrailed* stripes on **both** sides of the belt. These two zones of naked cuticle flanking the belt probably represent a duplication of the naked zone, which normally lies, in the wild type, between the posterior edge of an *engrailed* stripe and the anterior edge of a thoracic set of denticles (Fig. 4A). Keilin's organs are missing, as is the T1 'beard'. This syndrome is stable and consistent: we call it the **mirror** phenotype.

The mirror phenotype can be produced by a single early pulse of heat shock. If *wingless*⁻ embryos that carry one or two doses of the HSwg construct are pulsed 4–6 hours after egg laying, all or almost all develop the pattern (in one experiment 45 of 60 *wingless*⁻ embryos, the expected figure, given the genotypes) — but if the pulse is delayed by only one hour, the frequency of these phenotypes drops. A batch of embryos pulsed when they were 7–8 hours old produced no mirror phenotypes: the embryos remain dwarfish and acquire a pattern resembling an *engrailed*⁻ phenotype. This result is consistent with observations on the *wingless* temperature-sensitive allele (*wingless*^{ts}). If the temperature is shifted from 25°C to 18°C after 5½ hours of development, it is too late to restore segmentation and the phenotype of the embryo is also dwarfish and also resembles that of *engrailed*⁻ embryos (Bejsovec and Martinez-Arias, 1991).

The mirror phenotype is very stable; additional heat pulses do not produce any change in the pattern. We have tried several different regimes without effect. An example of an embryo that received two heat shocks 2 hours and 4 hours following the initial heat shock is shown in Fig. 2D.

The mirror phenotype is not a consequence of generalised, as opposed to local, *wingless* expression. If normally localised *wingless* function is provided up until 6 hours of development and then inactivated (by shifting up embryos carrying the *wingless*^{ts} allele from 18°C to 25°C), mirror phenotypes are almost invariably produced (Fig. 2E). The same phenotype is observed when rescuing *wingless*⁻ embryos with a *wingless* transgene (Fig. 2F, see Methods for details). This transgene expresses *wingless* antigen in a normal pattern until about 5 hours (Fig. 4D,E), when it starts to decay.

Persistence of *engrailed* stripes

The anterior boundaries of *engrailed* expression delineate the parasegment borders (Martinez-Arias and Lawrence, 1985; Vincent and O'Farrell, 1992). When a *wingless*⁻ embryo of about 5 hours old, which is losing *engrailed* expression in the epidermis, is given some ubiquitously expressed *wingless* protein, *engrailed* persists as stripes. The anterior borders of such restored *engrailed* stripes remain coincident, cell by cell, with the original parasegment borders (as independently defined by the stripes produced by an *even-skipped lacZ* construct (Fig. 4F–H).

By 4 hours after the heat shock, the *engrailed* stripes are still present in *wingless*⁻ embryos and their anterior margins are still coincident with the parasegment bound-

aries. However, they have widened up to about one third of the segments length; this is substantially broader than in wild-type embryos at the same stage. Also, ectopic

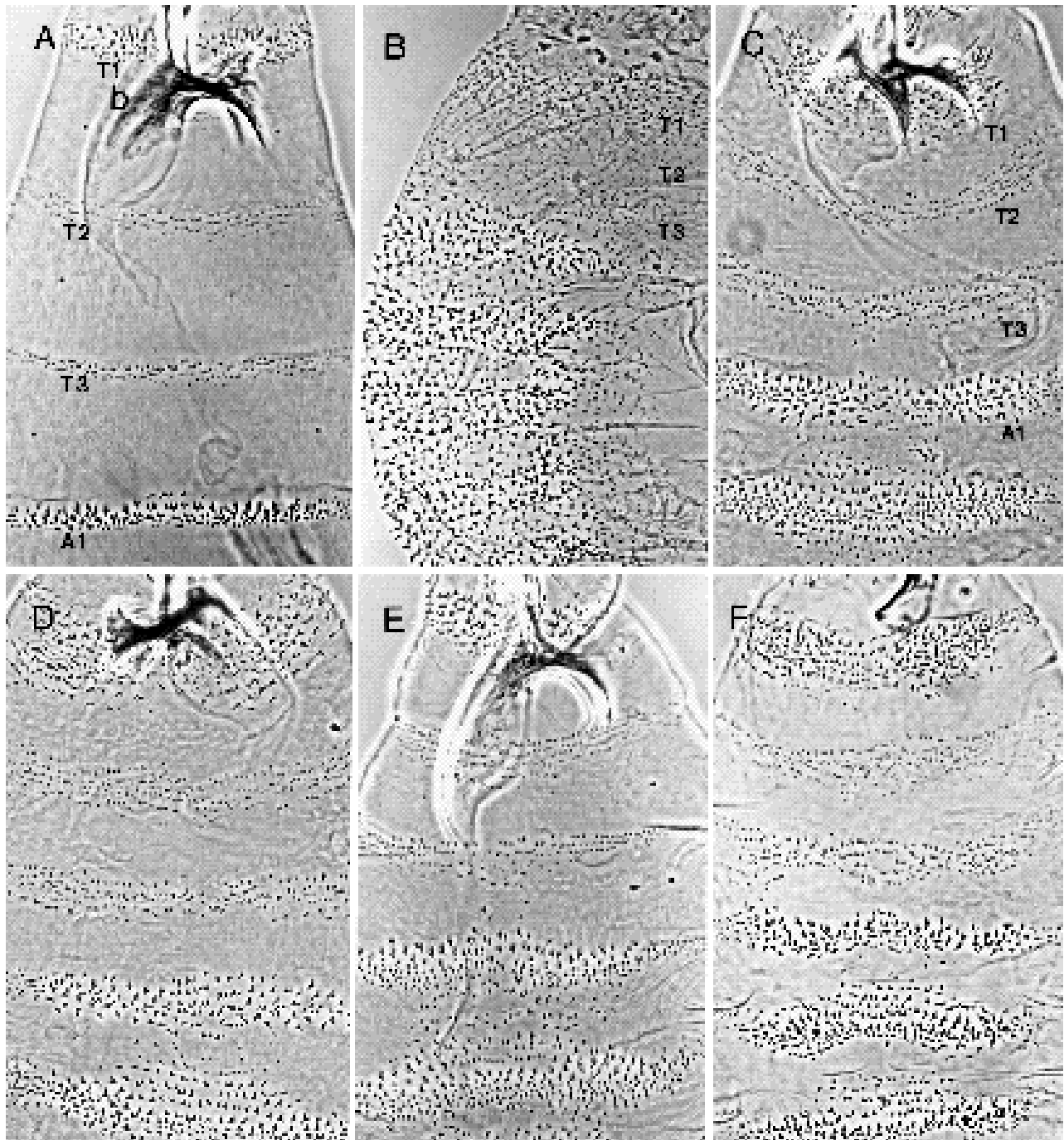


Fig. 2. Ventral views of the thorax; phase contrast, all to same scale. T1, T2 and T3 indicate the three thoracic segments. The wild-type pattern (A) can be contrasted with the *wingless*⁻ pattern (B). Note the *wingless*⁻ embryo is much smaller and there is an even field of denticles in which traces of metamerisation can be seen. Note also that zones bearing denticles characteristic each of the three thoracic (T1-T3) and abdominal segments are clearly demarcated and in the normal sequence. There must therefore be orderly expression of those genes of the bithorax and Antennapedia complexes responsible for segmental characteristics — even in the absence of parasegmental borders (cf. Struhl et al., 1992). (C) The mirror phenotype in a *wingless*⁻ embryo carrying the HS ω g construct, produced by a single pulse of 20 minutes at 36°C, given at 4-5 hours after egg laying (see Methods). (D) The mirror phenotype in a similar embryo given three heat pulses at 4-7, 6-9 and 8-11 hours after egg laying. (E) The mirror phenotype in an embryo homozygous for *wingless*^{Δ5} that was shifted from 18°C to 25°C at 6-9 hours after egg laying (being equivalent to 4-6 hours of development at 25°C (cf. Baker, 1988; Bejsovec and Martinez-Arias, 1991)). (F) The mirror phenotype in a *wingless*⁻ embryo that also carried the *wingless*^{Δ5} transgene (See Methods).

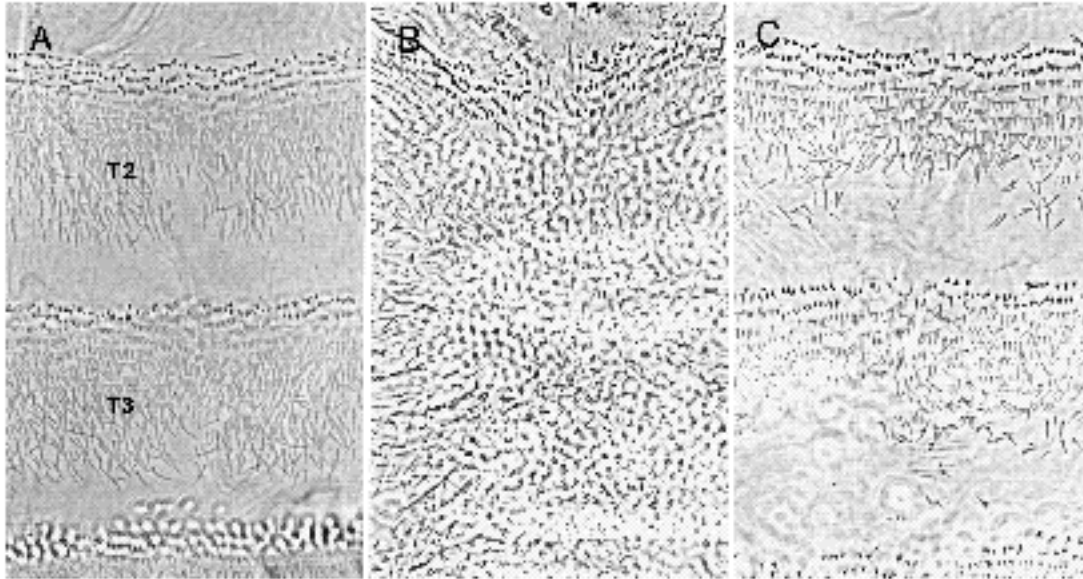


Fig. 3. Dorsal views of the thorax; phase contrast, all to same scale. T2 and T3 mark two thoracic segments. The wild-type pattern (A) can be compared with the *wingless*⁻ pattern (B) (actually the similar pattern of a *wingless*^{ts} embryo at 23°C which gives stronger, and therefore more photogenic, denticles). (C) The phenotype in a *wingless*⁻ embryo that carries a HSwg construct and was given three heat pulses at 4-7, 6-9 and 8-11 hours after egg laying.

grooves appear at the posterior margins of the *engrailed* stripes. In *wingless*^{ts} embryos that are shifted from 18° to 25° at 6 hours of development the *engrailed* stripes do not broaden and ectopic grooves are not seen (Bejsovec and Martinez Arias, 1991), and yet they also produce mirror phenotypes. It follows that the two classes of experimental embryos produce mirror phenotypes by different routes.

These experiments can be summarised as follows: mirror phenotypes result if the *wingless* gene is active during the early period of development (up until about 6 hours) but not later. It does not seem to matter whether the gene product is ubiquitous (as with HSwg) or normally localised (as with *wingless*^{ts}); the result in the cuticle is the same, although the routes followed are somewhat different.

Other segment polarity mutations

The majority of segment polarity mutations belong to what is usually referred to as the 'wingless class'. They have in common a phenotype in which the abdomen is covered with denticles, but they can be usefully separated into two groups of different strengths. The 'strong' group includes the maternal effect mutations in *dishevelled*, *porcupine* and *armadillo* (when mutated maternally) and the zygotic mutations of *hedgehog* and *smooth* (Fig. 5E, B) (Nüsslein-Volhard et al., 1984; Klingensmith et al., 1989; Mohler, 1988; Perrimon et al., 1989). They have phenotypes very similar to *wingless*⁻. The 'mirror' group includes *gooseberry*, *cubitus-interruptus*^D (Fig. 5C, F) and *fused* (Martinez-Arias, 1985). Some weaker mutations of the 'strong' group can also give mirror phenotypes; examples are *armadillo* (when mutated only zygotically, Fig. 5D) and weak alleles of *hedgehog* (such as *hh*^{10B}, Mohler, 1988). All these phenotypes are very similar to the mirror phenotype described above for partial rescue of *wingless*⁻ (compare Figs 2 and 5). HSwg can be used in princi-

ple to help establish a genetic hierarchy and we have examined examples of both groups.

(a) Strong group

We know that HSwg can rescue the strong *wingless*⁻ phenotype to produce a mirror phenotype. Therefore, if a given mutation of the 'strong' type produces its effect through inactivation of *wingless* transcription (i.e., it is 'upstream' of *wingless*), we would expect HSwg to rescue its phenotype and give a mirror pattern. However, if a gene product is needed for *wingless* to function, or is activated by it (i.e., it is 'downstream' of *wingless*) the mutant phenotype of such a gene should not be rescued by HSwg. Null alleles of *hedgehog* show the strong phenotype (Fig. 5E). They cannot be rescued to the mirror pattern by HSwg (see Methods for details), so it follows that the *hedgehog* product is a mediator of *wingless* action - it is 'downstream' of *wingless* in that sense, a conclusion that does not appear to fit with *hedgehog*'s role in the model of Ingham et al. (1991). There is however some effect: *hedgehog*⁻ HSwg embryos remain dwarfish and unsegmented but they lose denticles. The patterns can be arranged in a rank order with denticles being lost near the ventral midline in weaker cases, and spreading out laterally in more extreme examples (Fig. 5E). This type of patterned baldness is reminiscent of the phenotypes of HSwg in a wild-type background (Noordermeer et al., 1992). Thus, although *hedgehog* is essential for *wingless* function in segmentation, *wingless* appears to be still capable of some action in its absence.

A similar situation obtains with the *engrailed* gene. Since the *engrailed* phenotype is somewhat variable, this is better illustrated for the double mutant *wingless*⁻ *engrailed*⁻. This double mutant shows an extremely strong phenotype, with embryos even smaller, more spherical and less patterned than *wingless*⁻ (Bejsovec and Martinez-Arias, 1991 and Fig. 5G). The phenotype cannot be rescued to the mirror pattern by HSwg, from which we conclude that

engrailed is an essential mediator of *wingless* function. Once again, however, the embryos lose denticles and increase in size (becoming more elliptical (Fig. 5G)) as a consequence of HSwg, showing that the *wingless* protein can have effects independent of the *engrailed* gene. This augments the conclusion drawn by Bejsovec and Martinez-Arias (1991), who pointed out that the

wingless⁻ engrailed⁻ double phenotype differs from both *engrailed⁻* and *wingless⁻* single phenotypes.

Although the phenotype of *smooth⁻* is variable, we were unable to detect any clear rescue by HSwg; there may have been some loss of denticles. This result also places the *smooth* gene downstream of *wingless*.

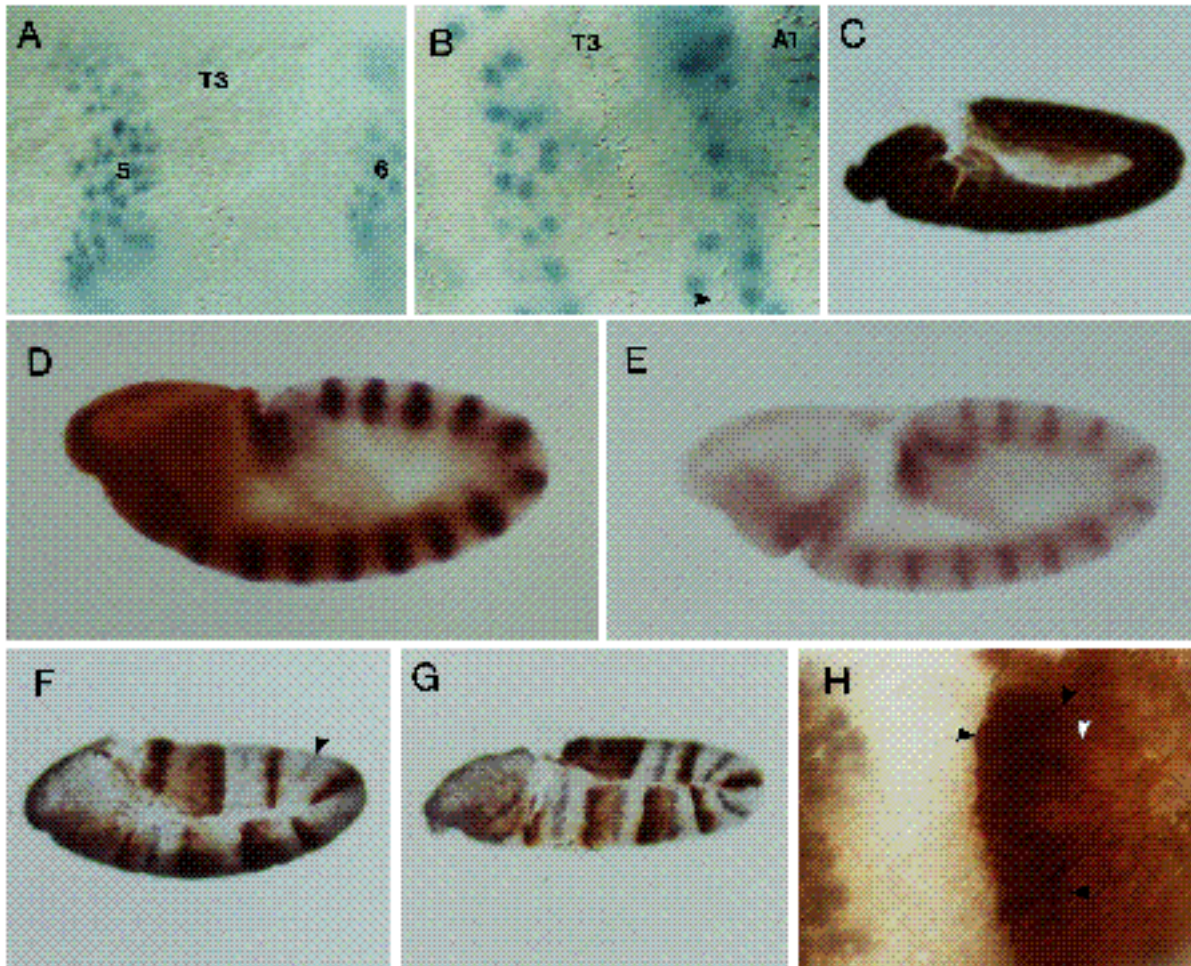


Fig. 4. (A) Preparation of ventral cuticle of newly hatched wild-type larva to illustrate cuticle pattern and *engrailed* expression (the blue stain shows individual nuclei that express *engrailed*). Note the gap between the *engrailed* stripe of posterior T2 (labelled 5, for parasegment 5) and the denticle band of T3. Part of the *engrailed* stripe of posterior T3 (6) is also shown. (B) A similar preparation of a mirror phenotype. Note that the T3 denticle band lies equidistant from the two *engrailed* stripes. Overlying the *engrailed* stripe that corresponds to parasegment 6 are two denticles; these are of the abdominal, not the thoracic type (arrow). The larva is *wingless⁻* and carries HSwg, it was heat shocked one pulse at 4–7 hours after egg laying. (C) The right half of an embryo at stage 11 which was bisected longitudinally because staining is so heavy. β -galactosidase shows in brown and *wingless* antigen in black. The embryo lacks the intense brown stain in the head due to the CyO *hb-gal* marker and is therefore *wingless⁻*. The very heavy and even *wingless* staining is due to one or two doses of HSwg and to the pulse given at 4–7 hours after egg laying, the embryo being fixed 45 minutes following the end of the pulse. At later times, even as the induced *wingless* protein fades, its distribution remains homogeneous. (D,E) Embryos at stage 10, stained for β -galactosidase in brown and *wingless* antigen in black. One individual (D) displays brown staining in the head (due to CyO, *hb-gal*) and is therefore *wingless⁺*. The other (E) does not and is therefore *wingless⁻*: The faint but correctly localised *wingless* staining is due to one or two copies of the *wingless^{ts}* transgene (see Methods). (F–H) *wingless⁻*, HSwg embryos at stage 10 stained for β -galactosidase in brown and *engrailed* in black. The embryos were bisected longitudinally and the left halves are shown. The stock also carries *even-skipped lacZ* and CyO *hb-gal*. These embryos lack β -galactosidase staining in the head and are therefore *wingless⁻*, they show brown staining in alternate parasegments due to *even-skipped lacZ*. (F) This embryo was not heat shocked and, being *wingless⁻*, shows no *engrailed* expression in the thoracic and abdominal epidermis — although there is some in the CNS (arrow). (G) This embryo was given one heat shock 2 hours prior to fixation. The embryo is the same stage as in F but the *engrailed* stripes have persisted due to the action of ubiquitous *wingless* protein. (H) Detail of G. The *engrailed*-positive cells can be distinguished in the lighter background (white arrow) of the β -galactosidase staining because they are darker brown (black arrows). Their anterior boundary coincides cell by cell with the parasegment boundary (5) as shown.

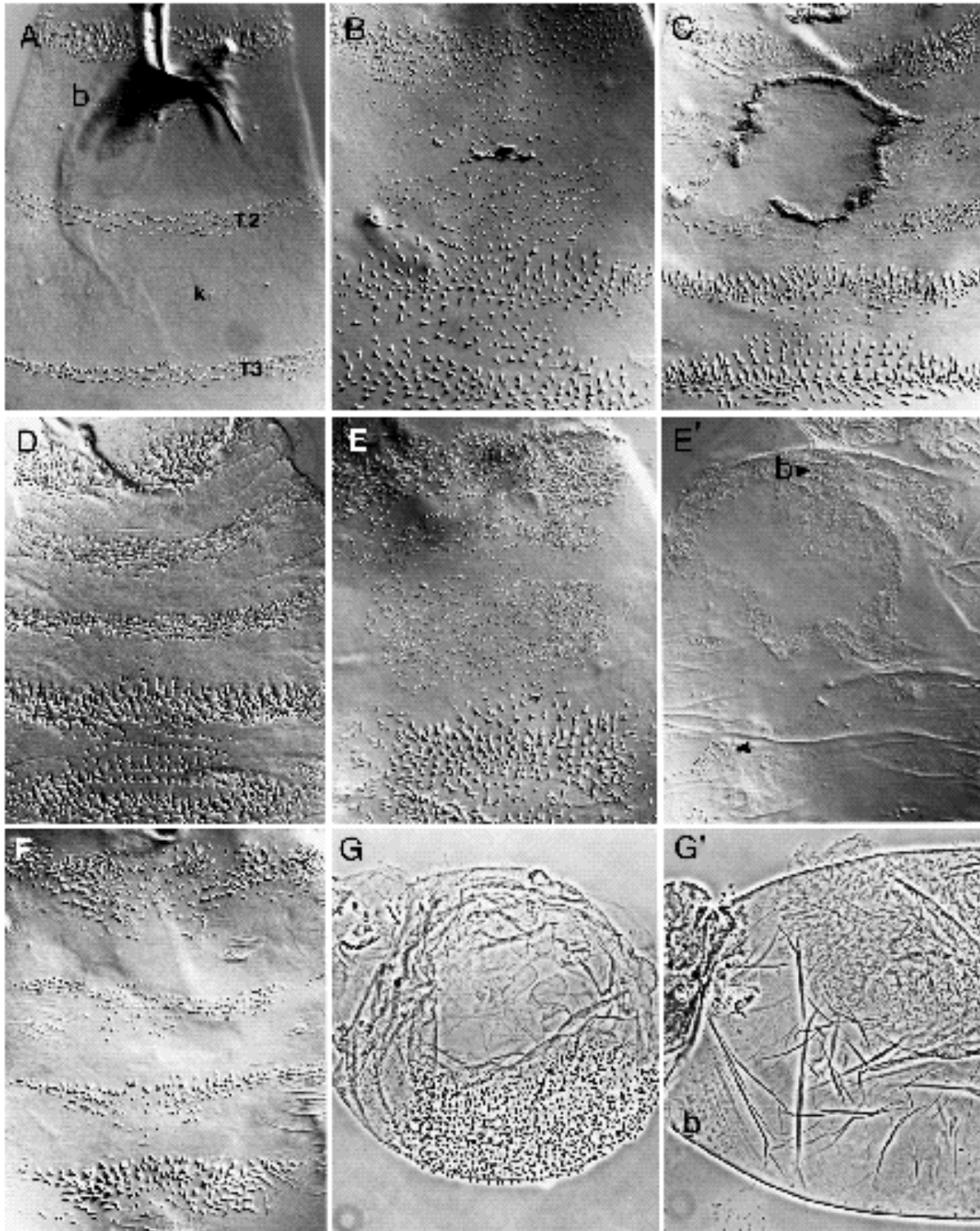


Fig. 5. (A-F) Ventral views of the thoracic region, interference contrast. All to same magnification. Anterior to top. (A) Wild type: Note T1 denticles, finer T2 and coarser T3 denticles. (*b*) beard; (*k*) Keilin's organ. (B) *smooth⁻* at 15°C. Phenotype approaches that of *wingless⁻*. (C) *gooseberry⁻* shows a typical mirror phenotype, plus the ventral hole characteristic of *gooseberry⁻*. (D) *armadillo*. This pattern is due to lack of the zygotic but not maternal function of *armadillo*. Shows a mirror phenotype. (E) *hedgehog⁻ Ubx⁻* embryo. (E') *hedgehog⁻ Ubx⁻*, Hswg embryo given three heat pulses at 5-7, 7-9 and 9-11 hours after egg laying. Note embryo is naked ventrally, apart from beard (*b*) and occasional patches of abdominal denticles (arrow). (F) *cubitus-interruptus^D*. This also gives a mirror phenotype. (G-G') Lateral views of embryos, phase contrast. Lower magnification than A-F. Anterior to left, dorsal to top. (G) *wingless⁻ engrailed⁻* embryo; (G') *wingless⁻ engrailed⁻* HSwg embryos given three heat pulses at 5-7, 7-9 and 9-11 hours after egg laying. Ventral cuticle is almost completely naked, apart from the beard (*b*).

(b) Mirror group

Testing the rescuing ability of HSwg to give the mirror pattern obviously makes no sense in the case of mutants that already have the mirror phenotype. Still, there could be another way in which to use HSwg to establish a hierarchy with genes in this group. HSwg can produce, in an otherwise wild-type background, a phenotype similar to that of the *naked*⁻ mutant (Noordermeer et al., 1992). If this effect of ectopic *wingless* expression requires the same downstream machinery as the normal *wingless* function,

one would expect mutations in downstream genes to block formation of the 'naked' phenotype. Our observation is that the mirror phenotypes of *gooseberry*, *cubitus-interruptus*^D and *armadillo* (zygotic) are not altered by HSwg. Although this might be taken as evidence that these three genes are downstream of *wingless*, there are grounds for caution: note that the mirror phenotype obtained by rescuing *wingless*⁻ embryos with an early pulse of HSwg is unaltered by further pulses of HSwg. It is possible that the mirror pattern is a stable developmental outcome, which

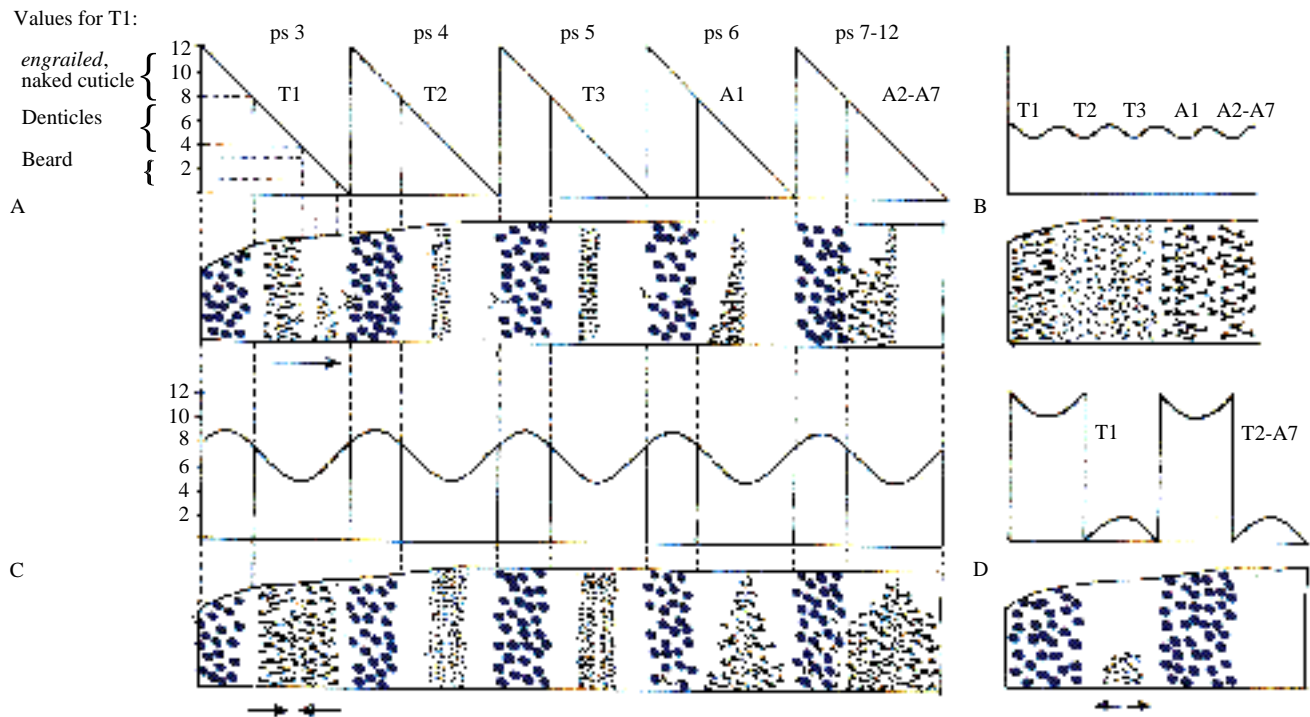


Fig. 6. A gradient model for the parasegmental patterns. For all the patterns the three tenets of the model are **first**, for each segmental unit, the differentiation of denticles always occurs between characteristic levels in the gradient; **second**, the orientation of pattern elements is determined by the slope of the gradient; **third**, within each of the four situations, every parasegment has an identical gradient landscape and pattern of *engrailed* expression. It is the observed consistency between these three tenets that constitutes the model's main virtue. (A) The wild type. We show the patterns of the parasegments, these are based as accurately as we can on the relative positions of the *engrailed* stripes and denticle bands shown in Fig. 4A. In the model, the scalar determines, from anterior to posterior, a stripe of cells expressing *engrailed*, a variable stripe of naked cuticle, a belt of denticles and a constant second stripe of naked cuticle. The transition between denticles and the second naked stripe occurs at the same position for all parasegments, so that the variation in size between denticle belts is due to differences in the depth of the anterior naked stripe. The parasegmental borders are coincident with a precipitous change in the level of the gradient. (B) The *wingless*⁻ phenotype. The level is confined to that corresponding to denticles in both thoracic and abdominal parasegments. There are some slight undulations (because the orientation of denticles shows traces of segmental repeats, Fig. 2B). (C) The mirror phenotype. As in A, the gradient landscape is drawn directly from the observed patterns. There is uncertainty about the shape of the gradient at the points of inflection. For example, the formation of scattered denticles throughout the *engrailed* zones of parasegments 7-12 suggests that the peak is not sharp at level 12 but rounded at about level 8-9 (which is appropriate to denticles of row 1 type). For symmetry one might therefore suspect that the troughs in the landscape would be correspondingly rounded as shown. The model explains with a simply reiterated landscape the characteristic mirror phenotypes of each parasegment (in particular, the presence of naked cuticle in the thorax but not in the abdomen), as well as the striking fact that the symmetry planes are centred in the mirrorized denticle belts (see Fig. 2), irrespective of the size of the belt. The parasegment borders, although they may not act as a complete barrier to passage of the morphogen, may still coincide with the anterior boundaries of *engrailed* and homeotic genes. The result of this is illustrated in parasegment 6 where occasional denticles are found in the *engrailed* territory. These are invariably denticles of the type normally found in A1 (see Fig. 4B), meaning that the *Ultrabithorax* gene is expressed at the level found in parasegment 6, rather than that of parasegment 5 (Struhl, 1984). (D) The HSwg phenotype. Note there is now a new and inverted parasegment border which has formed at the posterior boundary of the broadened *engrailed* stripe and therefore a new landscape. The missing levels (from approx. 3-10) correspond to those parts that make denticles in the wild type and no denticles, apart from the beard in T1a, form in these mutants. The model predicts that the beard should be enlarged and mirrorized, with anterior denticles pointing anteriorly and, mirabile dictu, this is the case.

cannot be affected by HSwg. This would mean that the 'naked' phenotype can be built over a wild type, but not over a mirror pattern situation.

DISCUSSION

Here we question the prevailing perception of the wild-type role of the *wingless* protein as a localised paracrine signal carrying an instruction from cells that secrete the protein to cells nearby that receive it. The evidence that leads to this perception as well as the background is summarised in the introduction.

It is clear that the *wingless* gene is essential for proper segmentation: in its absence most aspects of segmentation fail. We also know that in the wild type it is expressed in a precise but changing pattern. Previous analyses have suggested that *wingless* has different functions during embryogenesis (Bejsovec and Martinez-Arias, 1991; Heemskerk et al., 1991) and here we consider mainly the early function when *wingless* is thought to play a crucial role in the formation of parasegments. We show that an early pulse of generalised expression of *wingless* is able to emulate wild-type function to a considerable degree: generalised expression of *wingless* can maintain the expression of *engrailed* in *wingless*⁻ embryos. These restored *engrailed* stripes follow precisely the original parasegment border. An early pulse of generalised *wingless* expression also rescues the *wingless*⁻ cuticle phenotype to the same extent as the normal gene working around the same period: embryos recover in size and show clear signs of segmentation, normal dorsal patterns and a much improved ventral pattern (the 'mirror phenotype').

We conclude that, at least for this early function, *wingless* does not work as an instructive signal or as a morphogen. We mean by this that position-specific features (such as the anterior boundary of *engrailed* expression or the array of patterned elements in the cuticle) are not placed by a local source — or a differential distribution — of *wingless* protein.

Does *wingless* protein need to be localised?

If *wingless* protein is provided only during the first few hours of development, the outcome is a mirror phenotype, and this is true whether the *wingless* protein is localised as in the wild type or distributed universally. Does this mean that, in the early embryo, it is immaterial whether *wingless* protein is local or universal? Strictly speaking, it does not: the same final outcome could be reached in two different ways in these two situations and we have evidence that this is the case. Although universal *wingless* can restore *engrailed* stripes whose anterior margins follow precisely the parasegment borders, the stripes are slightly broadened and (at stage 11, or 4 hours after the pulse) flanked by new grooves at their posterior margins.

This observation can be related to the effect of generalised *wingless* which is added to wild-type embryos, when very broad *engrailed* stripes and ectopic grooves form and persist and a naked phenotype results (Noordermeer et al., 1992). Probably early generalised *wingless* expression

directs embryos down one pathway (with ectopic grooves) and early localised expression directs embryos down another (with no ectopic grooves). Lack of later *wingless* protein leads to the convergence of the two sets of embryos, both of which produce mirror phenotypes. These results show that *wingless* needs to be localised, but they rule out certain hypotheses as to why.

First, *wingless* protein might have to be on only one side of the parasegment border. Not so; the parasegment borders are unaffected when *wingless* protein is provided ubiquitously, both in *wingless*⁻ and *wingless*⁺ embryos (our results and Noordermeer et al., 1992).

Second, the differential distribution of *wingless* protein might determine which cells continue to express *engrailed*. Not so; when *wingless* protein is evenly distributed as in HSwg, *wingless*⁻ embryos, the *engrailed* stripe, even if slightly broadened, remains confined to the anterior part of the parasegment.

Third, the differential or graded distribution of *wingless* protein might act as a morphogen and determine the array of pattern elements in the cuticle. Not so; because normal patterns, such as fields of oriented hairs and alternating zones of naked cuticle and denticles can form following flat profiles of *wingless* protein concentration.

We believe that *wingless* protein does need to be localised but for a different reason: if it is present posterior to the *engrailed* stripe, it is instrumental in making an ectopic border there. It is therefore important to the wild type that *wingless* be restricted to the vicinity of the legitimate parasegment borders.

A gradient interpretation

We have seen that early function of *wingless*, provided either by the normal gene or by a heat-shock construct, produces a mirror phenotype in the ventral cuticle (Fig. 2). That the mirror phenotype represents a stable developmental outcome is suggested by two facts: **first**, several other segment polarity mutations give very similar or identical mirror patterns and, **second**, we have not been able to modify this phenotype by pulses of generalised *wingless* expression. Both the pattern and stability of the mirror phenotype can be easily modelled in terms of gradients. In these models, which have been postulated for the epidermis of other insects to explain its response to transplantation, pattern is thought to depend on a reiterated segmental gradient; the type of differentiation (e.g. denticles or naked cuticle) being determined by the scalar value of the gradient at each locale, and the polarity (e.g. orientation of denticles) by the local slope (reviewed in Lawrence, 1992). Although each segment has an identical gradient, the pattern of responses to it are segment specific. In Fig. 6 we present such a model as applied to the ventral cuticle of part of the thorax and abdomen. Note that the position and depth of the *engrailed* stripe is shown as determined by the landscape of gradient values. Also, that in the wild type (Fig. 6A) the parasegment borders are shown as if they were precipices interposing between one gradient and the next. We refer to such borders as 'sealed', meaning that they act as a barrier to the free flow of the substance of the gradient (e.g. a morphogen) - a flow that would otherwise tend to smooth out discontinuities.

In the strong segment polarity phenotypes (such as *wingless*⁻), parasegment borders fail to function and the gradient landscape becomes almost flat (Fig. 6B). In the naked phenotype (Noordermeer et al., 1992), two sets of borders, one normally positioned and oriented and one ectopic and abnormally oriented, lead to a new landscape with changes of pattern as shown (Fig. 6D). The mirror phenotypes (Fig. 6C) can be formed, we suggest, in two ways. In one experiment (*wingless*^{ts} shifts) only normal parasegment borders form, in the other (*HSwg,wingless*⁻) there are also additional ectopic borders. In later stages the lack of *wingless* product means that borders fail to 'seal' sufficiently, the morphogen 'leaks' through them and the gradient landscapes of both cases converge to the mirror phenotype.

It has been observed that initially *wingless* protein spreads across the parasegment border (van den Heuvel et al., 1989), but after about 5 hours the border appears to act as a barrier; *wingless* protein diffuses away from it but not across it (Gonzalez et al., 1991). This suggests that the parasegment border acquires some new property, which may relate to our more abstract description of it being 'sealed'.

Borders and junctions

We have interpreted the phenotype of *wingless*⁻ (and other 'strong' segment polarity mutants) as a complete failure of the parasegment borders and the mirror phenotype (characteristic of several 'weak' segment polarity mutants and partial rescues of *wingless*⁻) as a malfunctioning ('leaking') of the borders. The presence of ectopic *wingless* protein leads to extra borders. We envisage *wingless* protein as acting like a sealant to block the free passage of morphogen across the borders.

The molecular and cellular characteristics of the parasegment borders are largely unknown but there is evidence that intercellular communication can be restricted at compartment borders (Warner and Lawrence, 1981; Blennerhassett and Caveney, 1984). This could be due to the disposition or properties of intercellular junctions at or near the border. It would not be a complete surprise if the *wingless* protein had a role in these junctions. Although the *Wnt* proteins are secreted and associated with the **extracellular** matrix (van den Heuvel et al., 1989; Papkoff and Shryver, 1990), the *wingless* protein appears to affect the **intracellular** localisation of, or stabilise, the protein product of the *armadillo* gene. The *armadillo* protein is homologous to β -catenin and plakoglobin, two mammalian proteins that are constituents of junctions (Peifer and Wieschaus, 1990; McCrea et al., 1991; Peifer et al., 1992). Of course, our results and arguments fall short of establishing a wild-type role for *wingless* but we hope they will broaden the discussion about the function of the *Wnt* genes which, in our opinion, has become too influenced by the hypothesis that the *wingless* protein carries an instructive signal from one cell type to another.

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