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Role of the *gooseberry* gene in *Drosophila* embryos: maintenance of *wingless* expression by a *wingless*–*gooseberry* autoregulatory loop

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During *Drosophila* embryogenesis, segment polarity genes, such as *engrailed* (*en*), *wingless* (*wg*) and *gooseberry* (*gsb*) show complex interactions that provide positional information along the antero-posterior axis within each segment. Little is known about the specific role of each of these genes in this pattern determining process. Here we demonstrate that the main function of *gsb*, which encodes a transcription factor containing a paired-domain and a *prd*-type homeodomain, is the maintenance of *wg* expression by a *wg*–*gsb* autoregulatory loop after 6 h of development. The function of *wg*, the homologue of the murine *Wnt-1* gene, is to specify the denticle pattern by repressing a default state of ubiquitous denticle formation in the ventral epidermis. This repression of denticles by the *wg* signal is different from the wingless signalling pathways that activate *gsb* or *en*. Mutual activations involving *gsb*, *wg* and *en* show temporal asymmetries that lead to their different mutant phenotypes. A general model is proposed for the generation of morphogenetic fields by self-propagating autoregulatory loops.

Key words: autoregulatory loop/denticle formation/*gooseberry*/positional information/*wingless*

Introduction

Morphogenesis and pattern formation depend on the establishment of positional information in the embryo (Wolpert, 1971). In *Drosophila*, position along the antero-posterior axis is specified within each segment by the products of the segment polarity genes (for reviews, see Hooper and Scott, 1992; Ingham and Martínez-Arias, 1992; Nusse and Varmus, 1992; Peifer and Bejsovec, 1992). Their role in positional specification is reflected by the segmentally repeated aberrations in the larval cuticle of segment polarity mutants (Nüsslein-Volhard and Wieschaus, 1980). For example, the parasegmental grooves that initially divide the embryo into metameric units, the parasegments (Martínez-Arias and Lawrence, 1985), fail to form in *wingless*$^-$ (*wg*$^-$) embryos (Perrimon and Mahowald, 1987) while in *gooseberry*$^-$ (*gsb*$^-$) embryos the naked posterior portion of each segment is replaced by the denticle pattern of its anterior part in reversed polarity (Nüsslein-Volhard and Wieschaus, 1980). In addition, the striped expression at single segment periodicity of many segment polarity genes, such as *engrailed* (*en*), *wg*, *gsb* and *hedgehog* (*hh*), is consistent with the idea that segment polarity genes provide segmentally repeated positional information. Other segment polarity genes like *armadillo* (*arm*) are expressed ubiquitously in the embryo and specify cell fates by their interaction with localized segment polarity gene products as, for example, wg (Riggleman *et al.*, 1989, 1990; Peifer *et al.*, 1991).

The striped expression of segment polarity genes originates from their initial activation by combinations of pair-rule proteins during late blastoderm at ~3 h after egg laying (AEL) (for a review, see Ingham, 1988). Subsequent to cellularization, during germ band extension, the pair-rule proteins decay and, from ~4 h AEL, the established positional information is maintained by the segment polarity genes themselves as they interact with each other by complex mechanisms. For example, *wg* and *en* are expressed in neighbouring stripes of cells demarcating the parasegmental boundaries. However, in the absence of a functional product of one of these two genes, expression of the other decays prematurely (DiNardo *et al.*, 1988; Martínez-Arias *et al.*, 1988; Bejsovec and Martínez-Arias, 1991; Heemskerk *et al.*, 1991). The mutual activation of segment polarity genes implied by these observations is thus a mechanism that ensures their continued expression which is a prerequisite for their function in the specification of cell fates.

The mutual dependence of the segment polarity genes renders an analysis of the regulation and function of individual segment polarity genes difficult. For example, as a consequence of all examined segment polarity mutations the expression of *wg* and *gsb* decays, while conversely in *wg*$^-$ embryos it is the other segment polarity genes whose expression is disrupted prematurely (DiNardo *et al.*, 1988; Martínez-Arias *et al.*, 1988; Hidalgo and Ingham, 1990; Bejsovec and Martínez-Arias, 1991; Heemskerk *et al.*, 1991; Hidalgo, 1991; Peifer *et al.*, 1991; Lee *et al.*, 1992; Mohler and Vani, 1992; Tabata *et al.*, 1992; Ingham and Hidalgo, 1993; Li *et al.*, 1993; our unpublished results). The question then arises which segment polarity genes interact directly with each other and whether they may be ordered into an epistatic sequence. Furthermore, while their mutual activation explains how segment polarity genes maintain positional information, it is not obvious why mutations in these genes produce different phenotypes. For it would be expected that inactivation of any one segment polarity gene results in the inactivation of its dependent partners. For example, although *gsb*, *wg* and *en* interact with each other, formation of the parasegmental boundary depends on both *en* and *wg*, but remains unaffected in *gsb*$^-$ embryos (Perrimon and Mahowald, 1987). In contrast, *en*$^-$ embryos do not display a denticle lawn phenotype like *wg*$^-$ or *gsb*$^-$ embryos (Nüsslein-Volhard and Wieschaus, 1980).

To answer some of these questions, we analysed the function of *gsb* which encodes a transcription factor containing a paired-domain and a *prd*-type homeodomain (Bopp *et al.*, 1986). We conclude that the main function of *gsb* is to maintain the expression of *wg*, the homologue of the murine *Wnt-1* gene, by the establishment at 6 h AEL of a *wg*–*gsb* autoregulatory loop whereas *wg* rather than *gsb* represses denticle formation. The wg signal thus specifies the denticle pattern by a pathway different from the one that

activates *gsb* or *en*. In addition, we show that a temporal asymmetry in the regulatory interactions among *gsb*, *wg* and *en* is the reason why these genes exhibit different mutant phenotypes. The discovery of the *wg*−*gsb* autoregulatory loop suggests a general model for the establishment of positional information over large distances by intercellular self-propagation rather than diffusion.

Results

Maintenance of gsb expression by the wg signal

Although the expression of *gsb* is altered in all segment polarity mutants examined, several lines of evidence suggest that the maintenance of *gsb* expression depends on the *wg* product. First, loss of *wg* function after 6 h AEL results in a denticle lawn phenotype (Bejsovec and Martínez-Arias, 1991) very similar to that of gsb^- embryos. Second, the expression pattern of *gsb* (Gutjahr *et al.*, 1993) evolves in parallel to that of *wg* during embryogenesis (van den Heuvel *et al.*, 1989; González *et al.*, 1991). Third, gsb protein begins to disappear after 4 h AEL in wg^- embryos (Li *et al.*, 1993). In other segment polarity mutants, the change in *gsb* expression parallels that of the altered *wg* expression (Hidalgo, 1991; our unpublished results). Finally, we have shown that activation of the *gsb cis*-regulatory region responsible for the maintenance of *gsb* expression completely depends on *wg* (Li *et al.*, 1993).

Since the wg signal is required to maintain *gsb* expression after 4 h AEL, we expect *gsb* to be expressed in a region that also expresses *wg*. Figure 1 confirms that gsb protein is indeed restricted to *wg*-expressing cells and their immediate neighbours. As *wg* encodes a secreted extracellular protein (van den Heuvel *et al.*, 1989; González *et al.*, 1991), the wg signal activates *gsb* in a paracrine and autocrine fashion. In contrast, *en* is activated only by a paracrine wg signal (for a review, see Nusse and Varmus, 1992).

These results further predict a continuous requirement for wg to maintain *gsb* expression. To test this prediction, *gsb* expression was analysed in temperature-sensitive *wg* embryos shifted to the nonpermissive temperature at various stages of development. Indeed, as evident from Figure 2A and B, *gsb* expression is maintained only by the continuous presence of the wg signal at least until 8.5 h AEL (Figure 7). Since wg also activates *en* (DiNardo *et al.*, 1988; Martínez-Arias *et al.*, 1988) and *gsb* expression decays in *en* mutants (Figure 4O), *wg* might activate *gsb* via *en*. However, this possibility is ruled out because *en* activation depends on *wg* only between 4 and 5 h AEL (Bejsovec and Martínez-Arias, 1991; Heemskerk *et al.*, 1991). Rather, the decay of *gsb* expression in *en* mutants is explained by the dependence of *wg* on *en* after 5 h AEL (Bejsovec and Martínez-Arias, 1991; also compare with Figure 7).

Maintenance of wg expression by gsb protein

Maintenance of *wg* expression also requires *gsb* (Hidalgo and Ingham, 1990). In contrast to *gsb* expression that depends on *wg* after 4 h AEL, wg protein begins to decay only at ~6 h AEL in gsb^- embryos (not shown; Figure 7). The segment polarity genes *en*, *hh*, *arm* and *fused* (*fu*) are required for the maintenance of *wg* expression as well (Martínez-Arias *et al.*, 1988; Hidalgo and Ingham, 1990; Limbourg-Bouchon *et al.*, 1991; Peifer *et al.*, 1991).

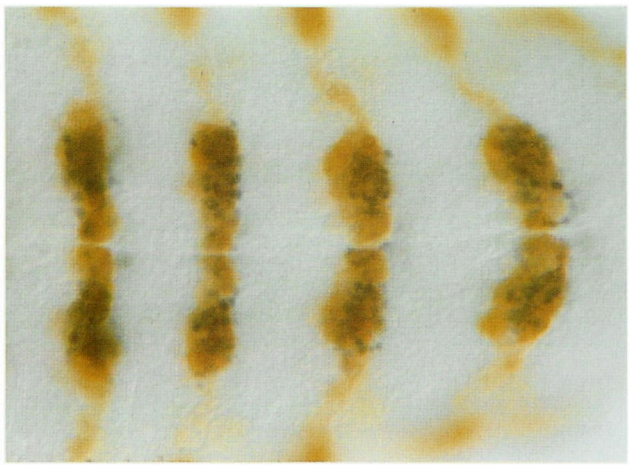

Fig. 1. Coexpression of *gsb* and *wg* in wild-type embryos. Embryos carrying a *wg*−*lacZ* transgene, which is expressed in the same manner as *wg* (Ingham *et al.*, 1991; Siegfried *et al.*, 1992; Couso *et al.*, 1993), have been stained for lacZ (brown) and gsb (dark blue), using an anti-lacZ monoclonal antibody and a rabbit anti-gsb antiserum. The ventral region between T3 and A3 (anterior to the left) or an early stage 12 embryo (7.5 h AEL) is shown. Note that *gsb* (dark blue) and *wg* (brown) are expressed in the same cells and that *gsb* is also detectable in cells adjacent to wg-expressing cells.

However, they cannot be direct activators of *wg* because they are not expressed in *wg*-expressing cells or do not encode transcription factors. In contrast, *gsb* encodes a transcription factor that is coexpressed with *wg* (Figure 1) and hence might directly activate *wg* after 6 h AEL. Indeed, activation of *gsb* by a 20 min heat shock, applied between 3 h 40 min and 6 h 20 min AEL to embryos carrying a heat-inducible *gsb* transgene (*Hsgsb*), induced an ectopic wg stripe anterior to the normal wg stripe in each segment (Figure 2C and D). This ectopic wg stripe is activated only after the heat-shocked embryos have developed for ~6 h AEL, indicating that gsb protein is not sufficient for the ectopic activation or to overcome repression of *wg* before this time. The ectopic *wg* expression in turn activates a similar ectopic gsb stripe in each segment (Figures 2E and F). It could be argued that the ectopic gsb stripes in *Hsgsb* embryos are activated directly by the heat-induced gsb protein without the cooperation of *wg*. However, this possibility is ruled out by the observation that the ectopic gsb stripes fail to appear after heat shock in *Hsgsb*; wg^- embryos (not shown). Therefore, after 6 h AEL, maintenance of *wg* expression depends on *gsb*, and *gsb* and *wg* expression become mutually dependent. As a result, an autoregulatory loop forms (Figure 7) that ensures the coexpression and maintenance of *wg* and *gsb* (Figure 1).

Since *en* expression depends on *wg* only between 4 and 5 h AEL (Bejsovec and Martínez-Arias, 1991; Heemskerk *et al.*, 1991), when *wg* activation is independent of *gsb*, it follows that *gsb* is not required for *en* activation (Figure 7). Indeed, no change of *en* expression is observed in gsb^- embryos throughout embryonic development (Figure 3; Hidalgo, 1991). Similarly, no ectopic *en* expression is detected after ectopic activation of *Hsgsb* after 3 h 40 min AEL (not shown). After 5 h AEL, *en* is autoregulated (Heemskerk *et al.*, 1991; Figure 7). Therefore, as summarized in Figure 7, each activation event in the mutual

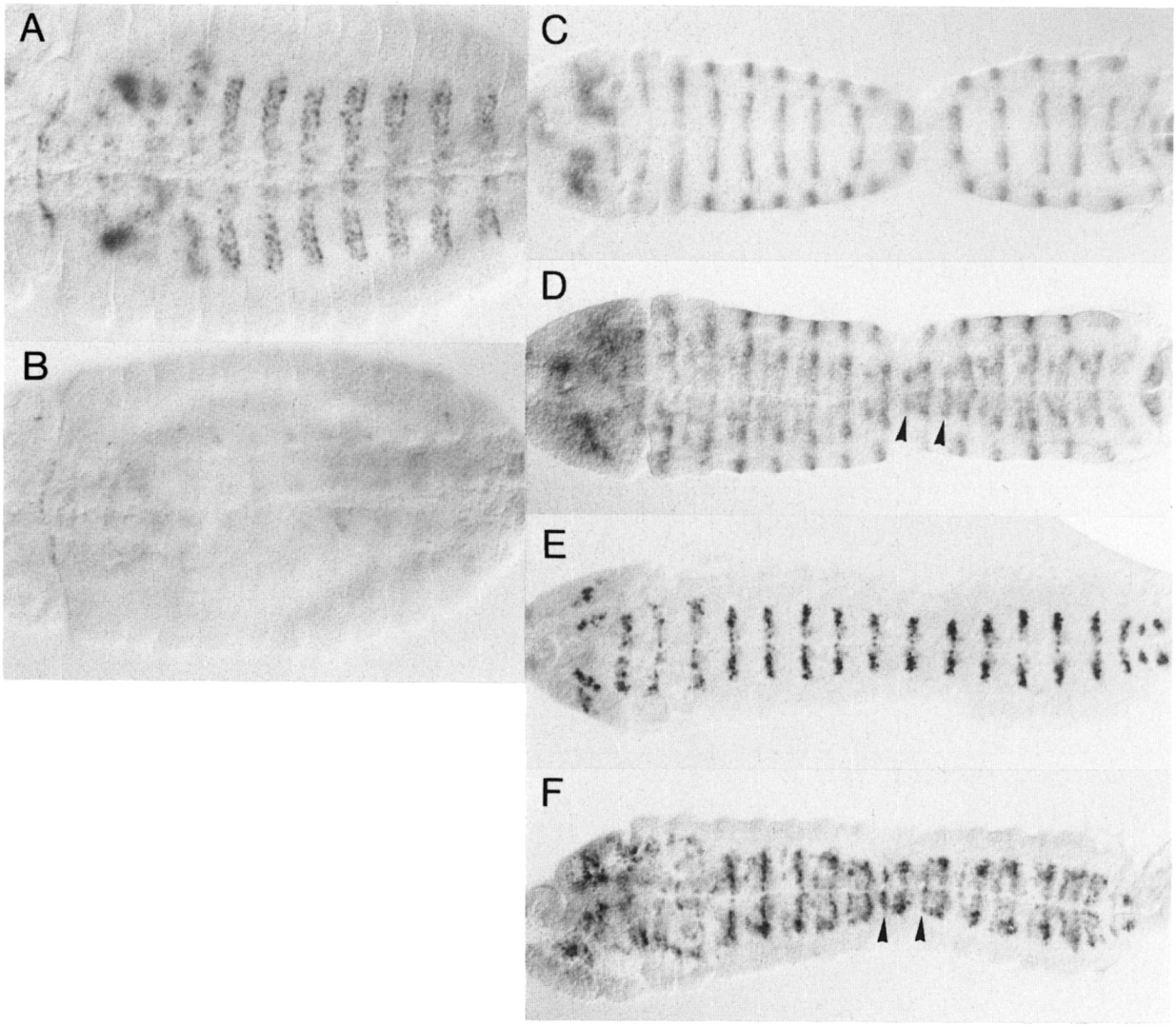

Fig. 2. Mutual activation of *gsb* and *wg*. (**A** and **B**) Dependence of *gsb* expression on *wg* until 8.5 h AEL. Ventral views (anterior to the left) of a wild-type (A) or homozygous wg^{IL114} (B) embryo [stage 13 (Campos-Ortega and Hartenstein, 1985); ~10 h AEL] stained with anti-gsb antiserum are shown. The temperature-sensitive wg^{IL114} embryo, shifted to the non-permissive temperature of 28°C at 8.5 h AEL, shows no gsb protein in the trunk while the wild-type embryo expresses *gsb* in epidermal stripes in the posterior portion of each segment. The remaining *gsb* expression visible in wg^{IL114} embryos is not epidermal but restricted to the CNS. (**C**–**F**) Ectopic expression of *wg* and *gsb* in heat-shocked *Hsgsb* embryos. Wild-type (C and E) or *Hsgsb* (D and F) embryos were stained with anti-wg (C and D) or anti-gsb (E and F) antiserum. *Hsgsb* embryos, between 3.5 and 4.5 h AEL, were heat-shocked for 20 min and allowed to recover for 3–4 h at 25°C. Note the ectopic wg and gsb stripes (arrowheads in D and F) anterior to the wild-type stripes and the close correspondence between the wg and gsb protein patterns in wild-type and *Hsgsb* embryos. Late stage 11 embryos (~7 h AEL) have been unfolded to show the entire set of stripes and are oriented with their anterior to the left.

regulation of *gsb* and *wg*, and of *wg* and *en* exhibits a different temporal requirement which results in an asymmetric flow of information from *en* via *wg* to *gsb*, but not in the opposite direction from *gsb* to *en*.

Correlation of wild-type and mutant gsb expression with repressed denticle formation

Comparing the cuticular phenotypes of wild-type or segmentation mutant embryos with the corresponding *gsb* patterns, we consistently find that *gsb*-expressing cells generate the naked regions of the ventral cuticle in each segment. All classes of segmentation mutant phenotypes show this correlation with respect to several types of altered *gsb* expression patterns (Figure 4). First, mutations abolishing *gsb* expression, such as gsb^-, wg^- and *even-skipped* $(eve)^{1.27}$, produce a lawn of denticles in the ventral cuticle (Figure 4C and D). In contrast, mutants displaying much broader gsb stripes in the ventral epidermis than wild-type embryos, like nkd^- embryos, have largely naked cuticles (Figure 4L and M). Third, mutations, that generate a pairing of gsb stripes—i.e. a reduced distance between odd-numbered and their anterior even-numbered stripes—show pair-rule phenotypes in which the extent of denticle repression in one set of denticles (e.g. in A2, A4, A6, A8 of *eve* embryos) depends on the degree of pairing (compare, for example, $eve^{3.77}$ with eve^{IIR} in Figure 4E–H). Fourth, mutations eliminating every other gsb stripe, such as the odd-numbered stripes in $paired^-$ (prd^-) embryos (Bopp *et al.*, 1989), give rise to pair-rule phenotypes as well (Nüsslein-Volhard and Wieschaus, 1980). The last two types of *gsb* expression patterns could also be induced by ectopic expression in early embryos of a pair-rule gene and are consistent

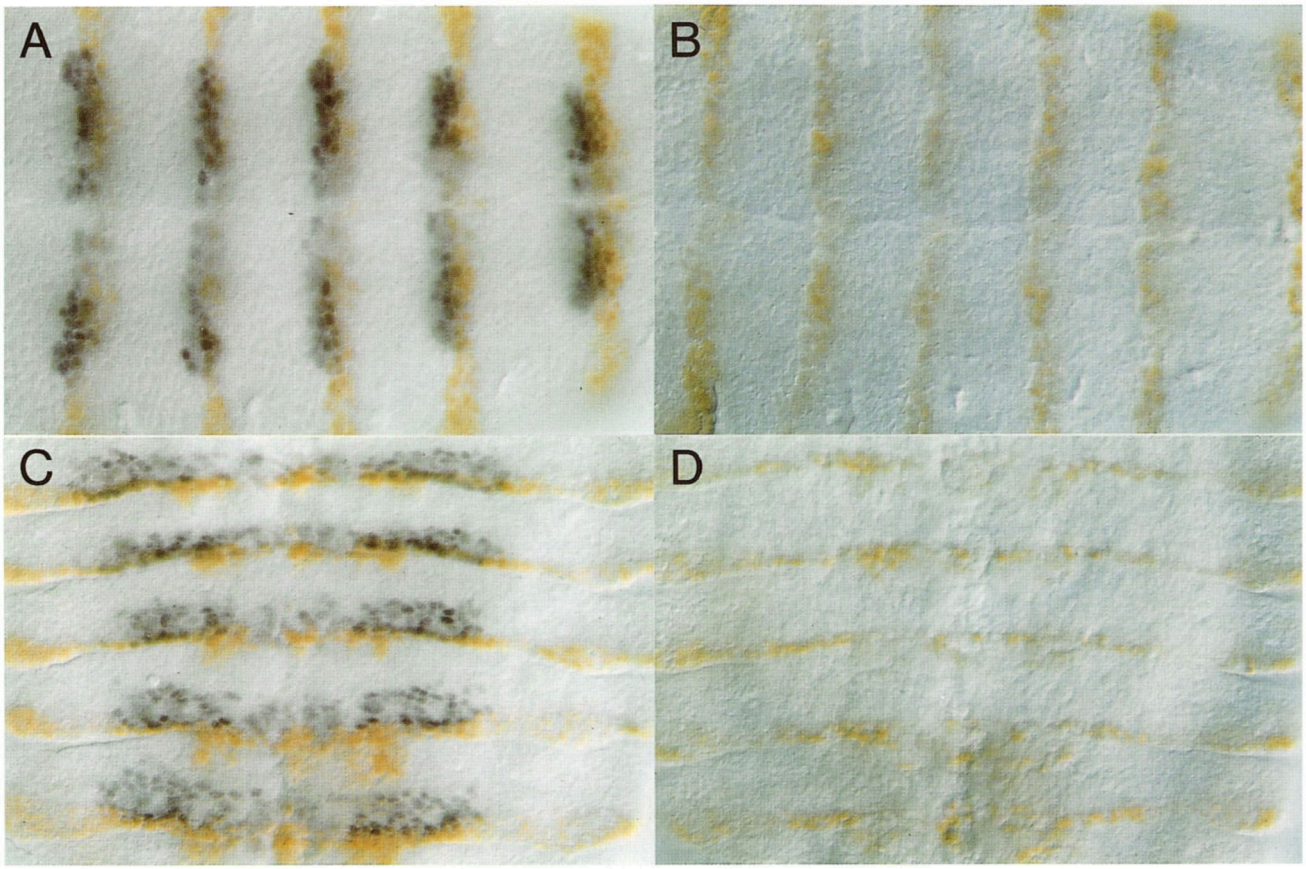

Fig. 3. Unaffected expression of *en* in gsb^- embryos. Wild-type (**A** and **C**) and homozygous gsb^- embryos (**B** and **D**) from $Df(2R)gsb^{IIX62}/CyO$ parents were double-stained with anti-gsb and anti-en antibodies at 6.5 h AEL (mid stage 11; A and B) or at 9.5 h AEL (early stage 13; C and D). Only five representative stripes are shown for embryos oriented with their anterior to the left (A and B) or up (C and D). Notice that the gsb (dark blue) and en (brown) stripes overlap in a narrow row of cells in wild-type embryos while, in gsb^- embryos, no gsb protein exists but en stripes have the same width and shape as in wild-type embryos. At the later stage, gsb stripes are wider whereas en stripes have narrowed to only one or two rows of cells.

with the observed pair-rule phenotypes. For example, ectopic expression of *ftz* in *Hsftz* embryos abolishes even-numbered gsb stripes (Figure 4I and K) while ectopic *prd* expression in *Hsprd* embryos generates pairing of gsb stripes (not shown). Finally, some mutants, like en^- and $Krüppel^-$ (Kr^-), reveal more irregular cuticular phenotypes which, however, are always preceded by *gsb* expression patterns that correlate with repressed denticle formation (Figure 4N–Q).

gsb protein represses denticle formation

The observed correlation between *gsb* expression and the absence of denticles suggests that *gsb* acts as a repressor of denticle formation. In agreement with this hypothesis, the ubiquitous expression of *gsb* in *Hsgsb* embryos results in the loss of denticle belts in most embryos subjected to a 20 min heat shock between 3 h 10 min and 6 h 20 min AEL (Figure 5A and H). After this period, heat shock has no effect on the cuticular phenotype of these embryos (Figure 5H). The heat-induced gsb protein is ubiquitously detectable between 10 min and 2 h after the heat shock (not shown). These results demonstrate that *gsb* is sufficient to repress denticle formation by overriding the denticle forming activity.

Evidently, *en* does not function in denticle repression since *en* expression is not affected in gsb^- embryos (Figure 3) and no ectopic *en* expression is induced by heat shock in *Hsgsb* embryos (not shown). Furthermore, the ubiquitous expression of *en* does not induce denticle repression in *Hsen* embryos (Poole and Kornberg, 1988). Moreover, the anterior-most row of each denticle belt develops from *en*-expressing cells (Hama *et al.*, 1990; Dougan and DiNardo, 1992).

wg acts downstream of gsb to repress denticle formation

Since *wg* and *gsb* are coexpressed (Figure 1) and depend on each other after 6 h AEL, we expect identical *wg* and *gsb* expression patterns in all other segmentation mutants after this time. Therefore, not only *gsb* but also *wg* could prepattern denticle formation. In fact, a model has been proposed recently in which *wg* functions as a repressor of denticle formation (Bejsovec and Martínez-Arias, 1991). Indeed, ubiquitous expression of *wg* also represses denticle formation (Noordermeer *et al.*, 1992; Figure 6A). The question then arises which of the two genes, *wg* or *gsb*, acts downstream of the other to repress denticle formation.

To answer this question, we first examined the effect of ectopic *gsb* activation in *Hsgsb* embryos in the absence of a functional endogenous *wg* gene. Since *wg* affects denticle formation only between 4 h and 9.5 h AEL (Bejsovec and Martínez-Arias, 1991), 3 h 40 min to 4 h 20 min old embryos were heat-shocked for 30 min at 37°C, allowed to recover

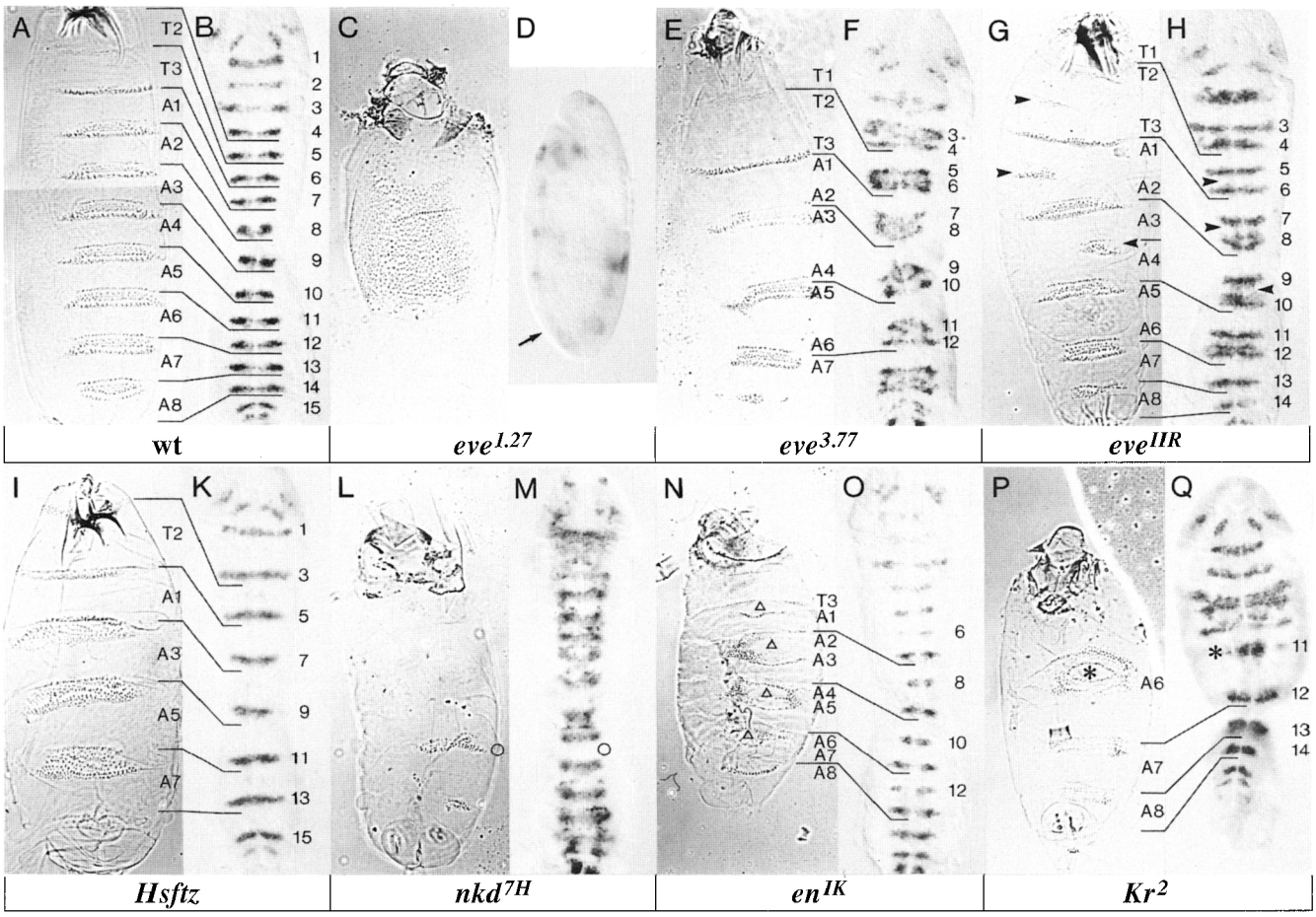

Fig. 4. Patterns of denticle repression in the ventral cuticle reflect earlier patterns of *gsb* expression. The gsb patterns of stage 11 (6 h AEL) embryos stained with anti-gsb antiserum (B, D, F, H, K, M, O and Q) are compared with the differentiated ventral cuticles (A, C, E, G, I, L, N and P) for various genotypes indicated below the panels. Phase contrast images of embryos, unfolded (except in panel D) to show the entire set of gsb stripes and of cuticle preparations are all shown at the same magnification as ventral views with the anterior up. Corresponding regions of stained embryos and cuticle preparations are connected by thin lines. In general, *gsb* expressing cells give rise to naked cuticular regions. (**A** and **B**) In wild-type embryos, the ventral cuticle of each segment consists of a naked and a denticle region. The segmental boundaries (horizontal lines) are located between the first and second denticle rows and are defined by the posterior boundaries of *en*-expressing cells (Hama *et al.*, 1990) which extend about two cells posteriorly to *gsb* expressing cells (cf. Figure 2A). Soon after the stage shown in (B), at mid-stage 11, the gsb stripes expand anteriorly to cover five or six rows of cells by early stage 12 (Figure 3C). A similar expansion of gsb stripes is also observed in mutant embryos (F, H, K, M and Q) except in those in which *gsb* expression fails to be maintained. (**C** and **D**) The decay of *gsb* expression in epidermal cells (arrow) of an *eve* null mutant results in a lawn of denticles. (**E** and **F**) The pairing of gsb stripes 5 and 6, 7 and 8, 9 and 10, 11 and 12 in a medium strong *eve* mutant represses the denticle belts of even-numbered parasegments (PS 6, 8, 10 and 12). (**G** and **H**) The pairing of gsb stripes 5 and 6, 7 and 8, and 9 and 10 (arrowheads) in a weak *eve* mutant is not as severe as in (F) and fails to completely repress the denticle belts of parasegments 6, 8 and 10 (arrowheads). (**I** and **K**) Ubiquitous expression of *ftz* in *Hsftz* embryos (Struhl, 1985) after a single 10 min heat shock at 3 h AEL induces a pair-rule-like *gsb* expression and denticle pattern. (**L** and **M**) Widened gsb stripes of a strong *nkd* mutant correspond to the considerably expanded naked region. The region marked by a circle (○) in panel M presumably develops into the similarly marked denticle belt in (L). (**N** and **O**) The decay of even-numbered gsb stripes in a strong *en* mutant precedes that of odd-numbered gsb stripes. The remaining even-numbered gsb stripes may develop into the naked regions of the fused denticle belts (△). (**P** and **Q**) In a strong *Kr* mutant, segments T1 to A5 are deleted. The reduced gsb stripe 11 coincides with the naked patch of cells between the duplicated denticles of A6 (marked by an asterisk).

at 25°C for 1−1.5 h, and subjected to two additional rounds of heat shock and recovery. Such a heat shock procedure provides ubiquitous *gsb* activity continuously from 4 h until at least 9 h AEL. However, in repeated experiments, none of hundreds of *Hsgsb*;wg^- embryos (see Materials and methods) exhibited repression of denticle formation but displayed the wg^- cuticular phenotype. In contrast, after the same heat shock treatment, ubiquitous expression of *gsb* is able to repress denticle formation in gsb^-, en^- or hh^- backgrounds (Figure 5B−G). Since ubiquitous *gsb* expression activates *wg* not before ~6 h AEL whereas *wg* expression begins to decay in these hh^- or en^- embryos as early as 4 or 5 h AEL (Bejsovec and Martínez-Arias, 1991; Ingham and Hidalgo, 1993), denticle formation in *Hsgsb*;en^- and *Hsgsb*;hh^- embryos is only partially repressed. Nevertheless, it follows that with respect to repression of denticles, *gsb* acts upstream of *wg*, but downstream of *en* and *hh*.

If *wg* acts downstream of *gsb* to repress denticle formation, one expects ubiquitous *wg* expression to repress denticles in gsb^- embryos or to suppress the gsb^- denticle lawn phenotype. We first tested the ability of ubiquitous *wg* expression to rescue the wg^- phenotype. As evident from Figure 6B and C, the wg^- phenotype (Figure 6D) is at least partially suppressed by three rounds of *Hswg* activation and recovery as described above. If the first heat shock was

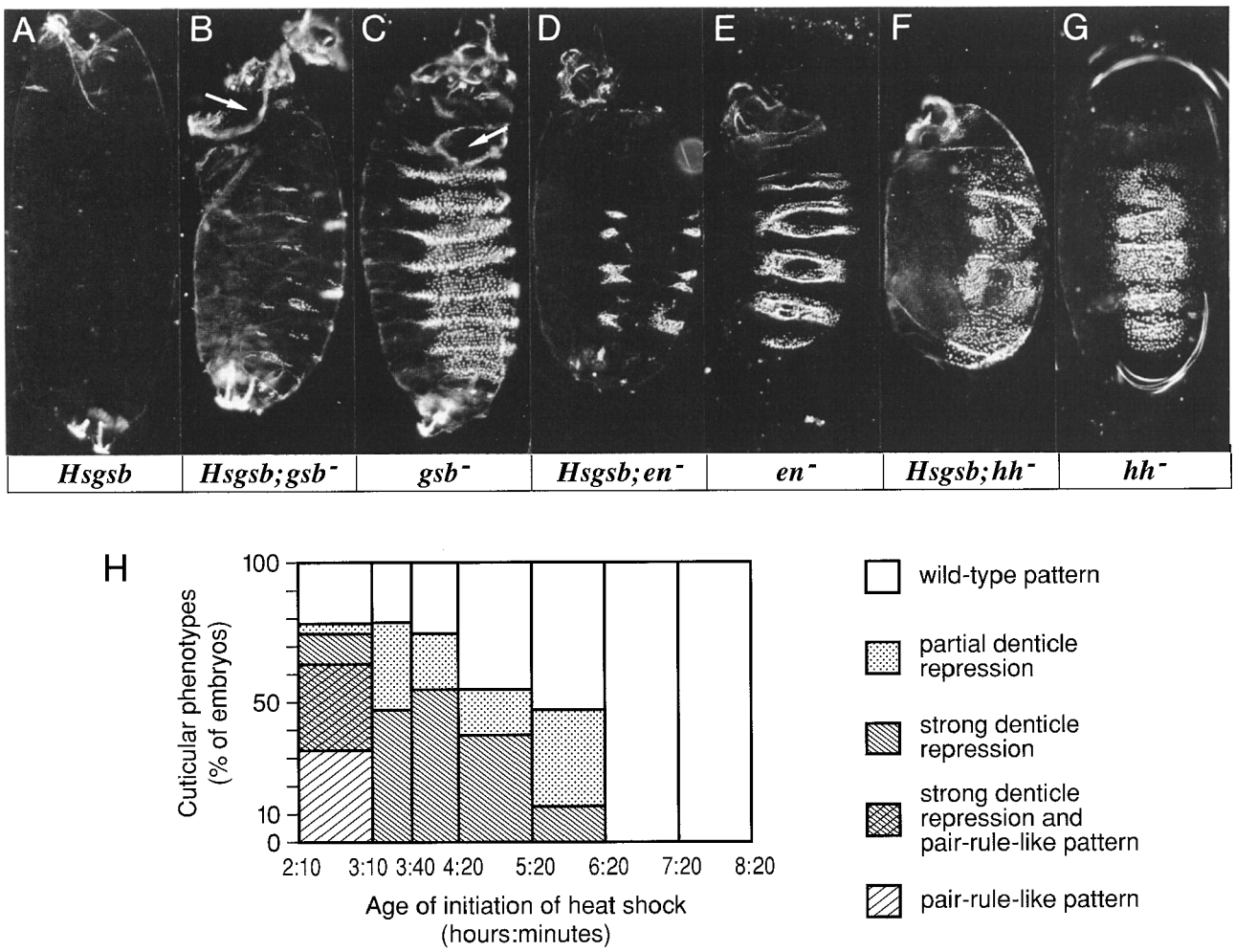

Fig. 5. Ubiquitous expression of *gsb* represses denticle formation. Panels A–G show ventral or ventrolateral views of cuticle preparations (anterior up) under dark field illumination. (**A**) Typical cuticle of *Hsgsb* embryos heat-shocked for 20 min at 3 h 10 min to 5 h 20 min AEL. Denticle formation is heavily repressed in thorax and abdomen. (**B–G**) Cuticles of *Hsgsb;gsb*$^-$ (**B**), *Hsgsb;en*$^-$ (**D**), *Hsgsb;hh*$^-$ (**F**) embryos, heat-shocked as described in Materials and methods, show repressed denticle formation as compared with cuticles of the corresponding homozygous mutant embryos *Df(2R)gsb*^{IIX62} (**C**), *en*^{IX57} (**E**) and *hh*^{IJ35} (**G**). Cuticles of *gsb*$^-$ embryos were identified by defects in head formation and dorsal closure (arrows) resulting from the inactivation of *zipper* in *Df(2R)gsb*^{IIX62} (Côté *et al.*, 1987). Cuticles of *Hsgsb;en*$^-$ and of *Hsgsb;hh*$^-$ embryos were identified on the basis of their remaining, although suppressed, *en*$^-$ or *hh*$^-$ phenotypes. (**H**) Distribution of different cuticular phenotypes of *Hsgsb* embryos as a function of developmental stage at which ubiquitous *gsb* expression was heat-induced. Cuticular phenotypes of *Hsgsb* embryos that had been subjected to a single heat shock at various times of development at 25°C (see Materials and methods) were scored as five classes: (i) wild-type, (ii) partial denticle repression (only part of each denticle belt is repressed), (iii) strong denticle repression (e.g. panel A), (iv) a mixture of classes iii and v and (v) pair-rule-like phenotypes. All five transgenic *Hsgsb* lines show essentially the same results, while none of these phenotypes were observed after heat shocking control embryos of the *w*^{1118} stock that had been used to generate the transgenic *Hsgsb* flies. The relatively large fraction of unaffected wild-type embryos has probably two main causes. The parental cross involved homozygous and heterozygous *Hsgsb* flies producing a significant fraction of embryos that did not carry a copy of the *Hsgsb* gene. In addition, some embryos that hatched and hence were scored as wild-type (see Materials and methods) actually showed a slight repression of denticles.

applied to *Hswg;wg*$^-$ embryos between 3 and 4 h AEL, most of the suppressed *wg*$^-$ embryos displayed a partial repression of denticles, resembling *gsb*$^-$ embryos in phenotype and size (Sampedro *et al.*, 1993; Figure 6B). In addition, some of the *wg*$^-$ embryos showed a strong repression of denticle formation but no rescue of the small size characteristic of *wg*$^-$ embryos (Figure 6C). This variability among suppressed *wg*$^-$ phenotypes may reflect different times of *wg* activity in these *Hswg;wg*$^-$ embryos. For example, if the embryos received their first heat shock at 3 h, wg protein is expected to persist until 8 h AEL. During this time interval wg specifies the dorsal and lateral patterns but is unable to complete the specification of the ventral denticle pattern (Bejsovec and Martínez-Arias, 1991) and thus generates *gsb*$^-$-like embryos (Figure 6B). However, if the first heat shock is initiated at 4 h AEL, wg activity would be prolonged to ~9 h AEL and repress most of the ventral denticles, generating a naked cuticular phenotype (Figure 6C). Consistent with this explanation, when *Hswg; wg*$^-$ embryos were heat-shocked after 4 h AEL (4 h 30 min to 5 h 30 min), no *gsb*$^-$-like and only partially naked cuticular phenotypes were observed.

Similarly, we examined the effect of ubiquitous *wg* expression on *gsb*$^-$ embryos. As expected, continuous wg activity after 3 h 40 min AEL, maintained by repeated heat shocks (see Materials and methods), rescues the *gsb*$^-$ denticle lawn phenotype at least partially (Figure 6E and F). This result, which has been obtained by the examination of hundreds of embryos, appears to be in conflict with an earlier report in which *Hswg* activation was unable to rescue the *gsb*$^-$

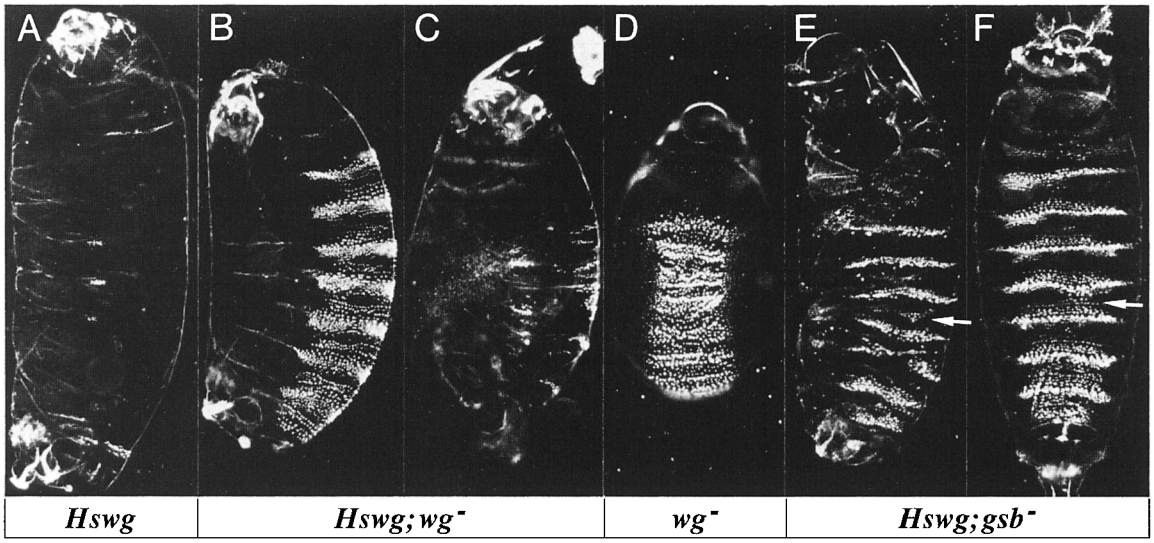

Fig. 6. Ubiquitous *wg* expression suppresses wg^- and gsb^- phenotypes. The panels show ventral or ventrolateral views of cuticle preparations (anterior up) under dark field illumination. (**A**) Cuticle of a heat-shocked *Hswg* embryo. (**B** and **C**) Cuticles of heat-shocked *Hswg*;wg^- embryos. The wg^- phenotype is suppressed to a gsb^--like (**B**) or a nearly naked phenotype (**C**). Note that a few denticles, irregularly distributed along the anterio-posterior axis, remain in ventral and ventrolateral regions of the embryo shown in panel C and thus permit the unambiguous identification of its genotype. The variability among suppressed wg^- phenotypes (B and C) probably reflects different times of *wg* activity in the *Hswg*;wg^- embryos as explained in the text. (**D**) Cuticle of the corresponding homozygous wg^{IG22} embryos. (**E** and **F**) Cuticles of heat-shocked *Hswg*;gsb^- embryos. The gsb^- phenotype is partially suppressed. Thus, the naked region frequently contains small patches of denticles (arrows) that allow these embryos to be identified as gsb^-. The same variability of cuticular phenotypes is expected to exist among *Hswg*;gsb^- (E and F) as among *Hswg*;wg^- embryos (B and C). However, for reasons explained in the text, phenotypes of *Hswg*;gsb^- embryos that would exhibit a stronger denticle repression than that shown in panel E could not be distinguished from *Hswg*;gsb^+ embryos.

phenotype (Sampedro *et al.*, 1993). However, in these experiments *Hswg* was activated only after 5–8 h AEL which might be too late to rescue the gsb^- phenotype and thus explain the apparent discrepancy. We do not know whether ubiquitous *wg* activity is able to completely repress denticle formation in *Hswg*;gsb^- embryos since it frequently generates head defects similar to those of gsb^- embryos and hence the observed naked embryos could be gsb^- or gsb^+. Therefore, we conclude that *wg* indeed acts downstream of *gsb* to specify the larval denticle pattern by the repression of denticle formation.

Discussion

gsb acts to maintain wg expression in a wg–gsb autoregulatory loop

The establishment and maintenance of segmentally repeated positional information that regulates segmental patterning depends on some 15 segment polarity genes (for reviews, see Hooper and Scott, 1992; Ingham and Martínez-Arias, 1992; Nusse and Varmus, 1992; Peifer and Bejsovec, 1992). Despite considerable progress in recent years, the specific roles of the individual segment polarity genes in this process are still poorly understood, mainly because of their complex interactions with each other. Here, we dissect the *gsb* function in segmental patterning. Our results demonstrate that the main, if not only, function of *gsb* in the specification of the cuticular pattern is to maintain the expression of *wg* by a *wg–gsb* autoregulatory loop after 6 h AEL.

The first indication for this *gsb* function is derived from the observation that the maintenance of *gsb* and *wg* expression becomes dependent on their mutual activation after 6 h AEL. While *gsb* is activated by a paracrine and an autocrine wg signal after 4 h AEL, *wg* expression begins to depend on *gsb* only after ~6 h AEL. The resulting autoregulatory loop between *gsb* and *wg* (Figure 7) thus ensures the continued synthesis of the wg signal and gsb transcription factor in the same epidermal cells (Figure 1).

The correlation of *gsb* expression with repressed denticle formation in wild-type and segmentation mutant embryos (Figure 4) suggests that gsb is a repressor of denticle formation (Figure 4), a conclusion corroborated by the observation that ubiquitous expression of *gsb* generates a naked cuticular phenotype (Figure 5). The same repression of denticle formation is also achieved by ubiquitous *wg* expression (Noordermeer *et al.*, 1992), supporting the view that wg is a repressor of denticle formation (Bejsovec and Martínez-Arias, 1991) as well. The existence of a *wg–gsb* autoregulatory loop raises the possibility that only one of these two genes is required for denticle repression. Indeed, we have shown here that with respect to repression of denticles, *wg* activity is epistatic over that of *gsb* (Figure 6). Therefore, *wg* represses denticle formation and *gsb* serves to maintain *wg* expression by a *wg–gsb* autoregulatory loop after 6 h AEL. Consistent with this mechanism, we note that gsb^- embryos exhibit a cuticular phenotype very similar to that produced by the loss of wg function after 6 h AEL (Bejsovec and Martínez-Arias, 1991).

Do different wg signalling pathways lead to denticle repression and gsb activation?

The most conspicuous feature of the segmental organization of the *Drosophila* larva is its ventral denticle pattern. During embryogenesis, both *gsb* and *wg* contribute to the specification of the larval denticle pattern by repressing denticle formation. However, as shown here, *wg* acts downstream of *gsb* in this process, while *gsb* serves to activate and maintain *wg* expression. As wg represses denticle forma-

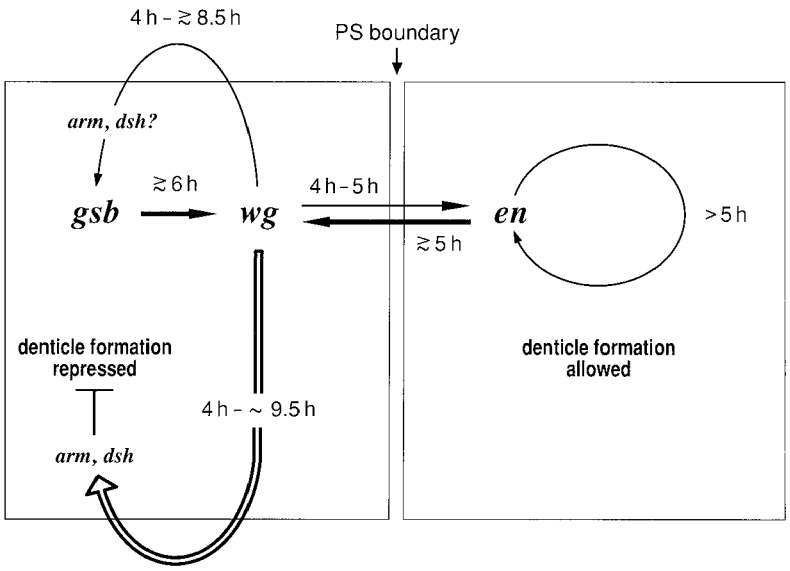

Fig. 7. Maintenance of *gsb*, *wg* and *en* expression by autoregulatory loops and role of *wg* in repression of denticle formation. *wg* and *en* are expressed in adjacent cells on either side of the parasegmental (PS) boundary while *gsb* is expressed in *wg*-expressing cells and their neighbours. For simplicity, the paracrine activation of *gsb* in the anterior neighbouring cell (not shown) and in the posterior *en*-expressing cell have been omitted. Arrows signify gene activation while the open arrow indicates an epistatic gene order.

After the initial activation of *gsb*, *wg* and *en* by pair-rule proteins, at ~4 h AEL, the wg signal activates *en* in a paracrine manner, whereas it activates *gsb* by a paracrine as well as an autocrine signalling pathway. However, *en* expression soon becomes independent of the wg signal which serves to initiate *en* autoregulation (Heemskerk *et al.*, 1991; Siegfried *et al.*, 1992). At about the same time, the homeobox gene *en* activates *wg* in adjacent anterior cells by a different signal transduction pathway that probably involves the hh signal and the ptc transmembrane protein (Ingham *et al.*, 1991; Ingham and Hidalgo, 1993). Thereafter, at ~6 h AEL, gsb transcription factor begins to activate *wg* independently of *en* and *hh*, presumably by binding directly to *cis*-regulatory elements of *wg*, and thus generates an autoregulatory loop between *wg* and *gsb*. The wg signal maintained by *gsb* serves as an output to specify the denticle pattern. In this pathway, the wg signal acts through the products of *arm* (and *dsh*) to antagonize a ubiquitous denticle forming activity. In addition to this function, *arm* is also involved in the regulation of *wg*, *gsb* and *en* since their expression decays in arm^- embryos (Peifer *et al.*, 1991; our unpublished results). While *arm* itself is regulated by *wg* and *en*, but not by *gsb* (Riggleman *et al.*, 1990), its role in the *wg*−*gsb* or *wg*−*en* autoregulatory loops is not yet known.

tion and since naked cuticular regions, in wild-type or segmentation mutant embryos, always result from *wg* expression, the default state of cuticular differentiation in the ventral epidermis corresponds to a ubiquitous denticle forming activity. The generation of any denticle pattern depends on where this activity is antagonized or repressed by the wg signal in wild-type and mutant embryos. The molecular nature of this denticle forming activity remains to be elucidated.

How does the wg signal repress denticle formation? Of all segment polarity genes examined, only *arm* and *dishevelled* (*dsh*) repress denticle formation in a cell-autonomous fashion (Wieschaus and Riggleman, 1987; J.Klingensmith and N.Perrimon, unpublished). Hence, although small arm^- (or dsh^-) clones receive the wg signal secreted by the neighbouring wild-type cells, the arm^- (or dsh^-) cells are unable to transduce the wg signal and to repress denticle formation. In contrast, very small wg^- or gsb^- clones do not form ectopic denticles in the naked cuticle (Wieschaus and Riggleman, 1987), suggesting that repression of denticle formation in these cells does not require the endogenous *gsb* or *wg* function as long as they receive the wg signal from the surrounding wild-type cells. These results thus provide independent evidence for our conclusion that *gsb* acts upstream of *wg* in the repression of denticle formation (otherwise gsb^- clones should fail to repress ectopic denticles).

Therefore, we have to distinguish between at least two wg signalling pathways. In one pathway that involves the cell-autonomous action of the *arm* and *dsh* products, wg represses denticle formation. By another pathway, wg activates *gsb*. The wg signal that activates *gsb* is simply a mechanism by which *wg* maintains its own expression (Figure 7). It is unclear whether *arm* and *dsh* are involved in these pathways as well. While *arm* encodes a protein related to vertebrate plakoglobin and β-catenin, which are components of adhesive junctions and associated with cadherins (Peifer and Wieschaus, 1990; McCrea *et al.*, 1991), the molecular nature of the *dsh* gene product is unknown.

Although *arm* RNA is uniformly distributed in wild-type embryos, arm protein accumulates at higher levels in regions that receive the wg signal (Riggleman *et al.*, 1990). This differential arm protein distribution depends on *wg* and *dsh* but not on *gsb* (Riggleman *et al.*, 1990). Since the gsb^- phenotype is virtually the same as the late (after 6 h AEL) wg^- phenotype, it follows that the uneven distribution of arm protein depends on the wg signal only before *gsb* is required for *wg* activation, i.e. before 6 h AEL. Therefore, the differential distribution of arm protein that is induced by the wg signal is not sufficient for denticle repression after 6 h AEL. It is not clear, however, whether the early denticle repression, which depends on *wg* but not on *gsb*, is also independent of the unequal accumulation of arm protein.

Since *gsb* expression decays in arm^- embryos (our unpublished results) while *arm* expression remains unaffected is gsb^- mutants (Riggleman *et al.*, 1990), *arm* is required for *gsb* activity and thus acts upstream of *gsb*. As the expression of the wg signal also depends on *arm* (Peifer *et al.*, 1991), it is unclear whether the arm protein is involved in

the transduction or the maintenance of the wg signal that activates *gsb*. In terms of denticle repression, however, *arm* acts downstream of *gsb* (and *wg*) which is also consistent with the observation that ubiquitous *gsb* expression fails to repress denticle formation in arm^- embryos (our unpublished results). This apparent contradiction is explained by and emphasizes again the existence of different signalling pathways that may share several components including *arm* (Figure 7).

Temporal asymmetries in autoregulatory loops of segment polarity genes

After their initial activation by pair-rule proteins, segment polarity genes maintain their expression throughout most of embryonic development. In the case of *en, wg* and *gsb*, at least two regulatory feedback loops coupled by *wg* serve to maintain their expression and thus the inherent positional information (Figure 7). Notably, mutual activations between *en* and *wg* and between *wg* and *gsb* are not synchronous but sequential (Figure 7). Activation of *gsb* requires the wg signal before *wg* begins to depend on gsb protein while the *wg*−*en* autoregulatory loop is disrupted by the direct autoregulation of *en* soon after it has originated (Heemskerk *et al.*, 1991; Siegfried *et al.*, 1992). This temporal asymmetry within both autoregulatory loops produces a flow of information via *wg* from *en* to *gsb*, but not from *gsb* to *en*, and explains why *gsb, wg* and *en* embryos display different phenotypes.

As a consequence of the temporal asymmetry of the coupled regulatory feedback loops, *gsb* does not interfere with the *wg*−*en* autoregulatory loop before 6 h AEL (Figure 7). Therefore, *gsb* does not affect pattern forming processes regulated by *wg* before this time and due to the uncoupling of *en* activation by *wg* after 5 h AEL, never affects such processes regulated by *en*. In contrast, we expect morphogenetic processes that are regulated by *wg* after 6 h AEL to be affected by *gsb*. For example, formation of the parasegmental groove (Martínez-Arias and Lawrence, 1985), which is regulated by *en* and *wg* (Perrimon and Mahowald, 1987; Martínez-Arias *et al.*, 1988), occurs before 6 h AEL and appears normal in gsb^- embryos (our unpublished results). In addition, the segmental groove, which forms at ~9.5 h AEL and depends on both *en* and *wg* (Kornberg, 1981; Perrimon and Mahowald, 1987), is not affected either in gsb^- embryos (Perrimon and Mahowald, 1987). As *wg* protein decays in *gsb* mutants after 6 h AEL, it follows that formation of segmental grooves must have been determined by this time. Independent support for this conclusion comes from temperature shift experiments with temperature-sensitive *wg* embryos, demonstrating that inactivation of *wg* after 6 h AEL has no effect on segment boundary formation (our unpublished observations).

The temporal asymmetries in the mutual interactions between *gsb, wg* and *en* also explain the differences among their mutant cuticular phenotypes that result from different patterns of *wg* expression. Since *wg* expression depends on *gsb* only after 6 h AEL, *gsb* is required for repression of denticle formation only after this time. However, *wg* is required for denticle repression already after 4 h AEL. Accordingly, wg^- embryos exhibit more ectopic denticles than gsb^- embryos. Similarly, the difference between the en^- and wg^- cuticular phenotypes is explained by the pair-rule-like decay of *wg* and *gsb* expression in en^- embryos (Figure 4O; Bejsovec and Martínez-Arias, 1991). It is presently not understood why even-numbered gsb and the corresponding wg protein stripes disappear first in en^- embryos, i.e. why *en* is required earlier for *wg* activation in these as compared with the complementary set of stripes.

Morphogenetic fields generated by self-propagating autoregulatory loops

The *wg*−*gsb* autoregulatory loop is clearly different from the previously described *wg*−*en* interaction in which a paracrine wg signal activates *en* only in neighbouring cells (DiNardo *et al.*, 1988; Martínez-Arias *et al.*, 1988; Bejsovec and Martínez-Arias, 1991; Heemskerk *et al.*, 1991). Conversely, the *en* product activates *wg* by another signal transduction pathway in which the proteins encoded by the segment polarity genes *hh* and *patched* (*ptc*) act as putative signal and receptor (Ingham *et al.*, 1991; Ingham and Hidalgo, 1993). It appears that *en* maintains *wg* expression only transiently after its initiation at 5 h AEL since ubiquitous *Hsgsb* expression is able to repress denticle formation in en^- and hh^- embryos, suggesting that *gsb* does not require *en* or *hh* to activate *wg* after 6 h AEL.

In the *wg*−*gsb* autoregulatory loop, expression of *gsb* is activated in the same as well as neighbouring cells by the autocrine and paracrine wg signal. The continued *wg* transcription, on its part, depends on *gsb* because *wg*, encoding a secreted extracellular protein (van den Heuvel *et al.*, 1989; González *et al.*, 1991), cannot directly maintain its own expression. In this way, the activation of these genes could be propagated from cell to cell over long distances. However, since in *Drosophila* embryos the wg signal has to travel only over a few cell diameters, a diffusion mechanism is adequate as has been found to be the case (van den Heuvel *et al.*, 1989; González *et al.*, 1991). In fact, mechanisms must exist in *Drosophila* to prevent the continuous expansion of the domain of *wg* expression by a self-propagating *wg*−*gsb* autoregulatory loop. Indeed, expansion of the *wg* domain in the posterior direction is prevented by *en* which represses *wg* (Heemskerk *et al.*, 1991) and overrules the activation by *gsb*. Similarly, the anterior expansion of the *wg* domain is limited by *ptc* which represses *wg* expression (Ingham *et al.*, 1991; Ingham and Hidalgo, 1993). These mechanisms also explain why *wg* is not activated ubiquitously by the activation of *Hsgsb* (Figure 2D). Hence, the *wg*−*gsb* autoregulatory loop is not self-propagating but required solely for the continued production of the diffusing wg signal.

However, the proposed mechanism of self-propagating autoregulatory loops might be important for the establishment of morphogenetic fields during embryogenesis of larger animals, such as mice and elephants. The essence of such an autoregulatory loop is that a secreted morphogen by signal transduction activates a gene encoding a transcription factor in the cell secreting the signal as well as in neighbouring cells and that the transcription factor in turn activates the gene generating the signal. Thus, this mechanism would be able to maintain and propagate the signal from cell to cell. Moreover, an attenuation of the signal along its path of propagation would lead to a gradient-like distribution of the signal which could thus act as a morphogen. Such a self-propagating autoregulatory loop thus provides a new

mechanism for the generation of morphogenetic fields not dependent on diffusion.

Recent experiments might indicate that autoregulatory loops similar to that of *wg* and *gsb* are conserved in vertebrates, supporting the view that they represent an ancient patterning mechanism (Noll, 1993). Thus, in zebrafish embryos for example, the *wg* homologue *wnt-1* and the paired-box gene *pax[zf-b]* are coexpressed at the midbrain–hindbrain boundary. Disruption of pax[zf-b] function by injection of anti-pax[zf-b] antibodies abolished the expression of both *wnt-1* and *pax[zf-b]* and results in malformation of this region in the brain (Krauss *et al.*, 1992).

Materials and methods

Generation of transgenic Hsgsb flies

Transgenic *Hsgsb* flies were generously provided by Koni Basler and Ernst Hafen and produced, as previously described by Dambly-Chaudière *et al.* (1992), by cloning a *gsb* cDNA, BSH9c2 (Baumgartner *et al.*, 1987), into the P-element vector pKB255 (K.Basler and E.Hafen, unpublished) and subsequent germline transformation of w^{1118} embryos according to standard procedures (Rubin and Spradling, 1982). Five independent lines were obtained.

Heat shock treatment of embryos

Hsgsb, Hswg or *Hsftz* embryos were collected and aged on agar plates for various time intervals at 25°C. Before heat shock treatment, embryos were collected and rinsed in a device prepared from a plastic vial with cut off bottom and a hole in its screw cap holding a fine nylon net. Embryos were heat-shocked by directly placing the vials into a 37°C waterbath for 10–30 min. Subsequently, the vials were transferred to a humidified chamber at 25°C either until the embryos reached 24 h AEL, when cuticles were prepared, or for a shorter time interval (1–5 h) when they were fixed and stained with antibodies.

To illustrate the window of *Hsgsb* function (Figure 5H), embryos at various stages were heat-shocked for 20 min, with the exception of embryos between 2 h 10 min and 3 h 10 min AEL which received only a 10 min heat shock because embryos of this stage are very sensitive to heat shock such that longer heat treatments block development of most embryos. Embryos were counted immediately after the heat shock. After 24 h AEL, hatched embryos were counted and scored as wild-type. From the unhatched embryos, cuticles were prepared and their cuticular phenotypes classified and counted. For each time point shown in Figure 5H, at least 100 embryos were heat-shocked. The fraction of each phenotype was calculated with respect to the total number of heat treated embryos.

To determine whether *Hsgsb* can repress denticle formation in gsb^-, wg^-, en^-, hh^- or arm^- backgrounds, the following repeated heat shocks were applied to embryos from heterozygous *gsb, wg, en, hh* or *arm* parents carrying one copy of *Hsgsb* to provide a continuous activation of *Hsgsb*. Embryos at 3 h 40 min to 4 h 20 min AEL were heat-shocked for 30 min at 37°C, allowed to recover at 25°C for 1–1.5 h and subjected to another two rounds of heat shock and recovery. Only embryos older than 3 h 40 min were subjected to the first round of heat shock because their treatment, in contrast to that of younger embryos (e.g. 3 h AEL), does not disrupt head development and hence allows the unambiguous discrimination of mutant (e.g. wg^-) from wild-type embryos. The same heat shock procedure was also applied to *Hswg*;wg^- and *Hswg*;gsb^- embryos, except that the time of the first round of heat shock treatment was varied between 3 h and 4 h 30 min AEL as specified in the Results.

Preparation of cuticles

Embryos aged until at least 24 h AEL were collected and dechorionated in a plastic collection tube, transferred to an Eppendorf tube filled with heptane–methanol (1:1) and briefly vortexed to remove the vitelline membrane. After one rinse with methanol, the embryos were fixed in a glycerol–acetic acid solution (1:4) at 60°C for 1 h and mounted in Hoyer's medium (Wieschaus and Nüsslein-Volhard, 1986).

Temperature shifts of wg^{ts} embryos

The *wg* mutation, wg^{IL114}, was confirmed to be temperature-sensitive as wg^{IL114} embryos raised at 18°C exhibited a wild-type cuticle and expressed *gsb* while, when raised at 28°C, they displayed the wg^- cuticular phenotype and *gsb* expression decayed prematurely. For temperature shift experiments, embryos were collected at 60 min intervals and aged for various time intervals at 18°C, shifted to the nonpermissive temperature of 28°C for 2 h, fixed and immunostained. As embryonic development at 18°C is twice as long as at 25°C, the times indicated in the text or figure legends have been corrected as if embryos had been raised continuously at 25°C.

Immunostaining of embryos

Immunostaining of embryos was carried out as described by Li *et al.* (1993). The double-labelling of *en* and *gsb* or *gsb* and *lacZ* was performed according to Lawrence *et al.* (1987). All stained embryos were photographed under Nomarski optics.

Fly stocks

The following fly stocks were used: *Hsftz* (K.Basler and E.Hafen, unpublished); *Hswg/TM3, hb-βgal* (Noordermeer *et al.*, 1992); *wglacZ/CyO,* en^{11} (Kassis *et al.*, 1992); arm^{XK22}, $Df(2R)gsb^{IIX62}$, en^{IK57}, $eve^{1.27}$, $eve^{3.77}$, eve^{IIR}, hh^{IJ35}, Kr^2, nkd^{7H16}, wg^{IL114}, wg^{IG22} from the Tübingen stock centre.

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