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Antagonism between EGFR and Wingless signalling in the larval cuticle of *Drosophila*

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SUMMARY

Signalling by the epidermal growth factor receptor (EGFR) plays a critical role in the segmental patterning of the ventral larval cuticle in *Drosophila*: by expressing a dominant-negative EGFR molecule or Spitz, an activating ligand of EGFR, we show that EGFR signalling specifies the anterior denticles in each segment of the larval abdomen. We provide evidence that these denticles derive from a segmental zone of embryonic cells in which EGFR signalling activity is maximal. Within each segment, there is a competition between the denticle fate specified by EGFR signalling and the naked cuticle fate specified by Wingless signalling. The final pattern of the denticle belts is the product of this antagonism between the two signalling pathways. Finally, we show that the segmental zones of high EGFR signalling activity depend on bithorax gene function and that they account for the main difference in shape between abdominal and thoracic denticle belts.

Key words: EGFR signalling, Wingless, segment polarity, larval cuticle, *faint little ball*

INTRODUCTION

During animal development, positional information is often conveyed to cells by extracellular signals that spread from localised sources. Typically, these signals stimulate cascades of transducing molecules to elicit specific changes in transcriptional activity in the receiving cells. These changes are thought to be the basis for stable developmental decisions and eventually lead to differentiation of particular cell types and whole structures.

Developmental decisions can be affected by multiple signalling pathways that stimulate cells simultaneously (e.g. Kimelman et al., 1992; Smith, 1993; Bienz, 1994; Greenwald, 1996; Cohen, 1996). Simultaneous multiple stimuli can act synergistically or antagonistically, and a receiving cell has the task of integrating these various stimuli. Signal integration is often achieved within the nucleus at the level of transcriptional enhancers (e.g. Hill and Treisman, 1995; Thüringer et al., 1993; Watabe et al., 1995).

The ventral epidermis of a *Drosophila* larva shows a highly characteristic pattern of denticle bands alternating with stretches of naked cuticle (Lohs-Schardin et al., 1979), which depends on the activity of segment polarity genes (Nüsslein-Volhard and Wieschaus, 1980). Two of these genes play a key role in conferring this pattern, namely *wingless* (*wg*) and *hedgehog* (*hh*), which encode extracellular signals that are secreted within each segment from localised adjacent sources (reviewed by DiNardo et al., 1994). Early on, these two signals act locally to stabilise each other's sources (Martinez-Arias et al., 1988; Bejsovec and Martinez-Arias, 1991; Heemskerk et al., 1991; Ingham, 1993; Ingham and Hidalgo, 1993; Heemskerk and DiNardo, 1994; we shall refer to this early phase as the stabilisation phase). Subsequently, each signal acts to specify various cell fates: *wg* specifies naked cuticle stretches between denticle bands (Baker, 1988; Bejsovec and Martinez-Arias, 1991; Dougan and DiNardo, 1992; Bejsovec and Wieschaus, 1993; Lawrence et al., 1996), whereas *hh* apparently specifies a number of distinct fates across the segment (Heemskerk and DiNardo, 1994; Bejsovec and Wieschaus, 1993; we shall refer to this second phase as the cell fate specification phase). It has been argued that the activity of the Wg and Hh signals can account for most if not all of the larval cuticle pattern, and that other segment polarity genes are involved either in transducing these signals or in controlling their localised production (DiNardo et al., 1994).

However, a third extracellular signal is known to affect segmental patterning of the larval epidermis, namely Spitz (Spi) (Mayer and Nüsslein-Volhard, 1988), a ligand for the *Drosophila* epidermal growth factor (EGF) receptor (DER) (Rutledge et al., 1992; Schweitzer et al. 1995a). For various reasons, the role of EGFR signalling in this patterning event is not well understood. Firstly, it is not clear whether there is a localised source of active ligand within the segment. Secondly, there is a discrepancy between the phenotype caused by *spi* mutation and that caused by DER loss-of-function mutations (Price et al., 1989; Schejter and Shilo, 1989), in that the latter is much more severe than the former (Clifford and Schüpbach, 1992; Raz and Shilo, 1992). However, the role of DER in the development of the ventral denticle belts remains somewhat unclear as it is hard to dissect from DER's other functions during embryonic development (Clifford and Schüpbach, 1992; Raz and Shilo, 1992). Also, in addition to Spitz, there are other

activating DER ligands: the ligand for DER in the germ line is Gurken (Neuman-Silberberg and Schüpbach, 1993), and a new putative DER ligand, called Vein (Vn), has recently been described with functions in the ventral epidermis as well as in wing development (Schnepp et al., 1996; Simcox, 1997).

We were struck by the observation that a key aspect of the segmental polarity phenotype of *spi* (loss of the first row of denticles in abdominal denticle bands; Mayer and Nüsslein-Volhard, 1988) is the opposite of that of *wg* removed during the phase of cell fate specification (an extra row of denticles anteriorly to abdominal denticle bands; Dougan and DiNardo, 1992). We thus decided to investigate more closely the role of EGFR signalling during the formation of the ventral cuticle. We used a dominant-negative form of DER to reexamine the function of this receptor in the ventral cuticle, and we show that DER plays a critical role in specifying denticle fates. We also present evidence that there is a localised source of EGFR signalling activity in a band of cells most anteriorly in each segment, which locally antagonises the effects of Wg signalling. Finally, we show that these segmental sources of EGFR signalling activity are upregulated in the abdominal segments by the bithorax genes, thus accounting for the major difference in shape between thoracic and abdominal denticle belts.

MATERIALS AND METHODS

Fly strains

The following mutant alleles were used: flb^{IF26} (Clifford and Schüpbach, 1992); $aos^{I\Delta 7}$ (Freeman et al., 1992a); wg^{cx4} (Baker, 1987); wg^{IL119} (Nüsslein-Volhard et al., 1984); arm^{XM19} (Peifer and Wieschaus, 1990); *Df109* (Lewis, 1978). A recombinant chromosome bearing both wg^{cx4} and flb^{IF26} was generated by standard genetic techniques. Mutant *armadillo* (*arm*) embryos lacking Wg signalling function were produced from homozygous mutant germ-line clones, using arm^{XM19} on an *FRT101* chromosome (Chou et al., 1993), as described by Peifer et al. (1994).

The following GAL4 driver and responder lines were used: arm.GAL4^4 and arm.GAL4-VP16 (Sanson et al., 1996); UAS.DN-DER (E. Raz and B. Shilo, personal communication; Freeman, 1996), UAS.sSpi (Schweitzer et al., 1995a); UAS.Dras^{V12} (Lee et al., 1996); UAS.Wg (Lawrence et al., 1996).

The following β-Galactosidase (*lacZ*) enhancer trap lines were used: X81 (Freeman et al., 1992b); rho^{lac1} (Bier et al., 1990); S^{5671} (Heberlein et al., 1993); *en-lacZ* (Hama et al., 1990).

Phenotypic analysis

Standard crosses were set up at 25°C (unless specified otherwise), producing a mixture of offspring. Homozygous mutant larvae and larvae overexpressing proteins from UAS constructs were readily identified by their mutant phenotypes. *aos* mutants (which show only a mild phenotype) were identified by the increased lateral width of their denticle belts (see Golembo et al., 1996). Mutant larvae expressing sSpi or DN-DER were identified on the basis of their novel phenotypes, in comparison to crosses producing simply mutants or overexpression phenotypes; in each case, the novel phenotypes were observed at the expected frequency. *Df109* homozygous embryos were identified by their midgut phenotypes (Bienz and Tremml, 1988).

In the case of temperature-sensitive alleles, the following conditions were used. To remove Wg during the cell fate specification phase, one hourly collections of embryos (collected at 18°C) were shifted to the restrictive temperature (25°C) after 24 hours at 15°C (equivalent to ~8 hours at 25°C). In the case of flb^{IF26} and flb^{IF26} wg^{cx4}, one hourly collections of embryos (collected at 18°C) were shifted to the restrictive temperature of 29°C after 12 hours at 18°C (equivalent to ~6 hours at 25°C). These conditions allow normal germ-band retraction and cuticle secretion (Raz and Shilo, 1992) but, in our hands, also allow development of 2-4 rows of denticles (Fig. 2A; see also Clifford and Schüpbach, 1992), rather than completely suppressing denticle formation (see Raz and Shilo, 1992).

For cuticle preparations, eggs were collected on apple plates and aged for 24 hours at 25°C. Unhatched larvae were devitellinised, using a methanol step (as for antibody staining of embryos), and larval cuticles were mounted in Hoyer's medium mixed with lactic acid (1:1; Wieschaus and Nüsslein-Volhard, 1986), incubated at 65°C overnight, and viewed under Nomarski optics. Fixation and staining of larval cuticles for *lacZ* activity was essentially done as described (Dougan and DiNardo, 1992).

Antibodies to the following proteins were used for staining of embryos: β-Galactosidase (β-Gal; Promega), Engrailed (En; Patel et al., 1989), Argos (Aos; Freeman, 1994b) or Rhomboid (Rho; Sturtevant et al., 1996). The protocol of Lawrence and Johnston (1989) was followed for single and double stainings, except that formaldehyde (instead of paraformaldehyde) was used for fixation. Care was taken to minimise exposure to methanol when anti-Aos was used.

In situ hybridisation to *aos* transcripts in embryos has been described (Freeman et al., 1992a).

RESULTS

Fig. 1 shows a slice across an abdominal segment of a young larva depicting a denticle belt with its six rows of denticles surrounded by naked cuticle (see also Lohs-Schardin et al., 1979; Campos-Ortega and Hartenstein, 1985). It also shows a projection of these denticle rows onto the embryonic cells that are the sources of various active extracellular signals required for this cuticular pattern. This projection is mostly based on the expression domain of *en*, which labels the posterior (P) compartment and which can be visualised in the larval cuticle by β-Gal staining of *en-lacZ* larvae (Hama et al., 1990; see also Dougan and DiNardo, 1992; Sampedro et al., 1993). Our work focuses on these abdominal denticle belts, which are fairly similar to one another (except for the first one; see below) but which are very different from the much narrower thoracic denticle belts. Whenever we mention denticle belts below, we shall refer to the belts of abdominal segments 2-8, unless specified otherwise.

According to Bejsovec and Wieschaus (1993), each row of abdominal denticles is different and has its own identity. By morphology alone, we find we can only confidently distinguish between three types of denticles (in the vicinity of the ventral midline where the variation is minimal): small denticles with hooked ends and flat bases in row 1-4, large tapered denticles that are less hooked in row 5, and tiny tapered denticles in row 6 (note that these are arranged in a strip rather than in an ordered line in young larvae; Figs 1, 2B). Orientation of denticles can serve as a further criterion for identification of denticle rows: denticles generally point towards the posterior, with the exception of those in rows 1 and 4 which point towards the anterior (Figs 1, 2B).

EGFR signalling specifies denticles in rows 1-4

A temperature-sensitive allele of DER, flb^{IF26}, has been used previously to dissect the various functions of DER in larval

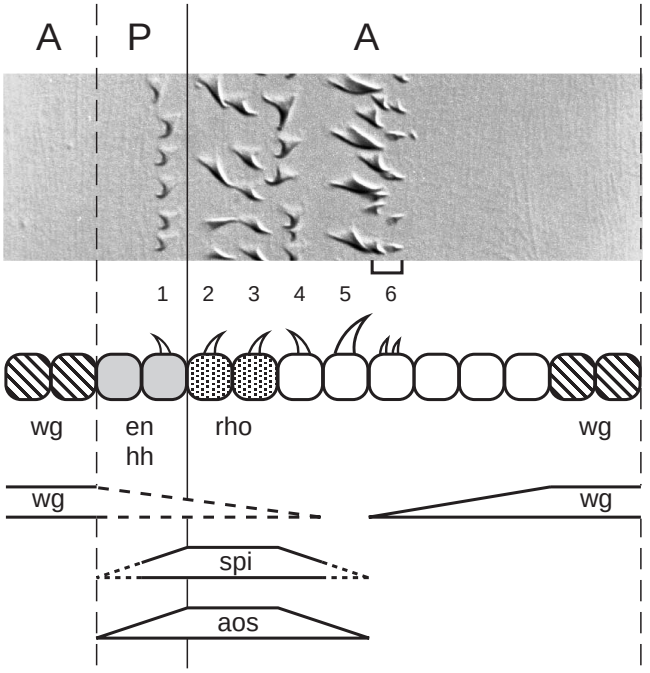

Fig. 1. Segmental sources of EGFR and Wg signalling in the embryo, and their projection onto the larval cuticle. Top, a slice of ventral cuticle across an abdominal segment of a young larva, showing a denticle belt with its six rows of denticles (numbered underneath; row 6 denticles form a strip rather than an ordered row at this stage; note anteriorward pointing of denticles in rows 1 and 4) and surrounding naked cuticle; A and P indicate anterior and posterior compartments; dashed lines mark parasegment borders, the solid line marks the segment border; anterior is to the left. Underneath, projection of denticles and naked cuticle onto an embryonic segment, about 12 cells wide, in which expression domains of *wg* (hatched), *en* (grey) and *rho* (stippled) are highlighted; *hh* is co-expressed with, and in response to, *en* (for references, see text). Bottom, reach of the extracellular signals Wg, Spi and Aos, based on their expression and phenotypes (if dashed, this signifies that the reach is inferred from mutant phenotypes of other genes; see text); note that 'reach' does not necessarily imply direct diffusion of signal. Our analysis predicts a symmetrical reach of Wg whose posterior effects are normally obscured by the antagonising activity of EGFR signaling activity (triggered by Spi, and maybe by Vn; see text).

cuticle formation (Clifford and Schüpbach, 1992; Raz and Shilo, 1992). These authors found that, if they disrupted DER function during a narrow window in mid embryogenesis, virtually no ventral denticles formed.

We reexamined DER function in the larval cuticle, using GAL4-mediated overexpression of a dominant-negative form of DER (DN-DER; E. Raz and B. Shilo, personal communication; Freeman, 1996). DN-DER lacks the cytoplasmic kinase domain and has been shown to interfere with endogenous DER function in eye development, presumably through dimerisation with endogenous DER (Freeman, 1996). We expressed DN-DER ubiquitously in the embryo with GAL4 linked to an *arm* promoter fragment (arm.G4; Sanson et al., 1996). This produced severely mutant larvae which did not hatch from their vitelline membranes. These larvae have strong head defects and also show very narrow denticle belts. Typically, the abdominal belts in these larvae consist of about four somewhat disordered rows of denticles, all of which are tapered and not so hooked (Fig. 2D,E, compare to Fig. 2A,B). Within these belts, there are usually one or two anterior 'rows' with a few largish denticles, followed by a strip of tiny denticles. The shape, size and position of these remaining denticles suggest that they correspond to row 5 and 6 denticles, respectively. These apparent identities of rows 5 and 6 can be confirmed by their substantial lateral stretching (in the wild type, row 5 and 6 stretch much more laterally than rows 1-4; Fig. 2A), and by their relative position to other cuticle markers, unaffected by DN-DER, such as the Keilin's organs (Campos-Ortega and Hartenstein, 1985) and the beard (see Sampedro et al., 1993). The second and third thoracic belts also lack most of their denticles, but the first belt, like the beard, seems unaffected by DN-DER (not shown).

We also reinvestigated the larval cuticles of flb^{IF26} mutants that had been shifted to the restrictive temperature at various stages during mid embryogenesis (see Materials and Methods). In our hands, even the most severely affected larvae (shifted to the restrictive temperature at 6 hours of development) still showed residual denticle belts in their abdomina, typically with 2-4 disordered rows of tapered denticles per belt (Fig. 2C; this mutant phenotype is very similar to that reported by Clifford and Schüpbach, 1992). These mutant belts are reminiscent of those caused by DN-DER overexpression (Fig. 2D), except that they are on the whole even narrower and that their denticles are smaller. They are virtually indistinguishable from denticle belts resulting from increased expression levels of DN-DER produced by a hyperactive version of GAL4 (arm.G4-VP16, not shown; G4-VP16 drives higher expression levels of UAS constructs than GAL4 alone; Sanson et al., 1996). The stretches of naked cuticle between the residual denticle belts in the flb^{IF26} mutants tend to be wider than in the wild type (Fig. 2C, compare to A), suggesting that the cells affected under these conditions adopt the naked cuticle fate instead of a denticle fate. Finally, neither the severe condition of increased DN-DER overexpression nor any of our temperature-shifting regimes with flb^{IF26} produced larval cuticles that lacked denticles altogether (cf. Raz and Shilo, 1992). These results indicate that DER is required for row 1-4 denticles in each abdominal belt and also for some of the thoracic denticles, and suggest that in the absence of DER function the prospective row 1-4 cells adopt the naked cuticle fate (see also below).

Next, we asked whether ectopic activation of DER throughout the embryonic epidermis would affect the ventral denticle belts. We used arm.G4 to ubiquitously express a secreted form of Spi (from UAS.sSpi; Schweitzer et al., 1995a; see also below). The resulting larval cuticles showed considerably widened ventral denticle belts with only narrow naked stretches between them (Fig. 2G). Within these belts, the normal rows 1-4 are still recognisable by the morphology and orientation of their denticles (Fig. 2H, arrowheads). However, posterior to these, we cannot see any row 5 or 6 denticles; these are replaced by a wide field of small denticles, apparently of the row 1-4 type (Fig. 2G,H). In addition, we see 1-2 extra rows of similar small denticles anteriorly to the normal row 1 (Fig. 2G,H). These extra anterior rows extend through the whole of the *en-lacZ* staining zone (not shown). The remaining short naked stretches (about as wide as the blue staining *en-lacZ* zones) therefore correspond approximately to the projected embryonic sources of Wg (see Fig. 1). We conclude

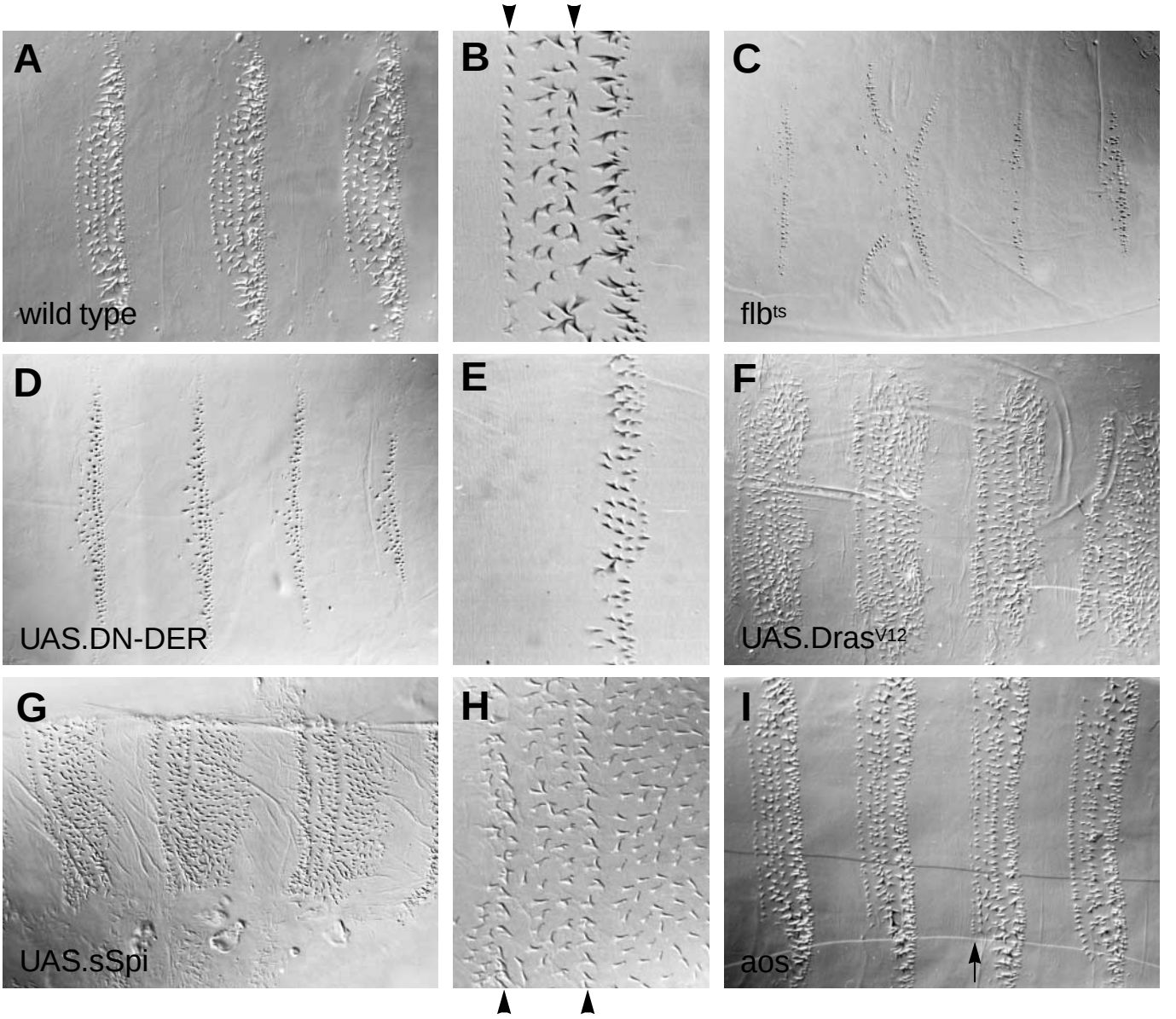

Fig. 2. Larval denticle belts in mutants with altered EGFR signalling activity. Views of ventral denticle belts in the abdomen; wild-type (A,B); *flb*^{ts} (C) or *aos* mutant (I; additional row of denticles indicated by arrow); expression of DN-DER (D,E), of Dras^{V12} (F) or of sSpi (G,H), in each case with arm.G4. (B,E,H) high magnification views of abdominal belts shown in the left-hand column, aligned with each other with respect to denticle rows (arrowheads pointing to rows 1 and 4; note also slight gaps posterior to these, consistently seen, especially under conditions of overactivation of EGFR signalling). Anterior to the left.

that ubiquitous activation of DER by secreted Spi can promote formation of row 1-4 denticles in most if not all regions of the ventral larval cuticle except those derived from the embryonic Wg sources. These extra row 1-4 denticles clearly arise at the expense of naked cuticle, again indicating a cell fate change.

We observed a similar phenotype of excessive denticles after ubiquitous expression of an activated form of Ras (UAS.Dras^{V12}; Lee et al., 1996). As above, additional small hooked denticles form anteriorly as well as posteriorly to the normal denticle rows 1-4 at the expense of row 5 and 6 denticles and of naked cuticle (Fig. 2F), although Dras^{V12} is slightly less potent than sSpi (Fig. 2G) in producing these extra denticles. This result is as expected, given that Dras^{V12} mimics constitutive activation of EGFR signalling in flies (Lee et al., 1996). Taken together, our results strongly indicate that activation of the Ras signalling pathway through DER is necessary and sufficient to specify ventral denticles of the row 1-4 type.

The segmental source of active DER ligand

Our results above predict that DER is activated in a localised fashion in each segment, implying a segmental source of active DER ligand. This putative source is likely to be within the prospective row 1-4 zone.

Both DER and *spi* are expressed fairly uniformly throughout the embryonic epidermis (Zak et al., 1990; Rutledge et al., 1992). However, Spi appears to be incapable of activating DER

unless it is processed into a secreted form (Freeman, 1994a; Schweitzer et al., 1995a), and there is genetic evidence that the membrane-spanning products of the *rho* and *Star* (*S*) genes may be mediating this processing event (Bier et al., 1990; Kolodkin et al., 1994; Schweitzer et al., 1995a; reviewed by Freeman, 1997). Indeed, both *rho* and *S* appear to be expressed during mid embryogenesis in segmental stripes abutting the segmental border (Bier et al., 1990; Freeman et al., 1992b; our own observations). Note also that the *rho* and *S* mutant phenotypes in the ventral cuticle are very similar if not identical to the *spi* mutant phenotype: they all lack row 1 denticles in their abdominal segments, and the polarity of their row 4 denticles is reversed (Mayer and Nüsslein-Volhard, 1988).

We decided to examine more closely the segmental expression pattern of *rho*, using two independent *rho* enhancer trap lines that show essentially identical β-Gal-staining patterns in the embryo (Bier et al., 1990; Freeman et al., 1992b). Below, we describe the β-Gal-staining pattern of the line X81 (Freeman et al., 1992b) whose *lacZ* insertion does not produce a mutant phenotype in the homozygous condition, unlike that in the line rho^{lac1} (Bier et al., 1990). We confirmed this pattern by examining rho^{lac1} embryos, and also by anti-Rho staining (Sturtevant et al., 1996).

In young X81 embryos (before the germ band is fully extended), there are initially two parallel longitudinal stripes of uniform β-Gal staining in the ventral region which extend throughout the embryonic trunk. This uniform staining resolves into distinct segmental stripes of β-Gal staining during germ-band shortening (7.5-9 hours of embryonic development). These stripes remain visible for about 7 hours until they fade away during stage 16 (for embryonic stages, see Campos-Ortega and Hartenstein, 1985). They become circumferential, and one cell wide in the dorsal half of the embryo. In the ventral half, they are also one cell wide in the thoracic segments, a bit wider in the first abdominal segment, and at least two cells wide in all other abdominal segments (Fig. 3A,B,E). They abut each segmental border (visible as a groove at this stage) at the posterior side, although transient traces of β-Gal staining can be seen in the row of cells just anteriorly to this border (Fig. 3C; this weak staining anteriorly to segment borders can no longer be seen after ~11 hours of development; see also Bier et al., 1990). Double-staining with anti-En confirms that the strong and sustained β-Gal staining in X81 embryos is in cells posteriorly abutting the cells expressing En (Fig. 3D,F).

The X81 stripes correspond roughly to the cells giving rise to denticle rows 2 and 3 (see Fig. 1), in other words, to the core of the zone in which we expect the DER signalling pathway to be active. Interestingly, *rho* mutation appears to affect cells bordering these X81 stripes, namely the cells giving rise to row 1 and 4 denticles (see above), suggesting non-autonomy of *rho*. Finally, the X81 stripes can first be discerned during the temporal window during which DER is required to specify denticle fates; however, neither this window nor the appearance of the X81 stripes have been sufficiently accurately defined to allow unambiguous temporal ordering of these events.

Expression and function of *argos*

aos is a known target gene of the EGFR signalling pathway; in the early embryonic ectoderm its ventral expression depends on EGFR signalling and is observed in, and adjacent to, cells expressing *rho* (Golembo et al., 1996). We asked whether *aos*, like *rho*, might also be expressed in a segmentally repeated pattern in the embryonic epidermis.

This is indeed the case: *aos* transcripts and anti-Aos staining can be seen in circumferential segmental stripes after completion of germ-band retraction (Fig. 3G-L). These stripes, about 2-3 cells wide, are in the most anterior parts of the segments, abutting and, in some regions, just crossing the segmental borders (Fig. 3H-J,L). They are strong in abdominal segments 2-8, slightly weaker in abdominal segment 1 (in this segment, strong staining is only visible in the ventralmost part of the embryo), but they are barely visible in the thoracic segments (Fig. 3G,H). These segmental stripes of *aos* expression are fairly similar to the *rho* stripes, though they may be a bit wider (e.g. compare Fig. 3K to E). Also, the difference in terms of expression levels between abdominal and thoracic segments are more pronounced in the case of *aos* (compare Fig. 3H to B). These segmental *aos* stripes, which we take to be an indication of EGFR signalling (see Freeman, 1997), support our notion that the EGFR signalling activity is maximal anteriorly within each segment, strong in abdominal segments, but weak in thoracic segments.

Aos is an inhibitor of DER function (Schweitzer et al., 1995b), so Aos reduction is expected to result in overactivation of EGFR signalling. To see whether *aos* functions in segmental patterning, we examined ventral cuticles of *aos* loss-of-function mutants. In these cuticles, we often find additional small denticles anteriorly to their abdominal denticle belts. In the most extreme cases, these show an entire additional row anteriorly to row 1 (Fig. 2I). Also, there is a subtle but consistent effect in the row 6 zone where there seem to be extra small denticles, giving the appearance of a slightly higher density of denticles compared to the wild type (Fig. 2I, compare to A). This phenotype, though a lot weaker, is reminiscent of the phenotypes produced by overexpression of sSpi (Fig. 2G) or of $Dras^{V12}$ (Fig. 2F). It provides evidence that, in the wild type, DER is inhibited by Aos anteriorly of the normal denticle belts, and probably in the row 6 zone within these belts. This implies that DER would be active in these positions in the absence of *aos*, suggesting that, in the wild type, there is activating ligand in these parts of the segments.

EGFR signalling antagonises Wg signalling

We have presented evidence that, in the absence of EGFR signalling, naked cuticle forms instead of row 1-4 denticles, whereas ectopic activation of EGFR signalling causes extra row 1-4 denticles to form at the expense of naked cuticle. Conversely, in the absence of Wg signalling, extra denticles form instead of naked cuticle (see Introduction, and below), whereas ectopic activation of Wg signalling causes naked cuticle to form instead of denticles (Noordermeer et al., 1992; Siegfried et al., 1992; Lawrence et al., 1996). Evidently, these two signalling pathways specify alternative cell fates, row 1-4 denticles or naked cuticle. Also, these signalling pathways can apparently override each other, and cells presumably choose between the two fates, depending on their relative stimulation. To gain further insight into how this choice is made, we asked what the cuticle phenotype would be in the absence of both pathways.

We first generated a ‘double-mutant’ condition, removing function of both signalling pathways after the stabilisation

phase. To do this, we expressed DN-DER with arm.G4 in wg^{ts} mutants which had been shifted to the restrictive temperature after 8 hours of development. From this cross, we obtained cuticles corresponding to the wg^{ts} mutants cuticles (Fig. 4A; see also Dougan and DiNardo, 1992), as well as a novel cuticle phenotype, which corresponds to the wg^{ts} mutants expressing DN-DER, the so-called double mutants (Fig. 4B; see also Materials and Methods). In the wg^{ts} mutant cuticles, we can still discern cores of normal denticle belts in which rows 1-5 are unaffected; in particular, rows 1 and 4 are readily identifiable since their normal anteriorward orientation is unchanged (Fig. 4C). However, these cuticles show an abundance of additional denticles both anteriorly and posteriorly to these belt cores, widening these considerably and leaving very little naked cuticle between them (Fig. 4A,C). Anteriorly, these extra denticles are apparently of the row 1-4 type, whereas posteriorly, they look like row 6 denticles. Apparently, late removal of Wg causes cells to change their fate from naked cuticle towards denticles. It does not affect the cells normally producing denticles; in particular, the anteriorward orientation of row 1 and 4 denticles is unchanged.

In contrast, the double-mutant cuticles show denticle belts with almost exclusively large denticles, and an apparently normal strip of row 6 denticles most posteriorly (Fig. 4B; for reasons not understood, loss of DER function also occasionally causes fusion of denticle belts, causing loss of row 6 denticles and of naked cuticle between belts; e.g. Fig. 2C, 4B; see also Mayer and Nüsslein-Volhard, 1988). Mostly based on their size, but also on their tapered shapes, we believe that the large denticles are of the row 5 type, although their hooks are somewhat reminiscent of the row 1-4 type (Fig. 4D; recall that

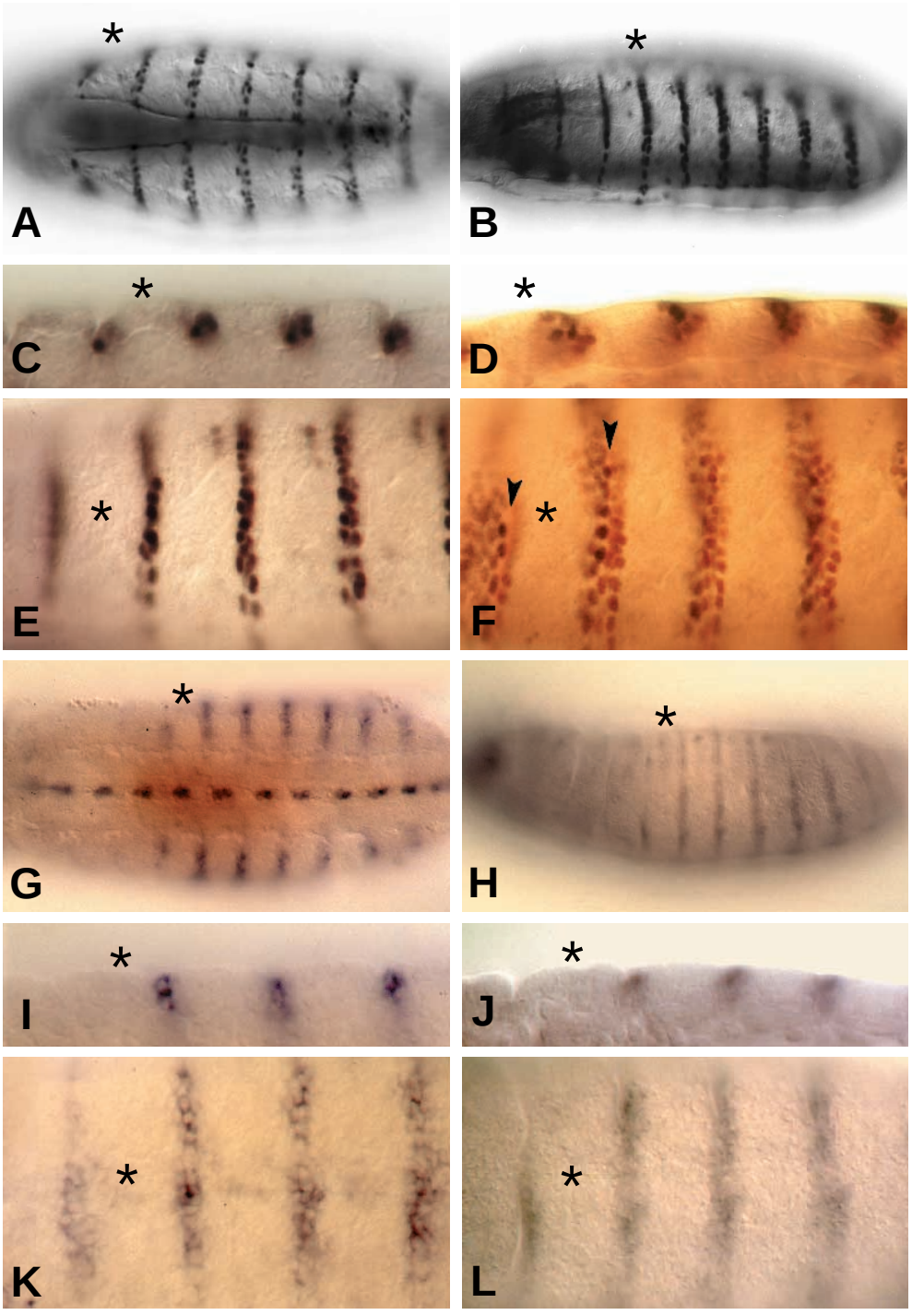

Fig. 3. Expression of *rho* and *aos* in the embryonic epidermis. (A-F) *rho* expression, visualised by anti-β-Gal staining of X81 embryos (A,B, ~16 hours; C, ~10 hours, to reveal the deep grooves corresponding to the segment borders; D-F, 13 hours; A, ventral view; B, E, F, side views; C, D, ventrolateral optical sections revealing segmental grooves; C-F, high magnification). Embryos in D and F are stained with anti-β-Gal (brown) and with anti-En (dark-grey), to show stripes of dark-grey nuclei abutting brown nuclei at segment borders (arrowheads in F pointing to the borders of T3/A1 and A1/A2 where nuclei of both stripes are in focus; note that the *rho* stripe is narrower in A1 than in A2). (G-L) *aos* expression in wild-type embryos (~13 hours; G, I, K, *aos* transcripts; H, J, L, anti-Aos staining; G, ventral view; H, K, L, side views; I, J, ventrolateral optical sections; I-L, high magnification). Note the anti-Aos staining mostly posteriorly to the segmental grooves (light lines in H and L), but in certain regions crossing these. Asterisks mark A1; anterior to the left.

there may be residual DER function under these conditions of DN-DER overexpression). Almost all denticles point posteriorly (Fig. 4D). This cuticle phenotype demonstrates that DER activity is responsible for the row 1-4 denticles that we see in the wg^{ts} mutants, including the anteriorward orientation of rows 1 and 4.

We wish to emphasise two points. Firstly, we had indications above that removal of DER function in the wild type seems to cause a cell fate change from small denticles to naked cuticle (see Fig. 2C,A). This is strongly confirmed by the double mutants which reveal a clear cell fate change caused by removal of DER function, in this case (i.e. in a *wg* mutant background) from small to large denticles (Fig. 4D,C). Secondly, the double mutants also reveal that DER function is strictly required for anterior orientation of row 1 and 4 denticles. This is consistent with the results of Mayer and Nüsslein-Volhard (1988) who first reported the requirement of *spi* group genes for this phenotype.

We found indications that there may be very little EGFR signalling in *wg* loss-of-function mutants: the segmental stripes of *rho* expression are considerably weaker, and those of *aos* expression completely disappear in these mutants, although both genes are still expressed apparently normally in wg^{ts} mutants after late removal of *wg* function (not shown). Thus, the segmental activation of EGFR signalling seems to be an indirect consequence of an early function of *wg*. To confirm this, we looked at the larval cuticles in a strain containing both flb^{ts} and a *wg* loss-of-function allele (using the same temperature-shift regimes as described above for flb^{ts}), and we also overexpressed DN-DER in these *wg* null mutants. In both cases, the denticle lawns were indistinguishable from denticle lawns in *wg* null mutants (Fig. 4E), showing almost exclusively large denticles of the row 5 type, with no interspersed naked cuticle (not shown). The absence of any row 1-4 denticles in either of these lawns demonstrates that there is very little or no EGFR signalling activity in *wg* null mutants. Note also that the *wg* null mutants are distinct from the wg^{ts} mutants in that they lack naked cuticle altogether, and they also completely lack row 6 denticles (compare Fig. 4A and E). The remnants of both these features in the wg^{ts} mutants can probably be explained by low Wg activity perduring from the early embryonic stages. It thus appears that row 6 denticles require *wg* (though not DER function; see Fig. 2C-E), but the lack of row 6 denticles in *wg* null mutants may be a secondary consequence of early loss of *wg* function.

The lack of EGFR signalling activity in *wg* mutants raised the question whether EGFR signalling would function to specify row 1-4 denticles in the complete absence of Wg signalling. To test this, we resupplied EGFR signalling activity in *wg* null mutants by expressing sSpi throughout the embryo. Among the progeny of this cross, we found two types of cuticles with denticle lawns, the *wg* mutants with the large denticles (Fig. 4E) and a novel denticle lawn type (Fig. 4F) cor-

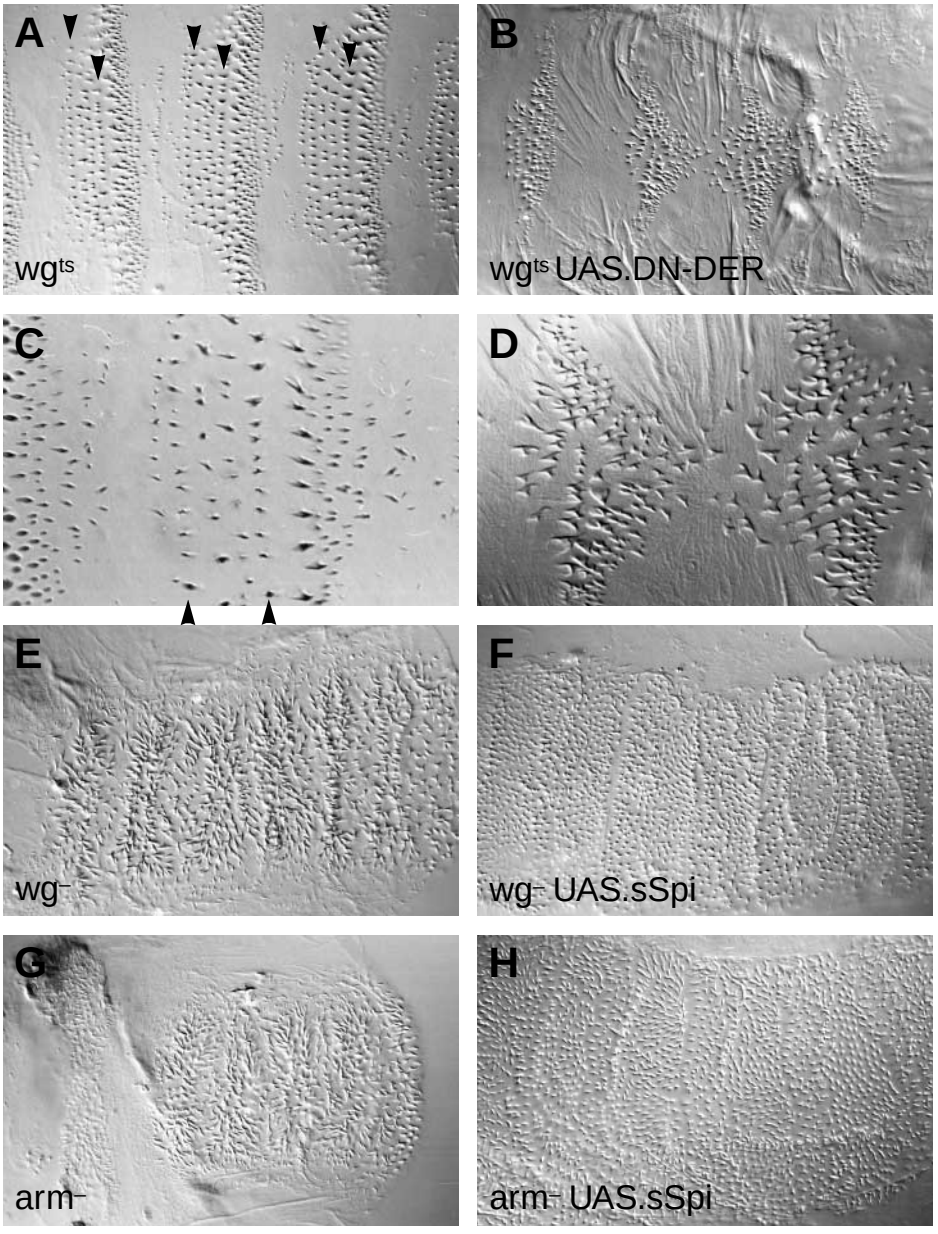

Fig. 4. Segmental effects of EGFR and of Wg signalling in the larval cuticle. Views of ventral denticle belts in the abdomen; (A-D)wg^{ts} mutants with (B,D) or without (A,C) expression of DN-DER; (E,F) *wg* null mutants with (F) or without (E) expression of sSpi; (G,H) *arm* mutant with (H) or without (G) expression of sSpi (in each case, with arm.G4). (C,D) High magnification views of abdominal belts shown above; arrowheads in A and C pointing to rows 1 and 4. Anterior to the left.

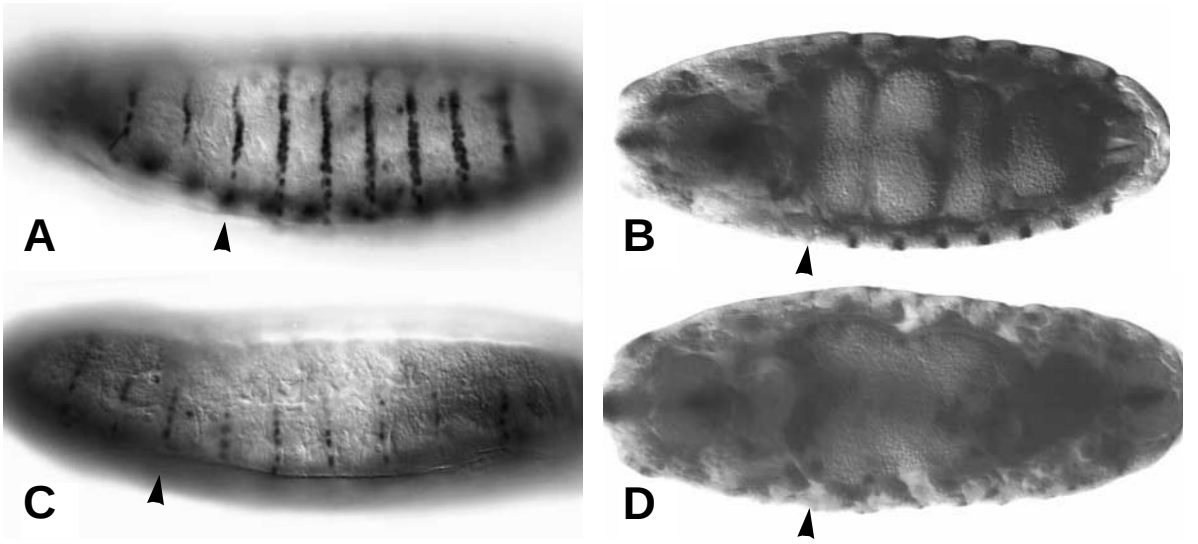

Fig. 5. Expression of *rho* and *aos* in a bithorax mutant. Wild-type (A,B) or *Ubx abd-A* mutant embryos (C,D) carrying X81, stained with anti-β-Gal to reveal *rho* expression (A,C) or with anti-Aos (B,D); (A,C) side views of early stage 14, (B,D) ventral views of stage 16. Arrowheads indicate T3/A1 borders; anterior to the left.

responding to the sSpi-expressing *wg* mutants (see Materials and Methods). These latter lawns consist of denticles that are clearly smaller than the ones in *wg* mutants (Fig. 4F, compare to E): they probably correspond to denticles of the row 1-4 type. Thus, EGFR signalling can function in the absence of Wg signalling to specify small denticles.

We wondered whether EGFR signalling would also be capable of specifying row 1-4 denticles in the absence of *arm* (*arm* encodes the ultimate cytoplasmic target molecule of the Wg signal transduction cascade; Peifer et al., 1994; reviewed by Perrimon, 1994). We thus expressed sSpi in *arm* mutant embryos that are defective in Wg signalling (see Materials and Methods). The cuticles from these *arm* mutants show a denticle lawn composed of large denticles (Fig. 4G; see also Peifer et al., 1994), much like *wg* null mutants (Fig. 4E). Moreover, these *arm* mutants, like the *wg* mutants in which sSpi is expressed ubiquitously, show exclusively small denticles, probably of the row 1-4 type (Fig. 4H). This demonstrates that EGFR signalling does not require *arm* function to specify these small denticles.

Segmental EGFR signalling activity depends on bithorax gene function

Segmental expression of *rho* and *aos* is modulated along the body axis: both genes are more strongly expressed in abdominal segments than in thoracic ones (see Fig. 3). We asked whether this anteroposterior modulation is controlled by homeotic genes, in particular by *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*) both of which are required for the formation of abdominal-type denticle belts (Lewis, 1978; Sánchez-Herrero et al., 1985).

We tested this by examining X81 staining in mutants lacking both *abd-A* and *Ubx*. We found that the X81 segmental stripes in these homozygous mutant embryos were very weak compared to the wild type, and that they were all one cell wide, just like the normal stripes in the thoracic segments (Fig. 5C, compare to A). The same was true for the anti-Rho staining patterns, which showed no difference between thoracic and abdominal segments in these mutants (not shown). Similarly, when we stained these embryos with anti-Aos, we observed barely any segmental expression in the homozygous mutants (Fig. 5D, compare to B).

This demonstrates that the two bithorax genes *Ubx* and *abd-A* are responsible for activating high levels of segmental *rho* and *aos* expression in abdominal segments and that they mediate *rho* expression in wide stripes in these segments.

DISCUSSION

Our experiments show that EGFR signalling is critical for cell fate specification in the ventral cuticle of the *Drosophila* larva: this signalling pathway is required and apparently sufficient to specify row 1-4 denticles in the abdominal belts. To specify these denticles, EGFR signalling antagonises Wg signalling in cells of the prospective row 1-4 zone; in the absence of EGFR signalling, these cells adopt the naked cuticle fate specified by *wg*. Our analysis also indicates that the EGFR signalling pathway is activated from a segmental source of EGFR ligand anteriorly within each segment, in a process depending on bithorax gene function.

Segmental activation of EGFR signalling

After the stabilisation phase during mid embryogenesis, *rho* begins to be expressed in stripes, one or two cell wide, most anteriorly within each segment, strongly in abdominal and weakly in thoracic segments. It is likely that EGFR signalling is activated in and adjacent to these stripes since *rho* expression generally coincides with EGFR activity (Bier et al., 1990; Ruohola-Baker et al., 1993; Sturtevant et al., 1993; Freeman, 1994a; Golembo et al., 1996). In support of this, *aos*, whose expression appears to be triggered by EGFR pathway activity in multiple developmental contexts (Golembo et al., 1996; Freeman, 1997), is expressed in segmental stripes similar to those of *rho* expression.

rho is thought to mediate cleavage of membrane-bound Spi (Schweitzer et al., 1995a). We have observed *rho* expression in embryonic cells of the prospective denticle rows 2 and 3. Thus, we propose that these cells, by virtue of their Rho activity, secrete Spi, which spreads in both directions away from its source in a graded fashion (Fig. 1). From the mutant phenotypes, we infer that the range of sSpi includes the cells of prospective denticle rows 1 and 4. However, the *aos* mutant phenotype which affects cells outside these rows strongly suggests that sSpi can reach cells beyond the zone of rows 1-4. Clearly, *aos* function itself reaches and affects these remote cells (Fig. 1). This remote inhibition by Aos resembles its role in other tissues. We believe that cells closer to the EGFR signalling source are refractory to Aos inhibition because they are already committed to their differentiation by the time local Aos reaches inhibitory levels (Freeman, 1997).

Why is the segment polarity phenotype caused by lack of DER function much stronger than that caused by *spi* and *rho*? We do not think that this is due to maternal products of these genes since there is no genetically detectable maternal contribution of *rho* (Mayer and Nüsslein-Volhard, 1988), and since removing *spi* function in the germ line does not enhance the segment polarity phenotype of zygotically mutant *spi* embryos (our unpublished observations). However, a possible explanation is provided by *vn*, a recently discovered putative DER ligand: the *vn*; *spi* double-mutant phenotype is much more severe than either the *vn* or the *spi* mutant phenotype (Schnepp et al., 1996), and looks very similar to the DER loss-of-function phenotype described here, indicating that Vn may contribute to the segmental activation of DER. *vn* encodes a secreted product which is not expected to require processing (e.g. by *rho*) in order to be diffusible (Schnepp et al., 1996). It is also possible that there is an as yet undiscovered DER ligand with a segment polarity function, or that DER has some ligand-independent activity.

Finally, we presume that the segmental source of sSpi is positioned by segment polarity genes which control *rho* expression. Although we have not investigated this in any detail, we know that *rho* and *aos* expression are virtually eliminated in *wg* null mutants which, as we have shown, exhibit little if any EGFR signalling activity. We also found that ubiquitous expression of *en* or *hh* causes *rho* expression to be widened substantially (not shown), providing an explanation as to why ubiquitous En leads to a lawn of small denticles (Lawrence et al., 1996). Thus, the segmental sources of EGFR activity are probably an indirect consequence of the interplay between the primary signals Wg and Hh during the early stabilisation phase (see Introduction). In the simplest scenario, the Hh signal spreading posteriorly from the *en*-expressing cells positions these sources of EGFR activity (see Fig. 1). EGFR activity therefore seems to be a secondary signalling system set up by Hh, and might explain some of the apparent morphogenetic properties of Hh (Heemskerk and DiNardo, 1994). It might correspond to the unknown signal that was proposed by Lawrence et al. (1996) to emanate from the side opposite to Wg (with respect to the segmental border) and to pattern the most anterior cells in the segment.

Antagonism between EGFR and Wg signalling

EGFR signalling apparently has two effects in the segmentation of the ventral epidermis: not only does it specify the denticles in rows 1-4, it also antagonises the activity of Wg signalling. The main piece of evidence for this is that, in the absence of EGFR signalling, the cells in the prospective rows 1-4 adopt a naked cuticle fate specified by Wg. Also, ubiquitous sSpi expression overrides the activity of Wg in specifying naked cuticle in most segmental regions. Significantly, sSpi cannot override Wg activity in the segmental zones corresponding to the Wg sources, strongly suggesting that there is competition between the two signals regarding subsequent cell type specification.

Our analysis predicts that this competition between the two signalling pathways normally occurs in P cells and in the most anterior A cells in abdominal segments 2-8 (Fig. 1) whose cells are exposed simultaneously to high levels of Wg and of sSpi (and probably Vn). As a result, only the most anterior P cells (close to the Wg source) adopt the naked fate, whereas the more posterior P cells and the anterior A cells (close to the sSpi source) adopt a denticle fate of the row 1-4 type. The denticle belts thus become wide. The first abdominal segment (which lacks the first row of denticles; Lohs-Schardin et al., 1979) shows less *rho* expression, and the level of sSpi is thus presumed to be lower. This not only explains the low *aos* expression levels in this segment, but also probably why all P cells in the A1 denticle belt adopt the naked cuticle fate. Finally, in the second and third thoracic segments, there is very little *rho* expression, and thus probably very little EGFR signalling (as witnessed by the very low levels of *aos* expression in these segments). This residual level of EGFR signalling is apparently insufficient to antagonise Wg in P cells and in the most anterior A cells, all of which adopt the naked cuticle fate. Thus, narrow denticle belts form with an anterior edge some cell diameters away from the P cells (see Sampedro et al., 1993). Our results cannot explain the shape of the first thoracic denticle belt.

An interesting implication of our findings is that the reach of the Wg signal is symmetrical, potentially determining a naked cuticle fate across several cells on both sides of the Wg source (Fig. 1). This follows mostly from the DER loss-of-function mutants in which the zones of naked cuticle are centered on the projected Wg sources (Fig. 1). This phenotype, and its comparison to that of the *wg*/DER loss-of-function double mutants, reveal that the prospective denticle row 1-4 cells can adopt the naked cuticle fate in a *wg*-dependent manner. Our evidence for a symmetrical reach of Wg in the segment seems to be at variance with earlier observations that Wg may spread from its source predominantly towards anterior (Gonzales et al., 1991), and with earlier proposals that Wg may form an asymmetrical gradient from its source, not spreading freely within, and not at all beyond, the P compartment (Sampedro et al., 1993; Lawrence et al., 1996).

Integration of simultaneous EGFR and Wg signalling

How do P and anterior A cells integrate EGFR and Wg signalling? We considered the possibility that this integration is achieved prior to the nucleus of the receiving cells, especially in the light of the finding that the mammalian homolog of Arm, β-catenin, associates with the EGF receptor and is phosphorylated by it upon its stimulation by EGF (Hoschuetzky et al., 1994). However, this possibility can be discarded since sSpi is capable of specifying row 1-4 denticles in the absence of *arm* function. Also, $Dras^{V12}$ mimics the activity of sSpi, strongly

suggesting that the specification of these denticle fates is mediated by DER triggering the Ras signalling pathway.

It is therefore very likely that the integration of the two signalling pathway is achieved within the nucleus, probably at the level of *cis*-control regions of Wg and EGFR target genes. With the possible exception of *aos*, none of the target genes for these signals that function in the segmentation of the ventral cuticle have been identified as yet. But we imagine that, in a simple scenario, there may be two selector genes, gene A specifying naked cuticle and gene B specifying row 1-4 denticles; gene A would be activated by Wg signalling, gene B by EGFR signalling. Candidates for transcription factors activating these genes in response to these signals are Pangolin/dTCF, a transcriptional activator of the lymphocyte enhancer-binding factor (LEF) type activated by Wg signalling (Brunner et al., 1997; Riese et al., 1997; van de Wetering et al., 1997), and Pointed (Pnt), a transcriptional activator containing an Ets domain activated by EGFR signalling (Brunner et al., 1994; O'Neill et al., 1994): *dTCF* null mutants show ventral cuticles similar to wg^{ts} cuticles (Fig. 4A,C, and not shown; van de Wetering et al., 1997), whereas *pnt* null mutants show ventral cuticles like *spi* null mutants (Mayer and Nüsslein-Volhard, 1988). Note, however, that Pnt cannot be the only effector of EGFR signalling in this case as *pnt* mutants still show denticles of the row 1-4 type (Mayer and Nüsslein-Volhard, 1988) and are thus less severe than DER loss-of-function mutants.

Our analysis indicates that cells stimulated by both signals simultaneously assume either one or the other fate (rather than intermediates), presumably depending on priority and/or on relative intensity of stimulation. To explain these phenotypic outcomes, we have to invoke repression. In the scenario outlined above, gene A may be repressed by EGFR, and gene B by Wg. Alternatively, gene A and B repress each other. The latter possibility is more likely since it explains more readily the apparent lack of intermediate fates. Also, both Pnt and dTCF act as transcriptional activators upon signal activation (Brunner et al., 1994; O'Neill et al., 1994; Riese et al., 1997; van de Wetering et al., 1997), but neither signalling pathway is known to activate transcriptional repressors. Most likely, however, these phenotypic outcomes are determined not just by the intial signals and their immediate consequences, but also by subsequent regulatory steps.

Homeotic genes setting up sources of EGFR signalling

According to our evidence, the main difference between thoracic and abdominal denticle belts is due to strong sources of EGFR signalling that arise as a consequence of bithorax gene function. To produce high levels of secreted Spi in abdominal segments, Ubx and Abd-A proteins may directly act through *cis*-regulatory regions of *rho* to stimulate its expression, or through intermediary genes to achieve this. Similarly, in the embryonic midgut, these two homeotic genes stimulate transcription of *decapentaplegic* and *wg* whose secreted products subsequently mediate regionalisation of the endoderm (Bienz, 1994). Clearly, one way by which homeotic genes create morphological differences along the body axis is by generating local sources of secreted signals.

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