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Compartments, wingless and engrailed: patterning the ventral epidermis of *Drosophila* embryos

Peter A. Lawrence, Bénédicte Sanson and Jean-Paul Vincent

Medical Research Council, Laboratory of Molecular Biology, Hills Rd, Cambridge CB2 2QH, UK

*Author for correspondence (e-mail: pal@mrc-lmb.cam.ac.uk)

SUMMARY

Recent experiments on the wing disc of *Drosophila* have shown that cells at the interface between the anterior and posterior compartments drive pattern formation by becoming the source of a morphogen. Here we ask whether this model applies to the ventral embryonic epidermis. First, we show that interfaces between posterior (engrailed ON) and anterior (engrailed OFF) cells are required for pattern formation. Second, we provide evidence that Wingless could play the role of the morphogen, at least within part of the segmental pattern. We looked at the cuticular structures that develop after different levels of uniform Wingless activity are added back to unsegmented embryos (*wingless*$^-$ *engrailed*$^-$). Because it is rich in landmarks, the T1 segment is a good region to analyse. There, we find that the cuticle formed depends on the amount of added Wingless activity. For example, a high concentration of Wingless gives the cuticle elements normally found near the top of the presumed gradient.

Unsegmented embryos are much shorter than wild type. If Wingless activity is added in stripes, the embryos are longer than if it is added uniformly. We suggest that the Wingless gradient landscape affects the size of the embryo, so that steep slopes would allow cells to survive and divide, while an even distribution of morphogen would promote cell death. Supporting the hypothesis that Wingless acts as a morphogen, we find that these stripes affect, at a distance, the type of cuticle formed and the planar polarity of the cells.

Key words: compartment, *Drosophila*, engrailed, morphogen, pattern formation, wingless

INTRODUCTION

Recent studies of *Drosophila* imaginal discs have produced a model for pattern formation, a model that may also apply more widely to animal development (reviewed in Lawrence and Struhl, 1996). The key elements of this model are the compartments, a short-range inducer and a long-range morphogen. In the anteroposterior axis of the wing disc, these elements are thought to drive pattern formation as follows:

(i) Two distinct populations of cells are specified by the *engrailed* selector gene, which is ON in the cells of the posterior (P) compartment and OFF in the anterior (A) (Morata and Lawrence, 1975; Kornberg, 1981).

(ii) The cells of the P compartment synthesise a short-range signalling molecule, Hedgehog to which only A cells are sensitive (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993).

(iii) A narrow strip of A cells responds to Hedgehog by synthesising Decapentaplegic (Dpp), a long-range morphogen (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994).

(iv) Dpp diffuses away both forwards and backwards into both compartments to set up two gradients (Zecca et al., 1995).

(v) In both compartments, cells respond to a gradient of Dpp, each compartment having its own specific set of responses, depending whether it is of A or P identity (Lecuit et al., 1996; Nellen et al., 1996).

Here, we explore the possibility that embryonic segments might be patterned in a manner analogous to discs. Since imaginal discs derive directly from embryonic cells, which are similarly divided by parasegmental boundaries (Garcia-Bellido et al., 1973; Vincent and O'Farrell, 1992), it seems logical to apply the disc model to the embryo (Lawrence and Struhl, 1996), particularly as many of the molecular components are found in both systems. In each embryonic segment, as in discs, P cells express *engrailed* while A ones do not. Again, as in discs, P cells synthesise Hedgehog. But the molecular correspondence ends here: Dpp does not appear to be activated by Hedgehog in embryos nor does it seem to act in embryonic segmental patterning. However, there is a gene that may be a good candidate to take the part of Dpp in embryos: not only is the secreted protein Wingless produced in a narrow strip of cells just anterior to the parasegment border (Baker, 1987; van den Heuvel et al., 1989), its expression also requires Hedgehog activity (Ingham and Hidalgo, 1993). These facts suggest that Wingless could be a morphogen and could act in embryos rather as Dpp does in discs. This suggestion contradicts earlier proposals (Sampedro et al. 1993; Diaz-Benjumea and Cohen, 1994; Vincent and Lawrence, 1994) and we have therefore reexamined the issue. We have also tested other key aspects of this model of pattern formation in the ventral epidermis of embryos. Specifically, we ask the following questions:

(i) Is the alternation of *engrailed* ON/*engrailed* OFF cells essential for segmentation of the embryo?

(ii) Does Wingless specify cell fates in a dose-dependent manner?

(iii) Is the response of A cells to Wingless distinct from that of P cells?

(iv) Does Wingless act at a distance in embryos?

For (i) (ii) and (iii), the answer is yes, while for (iv), it is probably, giving experimental support for the model. Also, our results add to previous evidence suggesting that Wingless can act as a morphogen (Bejsovec and Martinez Arias, 1991; Struhl and Basler, 1993; Hoppler and Bienz, 1995). In the embryo, the model is certainly an oversimplification as we show that Wingless patterns only part of the segment.

MATERIALS AND METHODS

Fly stocks

The following mutants were used: *Df(2R)enE* removes both *engrailed* and *invected* (Tabata et al., 1995), wg^{CX4} is a null mutation in Wingless (Baker, 1987) and wg^{IL119} is a temperature-sensitive allele of *wingless* (Nusslein-Volhard et al., 1984) referred to as wg^{ts} in the text. The following UAS-responder lines were used: UAS-Wingless (Lawrence et al., 1995), UAS-Engrailed (Guillen et al., 1995; Tabata et al., 1995), UAS-Hedgehog (Fietz et al., 1995) and UAS-Wingless^{ts} (Wilder and Perrimon, 1995).

The following Gal4 drivers were used: paired-Gal4 (made by L. Fasano and C. Desplan; see Yoffe et al., 1995), armadillo-Gal4VP16 (made by L. Seugnet and M. Haenlin: see Sanson et al., 1996) and armadillo-Gal4 (Sanson et al., 1996). The armadillo-Gal4 strain that we used mostly (armadillo-Gal4^4) was selected from a number of different inserts as it gave the strongest effects when tested with different UAS constructs (Sanson, unpublished). This strain consists of two separate inserts on chromosome III. When crossed to UAS-LacZ it gave strong universal expression of β-galactosidase that was detectable from early stage 9, this was initially spotty in appearance but rapidly became strong and fairly even.

Genetic crosses

Embryos of the different genotypes in a wild-type background were made by crossing homozygous stocks (e.g. armadillo-Gal4 to UAS-En) so there could be no doubt of the phenotype. Embryos made in a *wg⁻en⁻* background were made by crossing a balanced line to males from a cross, for example *wg⁻en⁻*/CyO; armadillo-Gal4 females to males *wg⁻en⁻*/+; UAS-En/+. There should therefore be three kinds of dead embryos in the progeny: *wg⁻en⁻* embryos, as well as *wg⁻ en⁻* and wg^+ en^+ embryos with universally expressed *engrailed*. In some cases, these three classes of embryos could be distinguished, in some not. In the example given above, there were only two classes of mutant embryos, and one was identical to those produced when armadillo-Gal4 and UAS-En homozygous flies are crossed, the other was identical to *wg⁻en⁻* embryos. We therefore concluded that ubiquitous Engrailed gives the same outcome in both *wg⁻en⁻* and wild-type embryos. In this and other cases, the validity of the allocation was checked by measuring the frequencies of the different classes of embryos.

Antibody stainings, cuticles

Rabbit anti-Wg (van den Heuvel et al., 1989) was used according to standard protocols. Stained embryos were dissected and mounted in Durcupan. Cuticle preparations were mounted in Hoyer's/lactic acid.

RESULTS

A ground state for the epidermal pattern

We sought to find embryos that would have as little segmental patterning as possible. These would then provide a ground state to assay the effect of adding back Wingless activity. Embryos that lack all maternal cues for anteroposterior patterning are ideal, and these can be obtained from females that are quadruply mutant for *bicoid*, *nanos*, *oscar* and *torso* (Struhl et al., 1992). However, these embryos would be too difficult to manipulate genetically. Fortunately, embryos that lack the *engrailed*/*invected* and *wingless* genes (Bejsovec and Martinez Arias, 1991) can be used instead. They differ from the progeny of the quadruple mutants in that the thoracic and abdominal identities are differentiated (Fig. 1) and this is expected since, unlike the quadruple mutants, they activate the homeotic genes. But in all other respects, the two types of embryos are very similar: they are small and spherical, they carry a lawn of unpolarised denticles and have no Keilin's organs, presumably because they have no functional parasegment boundaries. Each thoracic segment of *wg⁻en⁻* embryos is uniformly covered by a type of denticle which, in the wild type, is formed by A cells of that segment. This indicates that *wg⁻en⁻* embryos develop with all their cells taking the A identity – as would be expected, in the absence of Engrailed.

wg⁻en⁻ embryos are not completely unpatterned since the phenotype of *wg⁻en⁻hh⁻* triple mutants is slightly more severe (Bejsovec and Wieschaus, 1993). However, this is a small

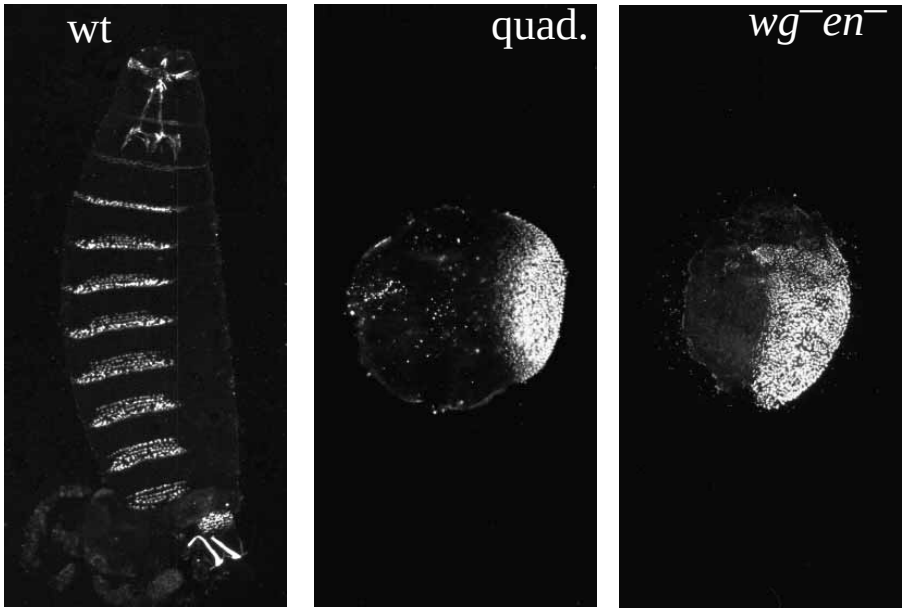

Fig. 1. The anteroposterior pattern in two types of unsegmented embryos compared with wild type (wt); note that the dorsoventral axis is more or less normal. The embryos from *bicoid nanos oskar torso* quadruple mutant females (quad.) were prepared as part of a previous study (see Struhl et al., 1992). The *wg⁻en⁻* embryo is wg^{CX4} *Df(2R)enE*. Dark field, all figures to same scale.

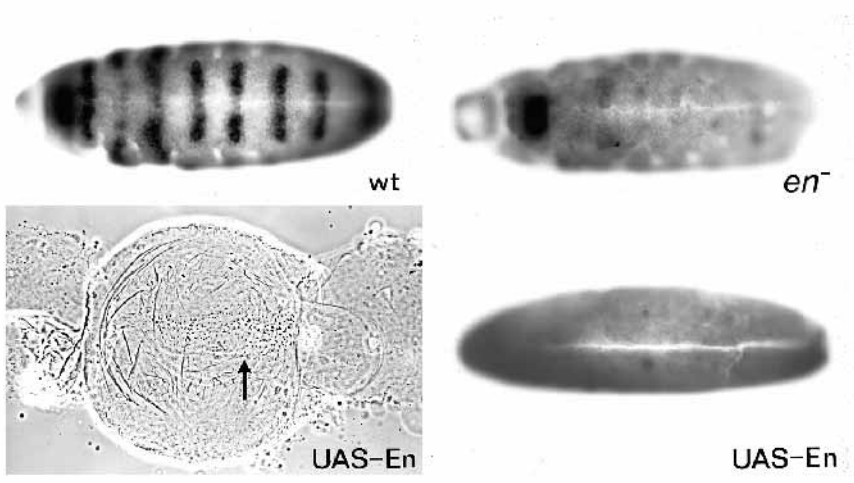

Fig. 2. Effect of loss of Engrailed or addition of uniform Engrailed on *wingless* expression and cuticle phenotype. Upper figures show late stage 11 wild-type (wt) and *Df(2R)enE* (*en*⁻) embryos. These embryos were stained with anti-Wingless antibody. They are dissected, only the ventral part of the embryo is shown. Lower panels show a cuticle and embryo preparation (late stage 11) of wild-type embryos to which uniform Engrailed has been added (armadillo-Gal4/UAS-En). The arrow marks the abdominal denticles that we consider to be row 1 in type. Note that there is no expression of *wingless* in the ventral epidermis both when *engrailed* is removed and when it is added uniformly, but Wingless disappears earlier and more completely in the latter.

effect and, for our purposes, we can use *wg*⁻*en*⁻ embryos as an unsegmented experimental base to which we can add extra gene products.

Alternation of *engrailed* ON and *engrailed* OFF cells is necessary for segmentation

According to the disc model, ubiquitous Engrailed should eliminate the OFF state and transform all A compartment cells into P ones, while the loss of *engrailed* should eliminate the ON state and transform all P cells into A ones. If the transition between the ON and OFF state of *engrailed* is a prerequisite for the establishment of the segmental pattern, then either expressing *engrailed* ubiquitously or removing *engrailed* function should destroy segmentation.

We expressed *engrailed* uniformly with the Gal4/UAS system (Fischer et al., 1988; Brand and Perrimon, 1993). Flies carrying an armadillo-Gal4 transgene (that gives high and uniform Gal4 expression) were crossed to UAS-Engrailed (UAS-En) flies and the progeny studied. All the embryos are small and spherical and unsegmented with a central stripe of weak denticles (Fig. 2); a phenotype resembling the most extreme obtained with a heatshock-Engrailed transgene (Poole and Kornberg, 1988).

The following arguments suggest that these denticles are of row 1 type which are, in the wild type, the denticles made by cells of P identity (Dougan and DiNardo, 1992; Bejsovec and Wieschaus, 1993; see these papers for the definition of denticle types):

(i) the denticles are confined to the abdomen (there are no P denticles in the wild-type thorax, but A2-A8 segments of the abdomen have P row 1 denticles)

(ii) the denticles are small and weak (this is a characteristic of row 1 denticles).

iii) the band of denticles is narrow (row 1 is the narrowest of the denticle rows).

Row 1 denticles are normally made by the *engrailed*-expressing cells located near the P/A (segment) boundary (Dougan and DiNardo, 1992; see also Fig. 4). Therefore, the formation of a continuous band of row 1 denticles indicates that all the cells develop as if they were at the posterior boundary of the *engrailed* ON (P) compartment. Our interpretation is that the trunk of the embryo consists of a homogeneous field of cells with no segmentation. All the cells are of the P type and thus there can be no A/P interfaces. In the absence of these interfaces, we would expect that the expression of *wingless* would not be maintained, which is exactly what we find in armadillo-Gal4/UAS-En embryos (Fig. 2).

Likewise, we expect removal of *engrailed* function to lead to unsegmented embryos, as all the P compartments should be converted to A ones, and the *engrailed* ON/*engrailed* OFF interfaces should again be eliminated and expression of the morphogen should not occur. To ensure removal of all *engrailed* function, we have used a deficiency that uncovers both *engrailed* and *invected* (a homologue of *engrailed* that is located nearby on the chromosome; Coleman et al., 1987), which we refer to simply as *en*⁻. Such embryos are smaller than wild type but longer than armadillo-Gal4/UAS-En embryos and display some alternating naked and denticulate cuticle (Tabata et al., 1995).

These alternating naked stripes are presumably due to Wingless (Bejsovec and Martinez Arias, 1991) for, even in *en*⁻ embryos, there is early striped expression of *wingless* that is activated by the pair-rule genes (Ingham et al., 1988; Ingham and Hidalgo, 1993). Note that *en*⁻ embryos are slightly longer than the *wg*⁻*en*⁻ double mutants. They resemble American footballs while *wg*⁻*en*⁻ embryos look like real footballs (see Discussion).

Sustained *wingless* expression in wild-type embryos depends on Hedgehog (Ingham, 1993; Ingham and Hidalgo, 1993). This is consistent with the disc model, in which Hedgehog needs to cross over from P cells to maintain *wingless* expression in nearby A cells. Accordingly, in mutant embryos that lack *engrailed* and therefore P cells, *wingless* expression is not sustained (Martinez Arias et al., 1988; Bejsovec and Wieschaus, 1993; see also Fig. 2).

The response to Wingless depends on whether *engrailed* is ON (A identity) or OFF (P identity)

To compare the response of *engrailed*-expressing and non-expressing cells to Wingless, we used test unsegmented embryos lacking both *engrailed*/*invected* and *wingless*. To these embryos, we added back, with the Gal4 system, either Wingless alone, or Wingless and Engrailed together.

If uniform Wingless is added to unsegmented embryos that lack both *wingless* and *engrailed*, there is no rescue of seg-

mentation, the embryos lengthen only a little and no Keilin's organs form. However, instead of denticles as in the double mutants, the ventral abdomen now makes naked cuticle (Fig. 3). Most significantly, T1 becomes largely covered by fine denticles of the type normally found in the beard. The beard is found, in wild-type T1, in the posterior region of the A compartment (Figs 3, 4). Although the abdominal cuticle of these embryos lacks all denticles, they are not phenocopies of *naked* (Jurgens et al., 1984) mutant embryos: the beard in our embryos, which has no polarity and is so large that it appears to fill about a segment, differs from that of *naked* mutants in which it is small, localised to part of the T1 segment (Fig. 3) and usually has mirror symmetric polarity (Sampedro et al., 1993).

If both *engrailed* and *wingless* are uniformly expressed (armadillo-Gal4 driving UAS-Wg and UAS-En, in $wg^{-}en^{-}$ embryos), the embryos are near-spherical and unsegmented (Figs 3, 4). However, they lack the beard denticles in T1. This differential effect on the beard clearly demonstrates that the response to Wingless is determined by the presence or absence of Engrailed.

Dose-dependent activity of Wingless

We assessed the dose response to Wingless for A (*engrailed* OFF) cells. This was done by expressing uniformly a temperature-sensitive Wingless protein in embryos lacking both *wingless* and *engrailed*/*invected*. We systematically varied the activity of the protein added by changing the temperature. We added Wg^{ts} protein (armadillo-Gal4/UAS-Wg^{ts}) to $wg^{-}en^{-}$ embryos: At 28.5°C, the protein is ineffective and the embryos develop with the $wg^{-}en^{-}$ phenotype. At 17°C, the protein is maximally effective, producing the same phenotype as when wild-type Wingless is added (Fig. 3). We have changed the temperature one degree at a time between these two extremes and observed the resulting patterns (Fig. 5). Within this range, the embryo length changes little (see Discussion). However, the type of cuticle in the abdomen does change from denticulate at low activity (high temperature) to naked with high Wingless (with the midventral cuticle being somewhat more sensitive to Wingless). Although this transition from naked to denticulate is unequivocal, these are the only two states that we can distinguish. However, three types of cuticle can be formed in T1. At 28.5°C, T1 is covered with denticles of the typical T1 type (same as $wg^{-}en^{-}$ in Fig. 3), that is the denticles normally found in the anterior region of the T1 A compartment. At 17°C, T1 is covered by fine denticles of the type normally found in the beard, which belongs to the posterior region of the T1 A compartment (same as UAS-Wg in Fig. 3). Between 23 and 24°C T1 is naked, that is it makes the type of cuticle normally present in the middle part of the A compartment. These results suggest that, in a situation where there is no segmental pattern and no polarity, the level of Wingless activity determines the type of cuticle formed.

Note that all the cuticle structures arising in this experiment are those found within the A compartments of the wild type:

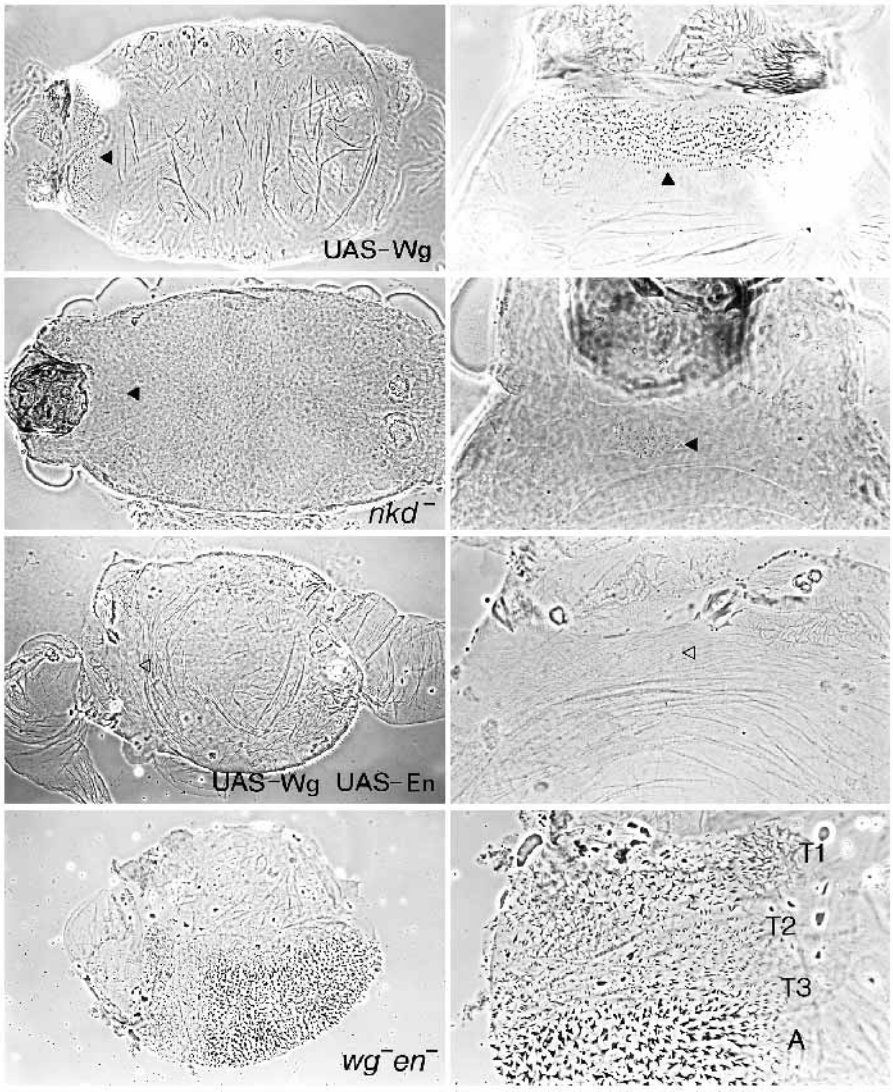

Fig. 3. Whole cuticles of unhatched larvae (left) with details of ventral T1 (right). All pictures within one column are at the same magnification. UAS-Wg = $wg^{-}en^{-}$; armadillo-Gal4/UAS-Wg. Adding uniform Wingless to an unsegmented embryo results in a naked cuticle, except for T1 which is covered with beard denticles (filled arrowhead). Note that adding uniform Wingless expression does lengthen the embryos somewhat (compare to $wg^{-}en^{-}$). We do not understand this; it may be a consequence of striped *hedgehog* expression, which, in combination with uniform Wingless, may restore some segmentation. nkd^{-} = a $naked^{89E}$ mutant embryo with a little beard (filled arrowhead). UAS-Wg, UAS-En = $wg^{-}en^{-}$; armadillo-Gal4/UAS-Wg, UAS-En. A small beardless embryo with extruded organs that we believe to be the foregut and hindgut; the empty arrowhead shows the approximate position where the beard might have been, but was not. $wg^{-}en^{-}$ = $wg^{-}en^{-}$ embryos. T1, T2, T3 and abdominal (A) denticles can be distinguished.

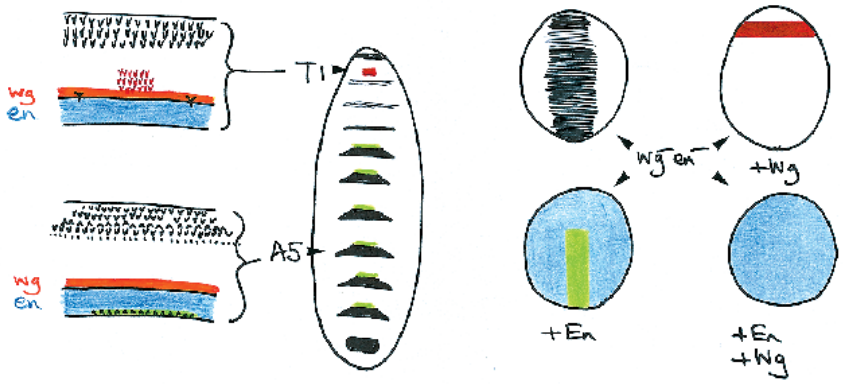

Fig. 4. A diagram of the ventral cuticle plus a summary of some results. On the left, a diagram of the ventral cuticle of the larva is shown with details of the T1 and A5 segments. Note the posterior compartments where *engrailed* is expressed (blue). Stripes of *wingless* expression are shown in orange. The beard (red) and row 1 denticles (green) are also shown. On the right are *wg*$^{-}$*en*$^{-}$ mutant embryos to which uniform Engrailed and/or Wingless have been added as indicated. For example, adding Engrailed produces a stripe of denticles that are presumed to be row 1 (green), while adding Wingless gives a naked embryo apart from an extensive beard in T1 (red). Embryos expressing engrailed uniformly are coloured blue to indicate that all cells have taken a posterior identity. Photographs of all these embryos are given in the figures.

this is expected as the embryos lack the *engrailed*/*invected* genes. Note also that in each segment (this is especially clear in T1), high levels of Wingless activity produce the type of cuticle normally found posteriorly while low levels produce the type of cuticle normally found more anteriorly. This is consistent with the gradient model since, in the wild-type embryo, *wingless* is expressed in the most posterior part of the A compartment (see Fig. 4).

By analogy with the experiments above, one way to assess the response of P cells to various levels of Wingless activity (and in the relevant range of concentration) is to express UAS-En and UAS-Wg^{ts} together under the control of armadillo-Gal4 at different temperatures. Unfortunately, there are not sufficient markers within the P compartment to look for a three-level response to Wingless as above. Nevertheless, we did find that P cells show a differential response to Wingless. At 17°C,

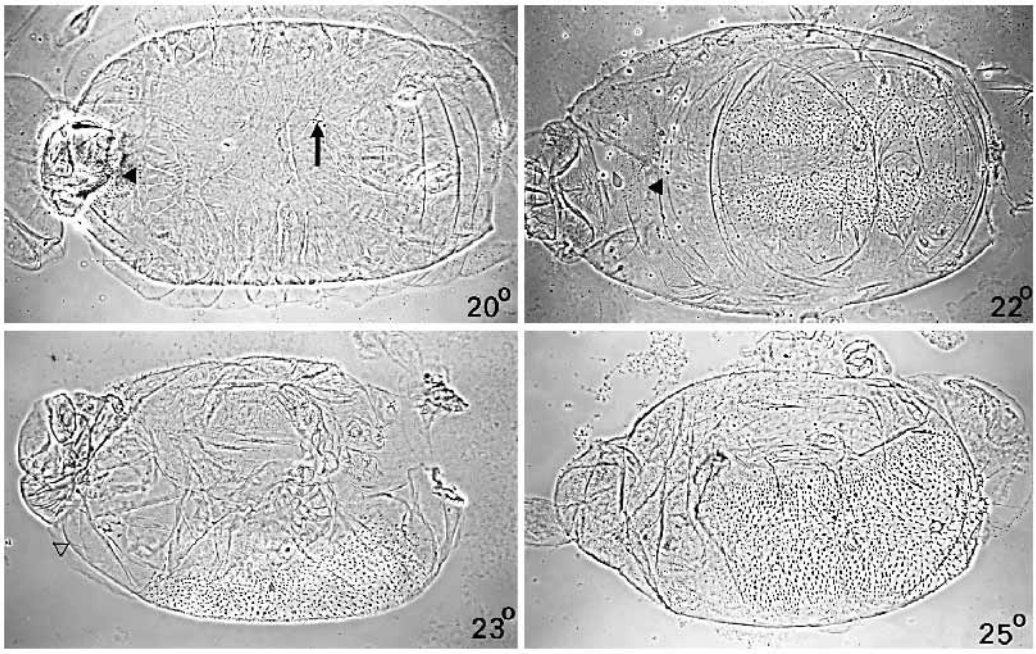

Fig. 5. Different amounts of uniform Wingless activity are added to *wg*$^{-}$*en*$^{-}$ embryos and cuticle preparations are shown. Note, as the temperature rises, less and less Wingless activity is added. At 17°C the embryos are indistinguishable from 'UAS-Wg' in Fig. 3. 20° = *wg*$^{-}$*en*$^{-}$; armadillo-Gal4/UAS-Wg^{ts}, 20°C, there are only a few ventral denticles (arrow), there is some beard in T1 (filled arrowhead). 22° = *wg*$^{-}$*en*$^{-}$; armadillo-Gal4/UAS-Wg^{ts}, 22°C, there are two lateral stripes of ventral denticles, with the mid ventral strip being naked. There is some beard in T1 (filled arrowhead). 23° = *wg*$^{-}$*en*$^{-}$; armadillo-Gal4/UAS-Wg^{ts}, 23°C, the abdomen is completely covered in denticles but there is no beard in T1 (empty arrowhead). 25° = *wg*$^{-}$*en*$^{-}$; armadillo-Gal4/UAS-Wg^{ts}, 25°C, the abdomen is completely covered in denticles; weak thoracic denticles are present, but are unprepossessing in the photograph. When the temperature is raised still further, to 28.5°C, these thoracic denticles become stronger — the embryo becomes indistinguishable from a *wg*$^{-}$*en*$^{-}$ mutant embryo (see Fig. 3).

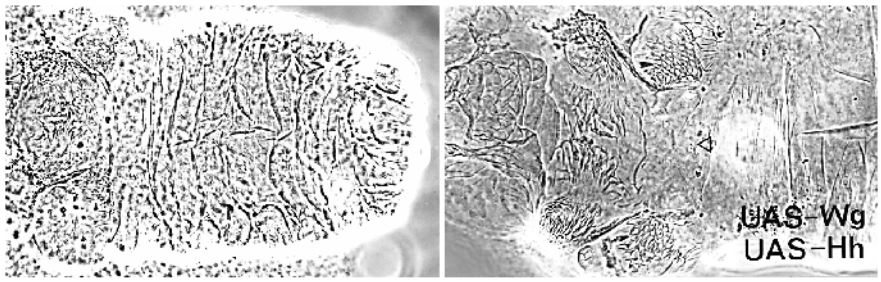

Fig. 6. Both uniform Wingless and Hedgehog are added to *wg⁻en⁻* embryos using the armadillo-Gal4 driver, resulting in no beard (empty arrowhead).

when the Wingless protein is functional, the outcome is as described above when wild-type *wingless* and *engrailed* are coexpressed, namely the embryos are unsegmented and abdominal cells make naked cuticle (cf. Fig. 3). But, at 25°C, when the Wingless protein is ineffective and only Engrailed is provided, it is no surprise that the embryos have a central band of denticles exactly as produced by ubiquitous Engrailed alone (see above and Figs 2, 4).

Thus, as shown previously (Dougan and Dinardo, 1992), at low or zero levels of Wingless, P cells make row 1 denticles while they make naked cuticle at higher levels.

Uniform expression of *wingless* and *hedgehog*.

In T1, the beard is found in the posterior region of the A compartment, that is near, but not at, the source of Wingless; between the beard and the Keilin's organs (which mark the parasegment boundary where A meets P) there is some naked cuticle (see Fig. 4). We were unable to 'raise' the cuticle type of *wg⁻en⁻* embryos 'above' beard, even with high levels of Wingless (we used an armadillo-Gal4VP16 driver which, in other experiments (Sanson et al., 1996) gives a strong effect). There is the possibility that regions of the A compartments that are close to the A/P borders are directly affected by Hedgehog; hence it might be the combination of Wingless and Hedgehog that would specify this position and, in T1, substitute naked cuticle for beard denticles. We therefore used armadillo-Gal4 to drive UAS-Wg and UAS-Hh at the same time. An example of the resulting embryos is shown in Fig. 6; we see that there is naked cuticle in place of the beard suggesting that, indeed, a combination of Hedgehog and Wingless is required to specify the most posterior fates of the A compartment.

Effects of Wingless at a distance

It has been clear for some time that Wingless does influence patterning at a distance: *wingless* expression is localised and, yet, loss of its activity has widespread effects throughout the embryo (Nusslein-Volhard et al., 1984; Baker, 1987; Gonzalez et al., 1991). Here we assay the effect of localised Wingless expression in unsegmented test embryos. We used the Gal4 system to express Wingless locally in *wg⁻en⁻* mutants. We chose paired-Gal4 as a driver; it is normally active in the test embryos and gives sharply delineated stripes of β-galactosidase when it drives UAS-LacZ (Yoffe et al., 1995). The outcome of these experiments is clear; the even lawn of denticles in *wg⁻en⁻* embryos is now interrupted by regularly spaced stripes of naked cuticle. In the legend of Fig. 7, we describe evidence that the naked stripes are slightly wider than the stripes of Wingless expression. Also, the denticles themselves are affected by the proximity of cells expressing *wingless*; they show changes in polarity of two kinds: firstly the types of denticles are arranged with mirror-image symmetry. At the most anterior and most posterior margins of the abdominal stripes, the denticles are small, and are similar to those found at the posterior margins of the wild-type denticle bands, (like row 6). In the centre of the bands, the denticles are larger more like those found further inside the denticle bands in the wild type (rather like row 5). Secondly, the denticles are oriented so that the anterior ones tend to point anteriorly and the posterior ones tend to point posteriorly. We think it may be significant that they point towards the nearest source of Wingless, that is up the presumed Wingless gradient. These observations suggest that the effects of a localised source of Wingless (producing naked cuticle, changing denticle type and reorienting the denticles) extend a few cell diameters beyond the apparent source, but they do not establish how direct the effect is.

Associated with these changes in pattern, the embryo gains length (Fig. 7, and see Discussion).

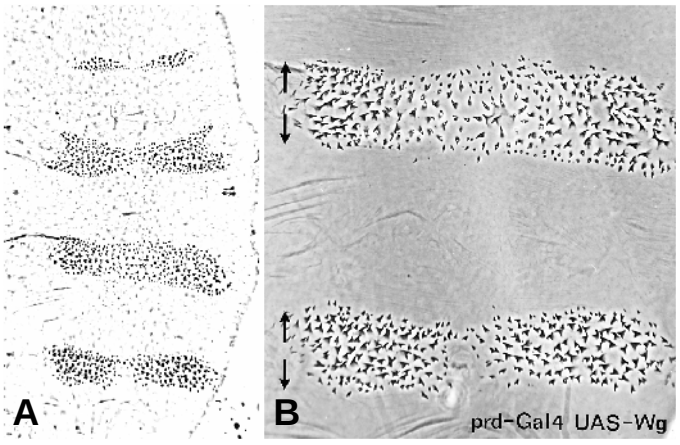

Fig. 7. Patterning in a *wg⁻en⁻* embryo by expressing UAS-Wg with paired-Gal4. (A) The whole embryo is lengthened (as compared to *wg⁻en⁻* embryos) and has alternating stripes of naked and denticulate cuticle. The domain of exogenous wingless expression is presumed to be within - and somewhat narrower than - the named region. This was estimated by two means. (1) paired-Gal4 in the wild type drives expression in about 1/3 of a repeated unit (Yoffe et al., 1995) while the naked cuticle in our experimental embryos occupies about 2/3. (2) X-Gal staining of cuticulate embryos that carry UAS-LacZ in addition (not shown). (B) At high magnification, it is clear that two types of denticles form in each band: small ones on the outside and larger ones inward. Also notice that individual denticles tend to point towards the nearest naked domain as shown (towards the source of Wingless).

DISCUSSION

A model for segmental patterning

The idea that pattern and polarity of the insect epidermis depends on a gradient that reiterates in each segment was based on transplanting small squares of epidermis in the bug, *Rhodnius* (Locke, 1959). This experimental work was extended by Lawrence (1966) and Stumpf (1966) who concluded that the gradient might be a diffusible morphogen whose scalar provided positional information and whose vector could polarise cells (Lawrence et al., 1972). It was later suggested that the slope of the gradient provides a measure of size which could be interpreted locally and determine probabilities of cell death and cell division (Bohn, 1970; Lawrence and Morata, 1976; Lawrence and Struhl, 1996).

In the Introduction, we summarise how a gradient model for the leg and wing discs could be applied to the embryonic epidermis with its topology conserved (Lawrence and Struhl, 1996) and why Wingless could, in the embryo, play the part acted by Dpp in the wing disc, or Wingless itself in the leg disc (Struhl and Basler, 1993; Basler and Struhl, 1994). We have put this model to test. We have looked at pattern formation within the segment when different amounts of uniformly distributed Wingless are added to unsegmented embryos.

As with patterning of discs, we propose that the key to segmental patterning in the embryo is the juxtaposition of A and P cells, a difference depending on the *engrailed* selector gene. This juxtaposition may lead indirectly to the sustained localised expression of a diffusible morphogen, Wingless.

A/P juxtaposition as the key to segmentation

We find that, if a high concentration of Engrailed is provided uniformly, all A cells acquire P identity and segmentation does not occur, as expected from the model. Similarly, one might expect removal of *engrailed* also to abolish segmentation – for, again, A/P interfaces should disappear. However, there is some residual pattern in *en*$^{-}$ embryos. This is certainly due to Wingless for when *wingless* and *engrailed* are both removed, a spherical, unpatterned and unsegmented embryo is produced (Bejsovec and Martinez Arias, 1991). Indeed, there is transient and striped *wingless* expression in young *en*$^{-}$ embryos. We interpret these results in light of the model for imaginal discs: production of the morphogen will normally be generated by the A/P interfaces, but if the morphogen is placed there by some other means, it should still be able to specify pattern elements in the absence of A/P alternation**.**

In *en*$^{-}$ embryos, patterning is rudimentary because expression of the morphogen is transient and because only anterior cells are present – thus only the A set of responses to the morphogen are possible. In our experiment where Wingless is expressed under the control of paired-Gal4 in *wg*$^{-}$*en*$^{-}$ embryos, more patterning occurs because exogenous expression of *wingless* is longer lasting. Still, here too *wingless* expression cannot generate P pattern elements as Engrailed is not present. These experiments illustrate two functions of Engrailed in segmental patterning, the same two functions that occur in imaginal discs (see also Heemskerk and DiNardo, 1994). First, the interfaces between *engrailed* ON and *engrailed* OFF compartments ensure sustained expression of the Wingless morphogen. Second, the presence or absence of *engrailed* product determines the cells as P or A and selects the responses to the morphogen (see Fig. 4). Supplying exogenous Wingless with paired-Gal4 only bypasses the need for the first function.

Wingless as a morphogen

One diagnostic for a morphogen is that it should specify cell fates in a dose-dependent manner. In our present test of whether Wingless qualifies as a morphogen, we have utilised the T1 segment which has three different types of cuticle in the A compartment, one at each level in the anteroposterior axis. We have used embryos that, having no Engrailed, are entirely made of A-type cells, and, having no Wingless, are unsegmented and unpatterned. We have then added back different amounts of uniformly distributed Wingless. Our results show a dose response to Wingless, a high dose giving T1 cuticle of the type normally found at the back, an intermediate dose giving cuticle normally found in the middle of the segment and a low dose that found at the front (Fig. 4). These results suggest that the normal sequence of pattern depends on a gradient of Wingless. Our best argument is based on results with T1 although we believe that the same argument applies to other segments as well. This constitutes additional evidence for an earlier hypothesis that Wingless could act as a morphogen in the embryonic cuticle (Bejsovec and Martinez Arias, 1991).

The experiment with paired-Gal4 demonstrates the effect of Wingless at a distance, as expected for a morphogen: outside the domains of striped Wingless expression, three denticle types are formed in order (naked, small and large denticles) suggesting a range of at least three cells; we cannot make a more precise estimate. We cannot be sure that this effect at a distance is direct and that the sequence of cuticle types represents a readout of the protein concentration, although this is the most likely explanation, particularly in light of contemporary results on imaginal discs using a membrane-tethered form of Wingless (Zecca et al., 1996).

Evidence against Wingless as a morphogen

Whether wingless is a morphogen or not has been extensively debated. Arguments in favour include the long-range effect of Wingless-expressing clones (Struhl and Basler, 1993) and a dose-dependent response in the embryonic gut (Hoppler and Bienz, 1995). Here we discuss the published evidence that argues against Wingless being a morphogen: with respect to its action on *engrailed* expression, Wingless has a range of only one cell in the embryo (Vincent and Lawrence, 1994), which would seem inconsistent with it being a morphogen. However, this range could be more a matter of response, *engrailed*-expression being maintained only in those cells that are close to the Wingless source and receive the highest concentration of Wingless. Lower concentrations of Wingless might have other effects further from the source. Alternatively, Wingless may not diffuse freely in the P compartment (see below) and thus would only reach adjoining cells.

Sampedro et al. (1993) argued against the hypothesis that Wingless is a morphogen. They postulated that homogeneous distribution of a morphogen should produce a homogeneous pattern and yet they found that a flat field of Wingless protein, when provided to *wg*$^{-}$*en*$^{+}$ embryos, rescued the embryo considerably. It became longer and had alternating bands of denticulate and naked cuticle. But things could be more complex

than they imagined: The stripes of early *engrailed* expression in *wg*⁻ embryos will make bands of A and P cells. This, in conjunction with uniformly added Wingless, may lead to sustained striped expression of Hedgehog or another factor; this in turn would lead to an undulating pattern of positional values.

Another result argues against Wingless being a morphogen: the *shaggy* gene is involved in the reception of Wingless; *shaggy*⁻ cells behave as if they receive a strong Wingless signal. Yet, in the leg, *shaggy*⁻ clones, produce pattern and polarity changes far beyond the clone itself in a manner similar to Wingless-expressing clones, that is, they show non-autonomy (Diaz-Benjumea and Cohen, 1994). This finding suggests that the diffusion of Wingless itself may not be responsible for the changes in pattern, unless of course *wingless* itself is expressed in *shaggy*⁻ clones as was shown recently by Jiang and Struhl (1996). It remains to be seen whether *shaggy*⁻ cells require Wingless activity to affect pattern at a distance.

To sum up, these three arguments fall short of proving their case.

Does Wingless act in a dose-dependent manner in the P compartment as well as in the A compartment?

In embryos that have uniform *engrailed* expression, the embryo is unsegmented and (we presume) consists entirely of cells of P identity; denticles resembling row 1 are formed in the mid-ventral abdomen. When uniform Wingless is now added to these embryos, the only change that we can see is that cells in the same position now make naked cuticle (see Fig. 4). Thus, in the abdomen, *engrailed*-expressing cells make naked cuticle in the presence of Wingless while they make row 1 denticles in its absence (or at low concentration) – exactly this conclusion was drawn earlier (Dougan and Dinardo, 1992). This may imply that a Wingless gradient patterns the P compartment in the embryo as it does in the disc (Zecca et al., 1995). Alternatively, as suggested by Dougan and DiNardo (1992), there could be only two levels of Wingless that matter in the P compartment: high and nil. Such a stepped distribution could arise if Wingless did not diffuse freely in the P compartment and could not reach beyond one cell. According to this view an asymmetric gradient of Wingless would be present in each segment: the peak would lie just anterior to the parasegment boundary with concentration trailing off anteriorly and dropping abruptly posteriorly (see Gonzalez et al., 1991).

Wingless alone does not pattern the whole segment

Even if, as we are suggesting, the imaginal disc is a model for the embryo, they are not equivalent. In the embryo we see the entire segment, whereas the disc is made by a restricted region that straddles the parasegment boundary (Cohen, 1993) and does not include cells close to the segment boundary, on either side. In our experiment, where we vary the level of uniform Wingless activity, we induce, in T1, three fates that are found in the central two-thirds of the A compartment (Fig. 4).

We now discuss the high and low end of the gradient: first, the high end. In the wild-type T1, naked cuticle forms at or near the source of Wingless, yet we did not observe naked cuticle even when we produced a high concentration of Wingless (with the armadillo-Gal4VP16 driver). However when we co-expressed *hedgehog* and *wingless*, naked cuticle did form in T1, consistent with the hypothesis that either a combination of the two proteins, or Hedgehog alone, make a value found at the high end of the gradient (Fig. 6; Lawrence and Struhl, 1996). Consider now the low end of the gradient: it is notable that in T2, T3 and A1 of wild-type embryos, the anterior ends of the A compartments form naked cuticle, with denticulate cuticle being made a little further back (see Sampedro et al. 1993 for mapping of these zones). Yet, *wg*⁻ *en*⁻ embryos make denticulate cuticle in these segments. In other words, structures made in the absence of Wingless do not correspond to the anterior limits of A compartments, suggesting that the patterning of these regions is dependent on something other than Wingless. Note also that in the prd-Gal4 experiment, only naked cuticle and, probably, row 5 and row 6 denticles are specified by exogenous wingless expression. It is as if Wingless alone does not specify anterior pattern elements such as rows 2-4 (although we do not know if a zero value for Wingless is generated in the experimental embryos). It could be that something else emanating from the side opposite to the Wingless source (possibly Hedgehog; Heemskerk and DiNardo, 1994) is involved in patterning anterior A cells, with or without the help of low level Wingless.

Size regulation by the morphogen gradient

We have noticed a correlation between embryo length and the extent of segmentation. *wg*⁻ *en*⁻ embryos are, like the progeny of *bicoid*⁻ *torso*⁻ *nanos*⁻ *oskar*⁻ females, unsegmented and very short (see Fig 1). *en*⁻ single mutants, which have alternating bands of naked denticle due to *wingless* activity, are longer. Intriguingly, in *wg*⁻ *en*⁻ embryos expressing *wingless* under paired-Gal4 control, a substantial amount of segmental patterning is restored as measured by the length of the embryo while, by contrast, uniform addition of various levels of Wingless activity (high or low) has a more modest effect on embryo size. This indicates that an uneven distribution of Wingless results in a larger embryo, presumably with more cells, than does homogeneous Wingless at any level of concentration. Thus it appears to be the slope of the gradient that matters: it could impinge on the cellular controls of apoptosis and/or proliferation to affect cell numbers. For example, steep slopes would encourage cells to survive and divide, while an even distribution of morphogen would lead to more cell death (Lawrence and Struhl, 1996).

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