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Separable regulatory elements mediate the establishment and maintenance of cell states by the *Drosophila* segment-polarity gene *gooseberry*

Xuelin Li, Thomas Gutjahr[1] and Markus Noll

Institute for Molecular Biology II, University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland
[1]Present address: Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, NY 10021, USA

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During *Drosophila* embryogenesis, position along the anteroposterior axis is specified within each segment by the products of the segment-polarity genes which include *wingless* (*wg*) and *gooseberry* (*gsb*). The striped expression of these genes in each segment is initially established by the pair-rule gene products during late blastoderm. This pattern is subsequently maintained after germ band extension by interaction among the segment-polarity genes themselves. Here we show that the maintenance of *gsb*, a PHox gene encoding a paired-domain and a homeodomain, is controlled by the *wg* signal, the homolog of the murine *Wnt-1* protein. A control element responsible for *wg*-dependent maintenance of *gsb* expression, *gsb*-late element, is separable from an element required for the initial activation of *gsb* by pair-rule transcription factors, *gsb*-early element. The significance of such a regulatory strategy is discussed with respect to the establishment and maintenance of cell states within each segment by segment-polarity genes.

Key words: *gooseberry*/segment-polarity stripe formation/transcriptional regulation/*wingless*

Introduction

The development of an organism critically depends on the initial generation of asymmetries along the body axes (for a review, see Melton, 1991). In *Drosophila*, the mother initiates asymmetries in the oocyte by four maternal signals or morphogens. Three of these provide positional information along the anteroposterior axis and one along the dorsoventral axis after fertilization (for a review, see St Johnston and Nüsslein-Volhard, 1992). These maternal cues are transcription factors which activate a zygotic gene regulatory cascade of the gap and pair-rule class of segmentation genes that progressively define position along the anteroposterior axis of the embryo before the nuclei are separated by membranes at cellular blastoderm.

In the last step of this cascade, when cells need to maintain and elaborate positional information during cell divisions and movements, the segment-polarity genes are activated by pair-rule gene products to specify cell states within each segment by complex mechanisms that involve communication between cells (for a review, see Ingham and Martinez-Arias, 1992). For example, the parasegmental boundary, which separates the anterior from the posterior compartment of segments (Martinez-Arias and Lawrence, 1985), is maintained by the action of the segment-polarity genes *wingless* (*wg*) and *engrailed* (*en*). Both *wg* and *en* are initially activated by the combinatorial action of pair-rule genes in neighboring cells on either side of the compartment boundary. Subsequently, they activate each other to maintain their expression by intercellular signals whose transduction involves several additional segment-polarity proteins. In contrast to the transiently active gap and pair-rule genes, segment-polarity genes remain active throughout development, presumably reflecting the requirement for a continuous specification of cell states.

We are studying the mechanism by which the stripes of segment-polarity gene products are established and maintained for the *gooseberry* (*gsb*) gene, which, with several other segment-polarity genes, specifies cell fates in the posterior portion of each segment (Nüsslein-Volhard and Wieschaus, 1980). The *gsb* locus consists of two closely apposed and divergently transcribed genes, *gsb* and *gsb neuro* (*gsbn*), previously named *gsb-BSH9* or *gsb-distal* and *gsb-BSH4* or *gsb-proximal*, respectively (Bopp *et al.*, 1986; Baumgartner *et al.*, 1987; Côté *et al.*, 1987). Both genes encode proteins with a paired-domain as well as a *prd*-type homeodomain (Bopp *et al.*, 1986), and hence belong to the PHox gene family (Bopp *et al.*, 1989). Since *gsb* rather than *gsbn* is mainly expressed in the epidermis and is able to rescue the *gsb* cuticular phenotype (T.Gutjahr, N.H.Patel, X.Li, C.S.Goodman and M.Noll, in preparation), we favor the idea that *gsb* is responsible for the specification of the posterior cuticular pattern (Bopp *et al.*, 1986).

Here we show that the establishment and maintenance of *gsb* stripes is under the control of two separable and consecutively acting *cis*-regulatory elements, *gsb*-early element (GEE) and *gsb*-late element (GLE). The GEE is activated by pair-rule proteins to establish the *gsb* stripes while the GLE controls their maintenance in response to the *wg* signal rather than to the *gsb* protein itself. We propose that separable *cis*-regulatory elements responding to pair-rule and segment-polarity gene products might reflect a general strategy of segment-polarity gene regulation.

Results

Upstream region controls gooseberry stripe formation

We used P-element-mediated germ line transformation with promoter–*lacZ* fusion constructs (Hiromi *et al.*, 1985) to analyze the *cis*-regulatory regions required for the segmentally repeated expression of *gsb* during embryogenesis. To this end, *gsb* DNA sequences were fused in-frame to the *lacZ* reporter gene as illustrated in Figure 1. The genomic *gsb* DNA consisted of different upstream regions adjacent to its promoter and transcribed 5′ end sequence up to the end of the paired-box. The expression patterns of these *gsb*–*lacZ* constructs were examined in embryos of corresponding transgenic lines. Embryos of *lacZ* lines containing 10 kb (9Z1), 6.5 kb (9Z2) and 5.7 kb (9Z3) of *gsb* upstream DNA (Figure 1B) exhibit segmentally

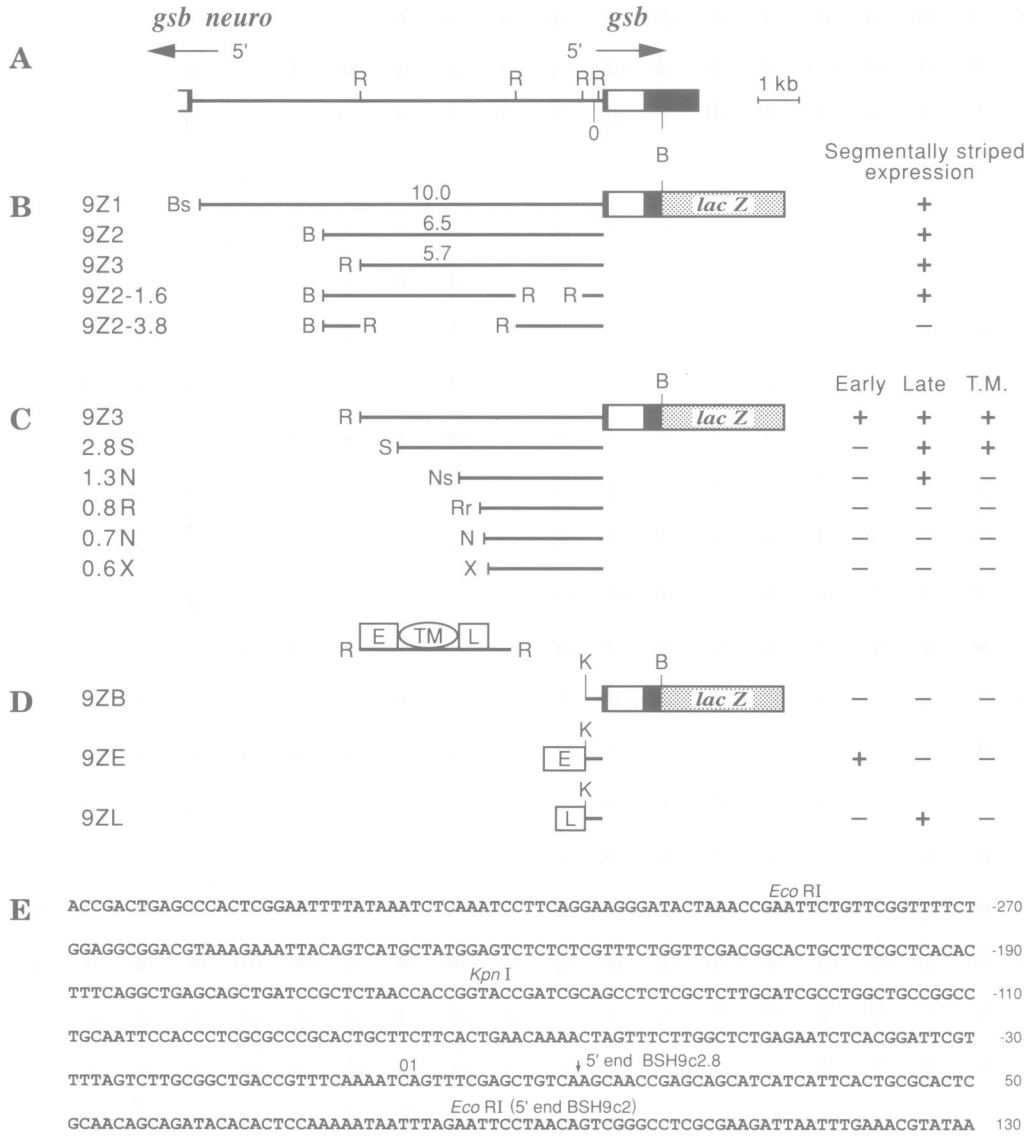

Fig. 1. *gsb*–*lacZ* constructs used for identification of *gsb cis*-regulatory elements. (**A**) *Eco*RI restriction map of *gsb* and its upstream region up to the first intron of the oppositely transcribed neighboring gene *gsbn*. The *gsb* transcriptional start site is marked by 0 while the directions of *gsb* and *gsbn* transcription are indicated by arrows (filled and open bars represent exons and introns, respectively). (**B–D**) Maps of three series of *gsb*–*lacZ* constructs. In all constructs, *gsb* sequences were fused in-frame to *lacZ* at the *Bam*HI site of the second *gsb* exon (Baumgartner *et al.*, 1987). Their ability to express *gsb*–*lacZ* in segmentally repeated epidermal stripes during early (Early; up to stage 11) and late development (Late; after stage 10) as well as in the thoracic mesoderm (T.M.) beginning with stage 12 is indicated on the right. The approximate lengths of *gsb* upstream sequences (in kb) are indicated above the 9Z1, 9Z2 and 9Z3 constructs. The first series of *gsb*–*lacZ* constructs (**B**) maps the control elements to the 3.8 kb *Eco*RI fragment. Within this fragment, the second series of constructs (**C**) delimits the control regions for early (E) and late (L) stripe formation and for expression in the thoracic mesoderm (TM). The third series of *gsb*–*lacZ* constructs (**D**) tests the independence of the *cis*-regulatory elements mapped in the second series by fusing them to the *Kpn*I site of the *gsb* promoter shown in E. (**E**) DNA sequence surrounding the transcriptional start site of the *gsb* gene. The start site of *gsb* transcription has been mapped by primer extension within a 372 bp *Eco*RI fragment that had escaped detection in a previous analysis and should be inserted into the *Eco*RI site at position 830 of the published *gsb* sequence (Baumgartner *et al.*, 1987). *gsb* is transcribed with equal efficiency from the adjacent C or A of the consensus transcription start site ATCAGTT (Hultmark *et al.*, 1986; data not shown). The sequence TTTTA, located between positions −26 and −30, might serve as TATA box. The 5′ end of a nearly full-length *gsb* cDNA, BSH9c2.8, is marked by a vertical arrow. Abbreviations of restriction sites: B, *Bam*HI; Bs, *Bss*HII; K, *Kpn*I; N, *Nhe*I; Ns, *Nsi*I; R, *Eco*RI; Rr, *Rsr*II; S, *Sal*I; X, *Xho*I.

repeated stripe patterns of *gsb*–*lacZ* expression which are very similar to the *gsb* protein patterns of wild-type embryos throughout embryonic development (Figure 2).

Expression of both *gsb* and *gsb*–*lacZ* is first observed when the odd-numbered stripes appear during late blastoderm. Subsequently, the even-numbered stripes arise during gastrulation and reach the intensity of the odd-numbered stripes during germ band extension. This process is slightly delayed for *gsb*–*lacZ* and is accompanied by some ectopic expression in the odd-numbered stripes and the head region (cf. Figure 2A and G). After completion of germ band extension (stage 11; Campos-Ortega and Hartenstein, 1985), expression of *gsb*–*lacZ* and *gsb* becomes laterally restricted to the more ventral region in stripes 4–17 (Figure 2B and H). Interestingly, stripe 16 is expressed only in 9Z1 (not shown), and not in 9Z2 and 9Z3 embryos (cf. Figure 2B and H), indicating that it is regulated separately from all other stripes and requires *gsb* upstream sequences between

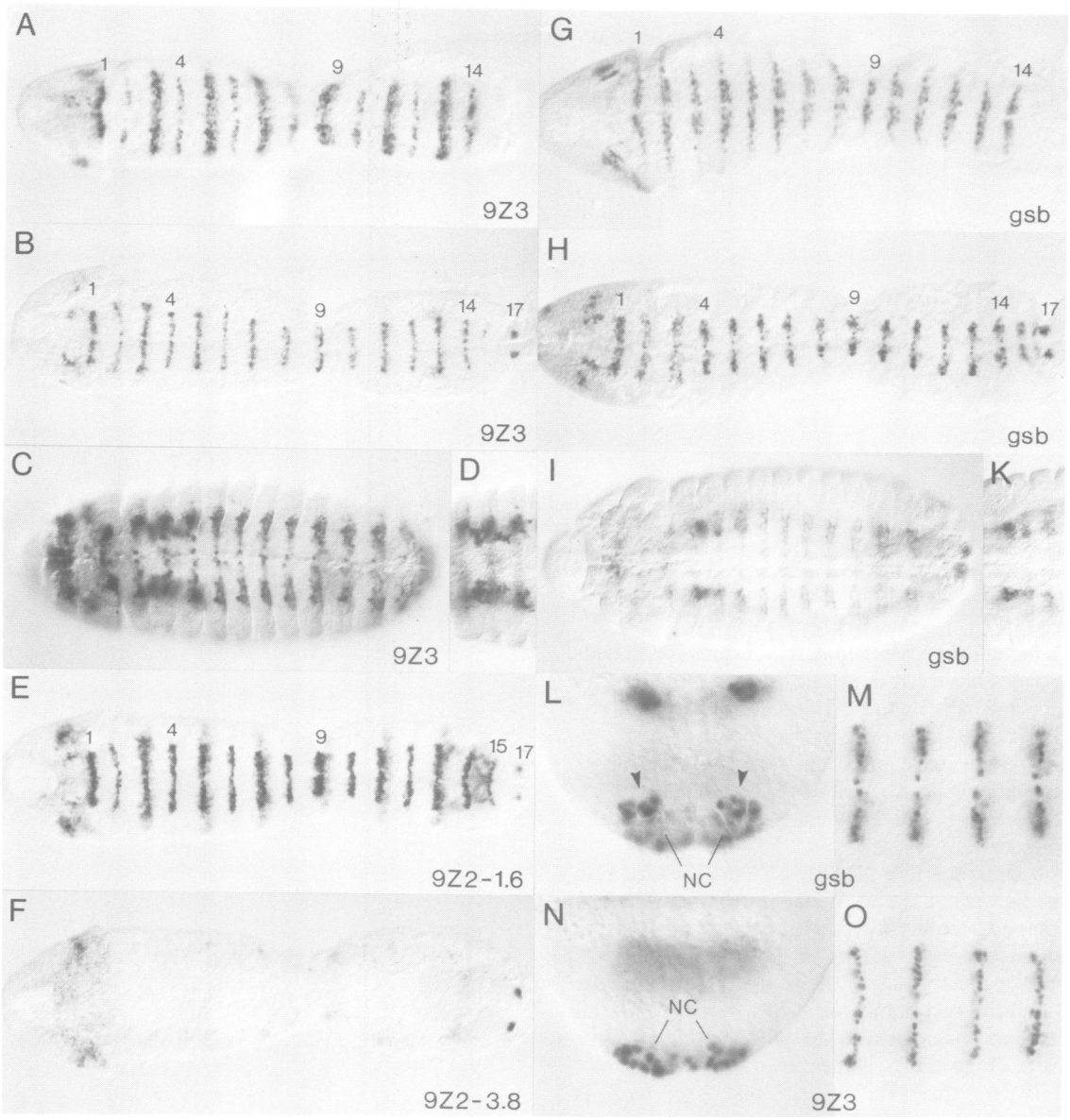

Fig. 2. Comparison of *gsb−lacZ* with *gsb* expression patterns in wild-type embryos. Expression of various *gsb−lacZ* constructs (9Z3 in **A−D**, **N** and **O**; 9Z2-1.6 in **E**; and 9Z2-3.8 in **F**) is compared with that of the endogenous *gsb* gene (**G−M**) in embryos at different developmental stages (A, G: beginning of neuroblast segregation, early stage 9; B, E, F, H and L−O: fully extended germ band, early stage 11; C, D, I and K: beginning of head involution, stage 14). Both *gsb−lacZ* and *gsb* are expressed in the posterior portion of each segment. Panels D and K focus on the expression in the thoracic mesoderm of the embryos shown in panels C and I. Panels M and O show an enlarged epidermal region comprising stripes 4−7 (T1−A1) in embryos at a stage similar to that of the embryos shown in panels H and B, respectively. The stripes appear narrower than at the lower magnification because stained non-epidermal cells do not remain in focus with epidermal cells at the higher magnification. Stained cells of mesodermal and neural origin are visible in panels L and N which focus on stripe 9 in A3, located at the posterior pole of stage 11 embryos. Both *gsb−lacZ* and *gsb* are expressed in neural cells (NC) while an internal cluster of cells stains only for *gsb* (arrowheads in L). Embryos during germ band extension have been unfolded to show the entire set of ventral and dorsal stripes (A, B, E-H). All embryos (except those in panels L and N) are oriented with their anterior end to the left. Embryos shown here and in all other figures were immunostained for *gsb−lacZ* and *gsb* proteins as described in Materials and methods.

−10 and −6.5 kb for its activation. At this time, the stripes consist of 1−2 rows of epidermal cells (Figure 2M and O), located on either side of the parasegmental boundary (Gutjahr *et al.*, in preparation), and of underlying neural cells (NC in Figure 2L and N). However, an internal cluster of cells beneath these neural cells (arrowheads in Figure 2L) does not express *gsb−lacZ* (Figure 2N). Double-labeling of embryos demonstrates that *gsb−lacZ* and *gsb* are expressed in the same set of epidermal cells (not shown; but see below).

Later, *gsb−lacZ* and *gsb* stripes broaden laterally but remain expressed in similar patterns in the posterior portion of each segment until the beginning of head involution (Figure 2C and I). During germ band retraction, for all three constructs, 9Z1, 9Z2 and 9Z3, a mesodermal expression pattern of *gsb−lacZ* (Figure 2D) very similar to that of *gsb* (Figure 2K) appears in the thoracic segments.

Embryos of lines transformed with a construct in which the 3.8 kb *Eco*RI fragment has been deleted from 9Z2 (9Z2-3.8 in Figure 1B) show no expression of stripes (Figure 2F). On the other hand, removal of the more proximal 1.6 kb *Eco*RI fragment from 9Z2 (9Z2-1.6 in Figure 1B) leaves the *gsb−lacZ* stripe pattern essentially

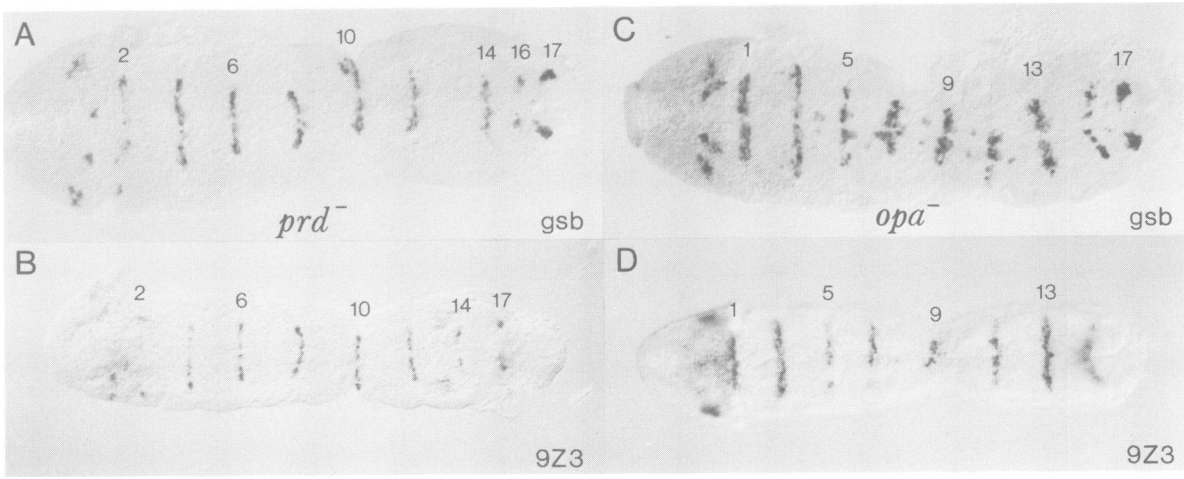

Fig. 3. Comparison of *gsb−lacZ* with *gsb* expression patterns in *prd*⁻ and *opa*⁻ embryos. *gsb* (**A** and **C**) and 9Z3 *gsb−lacZ* (**B** and **D**) expression patterns were analyzed in homozygous $prd^{2.45.17}$ (**A** and **B**) or opa^{IIP32} embryos (**C** and **D**). The $prd^{2.45.17}$ allele is a null mutant (Frigerio *et al.*, 1986) while opa^{IIP32} is a strong *opa* allele (Tearle and Nüsslein-Volhard, 1987). Embryos at stage 11 (A−C) or at late stage 10 (D; at this stage, stripe 17 is not yet expressed) are shown unfolded as in Figure 2. Note that *gsb* stripe 16 is also strongly reduced in *opa*⁻ embryos whereas stripe 17 remains unaltered in *prd*⁻ embryos.

unaffected although the ectopic epidermal expression in odd-numbered stripes observed at an earlier stage (Figure 2A) does not disappear (Figure 2E) as in 9Z3 embryos (Figure 2B). These results suggest that the main upstream *cis*-regulatory elements required for normal expression of *gsb* in segmentally repeated stripes (except stripe 16) are located in the 3.8 kb *Eco*RI fragment between −5.7 kb and −1.9 kb (Figure 1).

As for *gsb* (Baumgartner, 1988), *gsb−lacZ* expression depends on pair-rule proteins in the same manner. For example, in *prd*⁻ embryos, odd-numbered *gsb* protein stripes are not expressed (Bopp *et al.*, 1989), with the exception of the most posterior stripe 17 (cf. Figures 3A and 2H). Similarly, odd-numbered stripes of *gsb−lacZ* fail to be activated in *prd*⁻ embryos carrying the 9Z1, 9Z2 or 9Z3 construct (Figure 3B). In contrast, in *odd-paired*⁻ (*opa*⁻) embryos expression of *gsb* and *gsb−lacZ* is abolished or strongly reduced in even-numbered stripes (Figure 3C and D). The expression patterns of *gsb* and *gsb−lacZ* exhibit identical alterations in other pair-rule mutants as well (not shown). Therefore, both *gsb* and *gsb−lacZ* respond *in vivo* to pair-rule gene products in the same way, and the *cis*-regulatory elements mediating the response are located in the 3.8 kb *Eco*RI fragment shown to regulate *gsb* expression in stripes.

Two separate cis-regulatory elements direct gooseberry expression in stripes

To narrow down further the regulatory elements required for the activation of *gsb* in segmentally repeated stripes, the 3.8 kb *Eco*RI fragment was subjected to a series of deletions from its distal end. When a 1.0 kb *Eco*RI-*Sal*I fragment is removed (2.8S in Figure 1C), *gsb−lacZ* remains inactive during early embryonic development and is first expressed only just prior to completion of germ band extension (Figure 4A). During the extended germ band stage, *gsb−lacZ* appears in the characteristic *gsb* stripe pattern although the *gsb−lacZ* level is considerably reduced in the middle portion of the stripes (cf. Figure 4B with Figure 2B and H).

The additional deletion of a 1.5 kb *Sal*I−*Nsi*I fragment

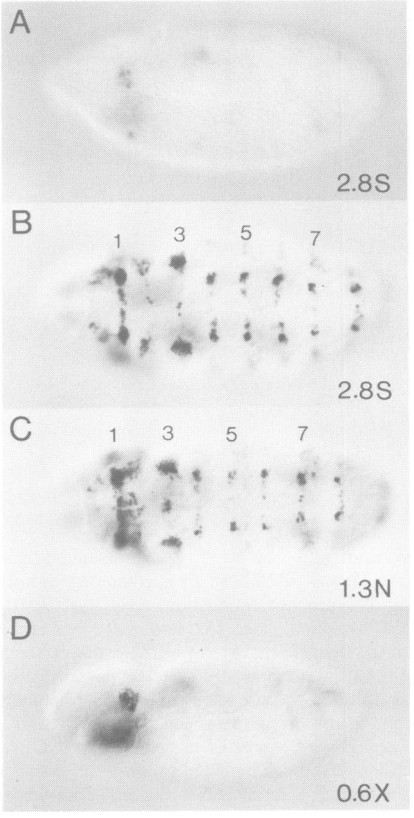

Fig. 4. Regulatory regions of *gsb* stripe expression within the 3.8 kb *Eco*RI fragment. (**A**) *gsb−lacZ* expression of 2.8S construct (Figure 1C) at late germ band extension (stage 10) and (**B**) at the fully extended germ band stage (stage 11). The *gsb−lacZ* expression pattern of segmental stripes is similar to that of *gsb* at stage 11. (**C**) 1.3N *gsb−lacZ* expression at stage 11. (**D**) 0.6X *gsb−lacZ* expression at stage 11 shows no striped expression. Embryos are shown with their anterior end to the left, either as lateral views with their dorsal side up (A and D) or as ventral views (B and C).

from the 2.8S construct (1.3N in Figure 1C) has no effect on this expression pattern (Figure 4C) but eliminates the thoracic mesodermal expression (not shown), which arises

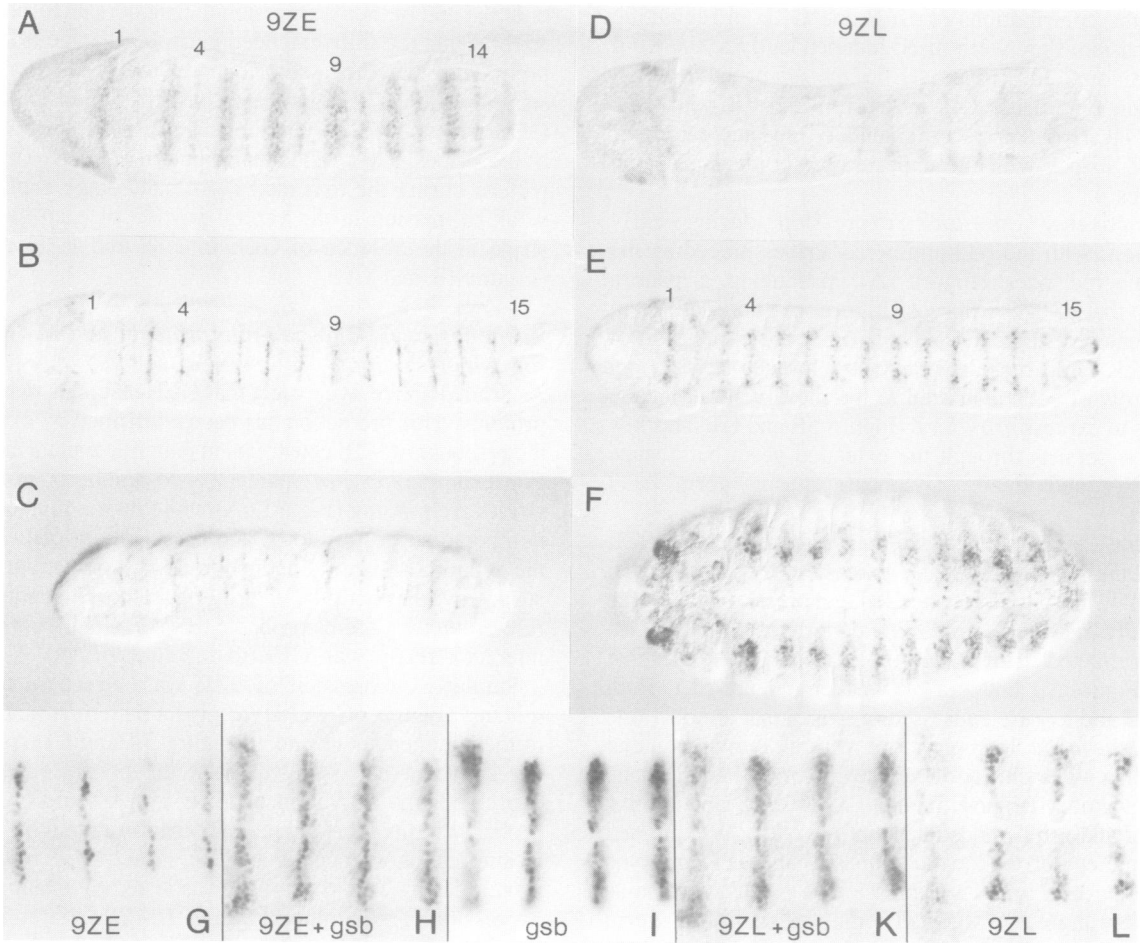

Fig. 5. Independent regulation of *gsb* by GEE and GLE. Expression of *gsb*−*lacZ* was analyzed in 9ZE (Figure 1D) embryos during early germ band extension (end of stage 8; **A**), during the extended germ band stage (mid stage 11; **B**), and shortly before germ band retraction (late stage 11; **C**). Similarly, expression of *gsb*−*lacZ* was studied in 9ZL (Figure 1D) embryos at late germ band elongation (stage 10; **D**), at the fully extended germ band stage (mid stage 11; **E**), and after germ band retraction (stage 13; **F**). Panels **G** and **L** are magnifications of the region comprising stripes 3−6 (parasegments 3−6) of the embryos shown in panels B and E, respectively, while panels **H**−**K** show the same region of 9ZE (H), 9ZL (K) or wild-type embryos (I) at a similar stage stained with anti-*gsb* (I) or with both anti-*gsb* and anti-*lacZ* (H and K). The double stainings shown in panels H and K in black and white were carried out with two differently coloured stains according to Lawrence *et al.* (1987) to demonstrate precise coexpression of *gsb* and *gsb*−*lacZ*. Embryos are oriented with their anterior end to the left and have been unfolded in panels A−E.

during germ band retraction (Figure 2D and K). Hence, the deleted 1.5 kb fragment contains elements regulating the mesodermal expression of *gsb* in the thorax (TM in Figure 1C) during germ band retraction.

Further deletion of a 0.7 kb *Nsi*I−*Xho*I fragment from the 1.3N *gsb*−*lacZ* construct (0.6X in Figure 1C) resulted in a complete loss of the segmentally repeated expression pattern of *gsb*−*lacZ* in embryos at the extended germ band stage (Figure 4D) as well as thereafter (not shown). Even the removal of 0.6 kb (0.7N in Figure 1C) or of only 0.5 kb (0.8R in Figure 1C) from 1.3N *gsb*−*lacZ* suffices to abolish its striped expression (not shown).

These results suggest that information required for the regulation of *gsb* expression in segmentally repeated stripes is contained in the 1.0 kb *Eco*RI−*Sal*I (GEE; E in Figure 1C) and the 0.7 kb *Nsi*I−*Xho*I regions (GLE; L in Figure 1C). While GEE is necessary for the early activation of *gsb* expression during late blastoderm, GLE may be required similarly after germ band extension. It is striking that the appearance of the pattern regulated by GLE coincides with a marked change in the pattern of *gsb* stripes retracting laterally, which might indicate that these lateral regions of *gsb* expression switch from GEE to GLE control at this time (see below).

Since *gsb* is initially activated by pair-rule gene products (Baumgartner, 1988) and since expression of 9Z3 is the same as that of *gsb* in pair-rule mutant embryos (Figure 3), it follows that GEE contains elements responding to pair-rule proteins. However, GLE responds to pair-rule proteins as well since the expression of the 2.8S and 1.3N *gsb*−*lacZ* constructs was altered in different pair-rule mutants in the same manner as that of the endogenous *gsb* gene (not shown). Because *prd* (Gutjahr *et al.*, 1993) and other pair-rule proteins are hardly detectable after germ band extension, this response of GLE to pair-rule gene products is probably indirect and mediated by segment-polarity genes.

GEE and GLE independently control early and late gooseberry expression

Since GEE was not tested in the absence of GLE, it is not clear whether GEE is also sufficient for the early activation of *gsb*. In addition, the formal possibility remains that GLE is required for late expression only in the absence of GEE but redundant in its presence. Hence, in an attempt to

separate the contributions of GEE and GLE in regulating *gsb* expression, they were fused independently to the *Kpn*I site at position −155 (Figure 1E) of the *gsb* promoter (9ZE and 9ZL in Figure 1D). As shown in Figure 5, both GEE and GLE clearly generate a segmentally repeated pattern of *gsb−lacZ* stripes with the expected temporal and spatial differences.

9ZE activates *gsb−lacZ* expression during early gastrulation, with the odd-numbered stripes preceding the even-numbered ones (Figure 5A), producing a pattern similar to that of 9Z3 embryos (Figure 2A) until late germ band extension (stage 10). At this time, the expression of 9ZE begins to decrease dramatically. In each stripe, only a single row of cells restricted to the more ventral portion continues to express *gsb−lacZ* (Figure 5B and G). This low expression persists through the extended germ band stage (Figure 5C) and finally disappears during germ band retraction.

In contrast, expression of *gsb−lacZ* is not detected in 9ZL embryos until late germ band extension (early stage 10; Figure 5D). During the extended germ band stage, the segmentally repeated expression pattern of *gsb−lacZ* in laterally retracted stripes (Figure 5E) closely resembles that of *gsb* (Figure 5I) but is a bit weaker (Figure 5L). Both *gsb−lacZ* and *gsb* are expressed in stripes that are only one or two cells wide in their more ventral region but end laterally in a wider cluster of cells, exhibiting a barbell shape in each segment (Figure 5I and L). Double-labeling for *gsb−lacZ* and *gsb* demonstrates that *gsb−lacZ* is expressed in the same epidermal locations of 9ZE and 9ZL embryos as *gsb* (Figure 5H and K). Expression of *gsb−lacZ* remains at relatively high levels in 9ZL embryos until after germ band retraction (Figure 5F). No segmentally repeated expression of *gsb−lacZ* was observed (not shown) with a control construct containing upstream sequences only up to the *Kpn*I site (Figure 1D).

Evidently, the striped expression of *gsb* is initially controlled by GEE until the end of germ band extension. Thereafter, GLE contributes to the specific transition of *gsb* stripes to the laterally retracted form and to their maintenance until the end of their expression during dorsal closure (stage 15). During the fully extended germ band stage (stage 11), both GEE and GLE contribute to the formation of *gsb* stripes. While GLE generates barbell-shaped expression, GEE activates *gsb* only in the narrow, more ventral region of the stripes. The residual contribution of GEE to the stripe pattern during the extended germ band stage explains the weak expression in the ventral portion of each *gsb−lacZ* stripe in the absence of GEE in 2.8S and 1.3N embryos (Figure 4B and C).

Response of GEE to pair-rule proteins and of GLE to the wingless signal

As argued above, we expect that GEE responds to pair-rule proteins. This prediction has been confirmed by examining the response of 9ZE constructs in pair-rule mutant embryos. For example, in *prd* mutants, odd-numbered *gsb−lacZ* stripes are missing (Figure 6A) while in *opa* mutants even-numbered stripes fail to be activated (Figure 6B). Because the temporal and spatial induction of odd- or even-numbered stripes overlap with the activity of the corresponding pair-rule proteins, it seems probable that some pair-rule proteins interact directly with GEE to regulate *gsb*.

Similarly, expression of 9ZL was altered in pair-rule mutants (Figure 6C and D) to give a pattern similar to that of the endogenous *gsb* gene (Figure 3A and C). As 9ZL is not active at early stages when the pair-rule genes are expressed, the activation of GLE by pair-rule proteins is probably indirect. A simple explanation would be that *gsb* is autoregulatory such that GLE is activated by the *gsb* protein itself. This possibility was tested by examining the expression of 9Z2 in homozygous $Df(2R)Kr^{SB1}$ embryos in which the *Krüppel* (*Kr*) and *gsb* genes are both deleted (Bopp *et al.*, 1986; Côté *et al.*, 1987). Since the gap gene *Kr* is required for the development of the thoracic and first five abdominal segments (T1−A5), these segments are absent in homozygous *Kr* embryos (Nüsslein-Volhard and Wieschaus, 1980), thus allowing the unambiguous identification of gsb^- embryos. In such embryos, *gsb−lacZ* expression is normal in segments anterior and posterior to

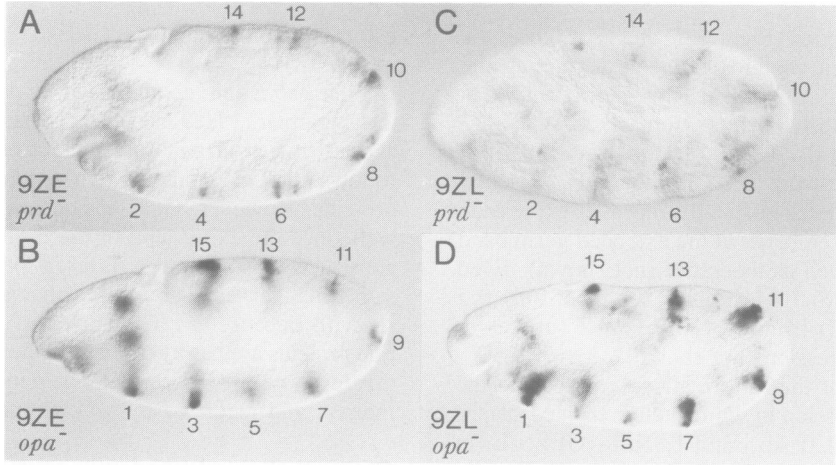

Fig. 6. 9ZE and 9ZL expression in prd^- and opa^- embryos. Expression of *gsb−lacZ* was analyzed in homozygous $prd^{2.45.17}$ (**A** and **C**) and opa^{IIP32} (**B** and **D**) embryos carrying the 9ZE (**A** and **B**) or the 9ZL construct (**C** and **D**). Only the even- (A and C) or odd-numbered bands (B and D) of 9ZE and 9ZL are expressed. The transgenic line carrying 9ZE on the third chromosome shown in (A) exhibited weak ectopic expression in a few cells which was also observed in wild-type embryos. One line carrying 9ZL (C) showed a slightly reduced expression of *gsb−lacZ* as compared with another line (D, Figure 5E). Lateral views of embryos with their anterior end to the left and their dorsal side up are shown at stage 10 (B) or stage 11 (A, C and D).

T1−A5 (Figure 7A and B), suggesting that *gsb* does not regulate itself. Similarly, no change in the 9Z2 *gsb−lacZ* expression was observed in homozygous *Df(2R)gsb*^{IIX62} embryos (Nüsslein-Volhard *et al.*, 1984), in which both *gsb* and *gsbn* are deleted (Bopp *et al.*, 1986; Côté *et al.*, 1987). Moreover, similar results were obtained when expression of 9ZE or 9ZL was analyzed in these deficiency mutants (not shown).

Since *wg* and *gsb* mutants exhibit similar cuticular phenotypes and since *wg* is also regulated by pair-rule genes (Ingham *et al.*, 1988) in a similar way to *gsb* (Baumgartner, 1988; X.Li, unpublished observation), the late activation of *gsb* might be mediated by *wg*. Furthermore, during the extended germ band stage, *wg* stripes are interrupted to generate ventral stripes and lateral patches (van den Heuvel *et al.*, 1989). During this process, the ventral *wg* stripes become laterally restricted in a strikingly similar way to the *gsb* stripes. Double-labeling with antisera against *wg* and *gsb* demonstrates that, after germ band extension, *gsb* is indeed activated in *wg*-expressing cells as well as in an immediately adjacent posterior row of cells which receive the *wg* signal (not shown). If this signal activates *gsb*, we would expect expression of *gsb*, 9Z1, 9Z2 and 9Z3 to be discontinued after germ band extension in *wg*$^-$ embryos. In addition, in the absence of the *wg* product, 9ZL should never be expressed while expression of 9ZE should remain unaffected. The experimental observations agree well with these expectations (Figure 7C−K). In homozygous *wg*

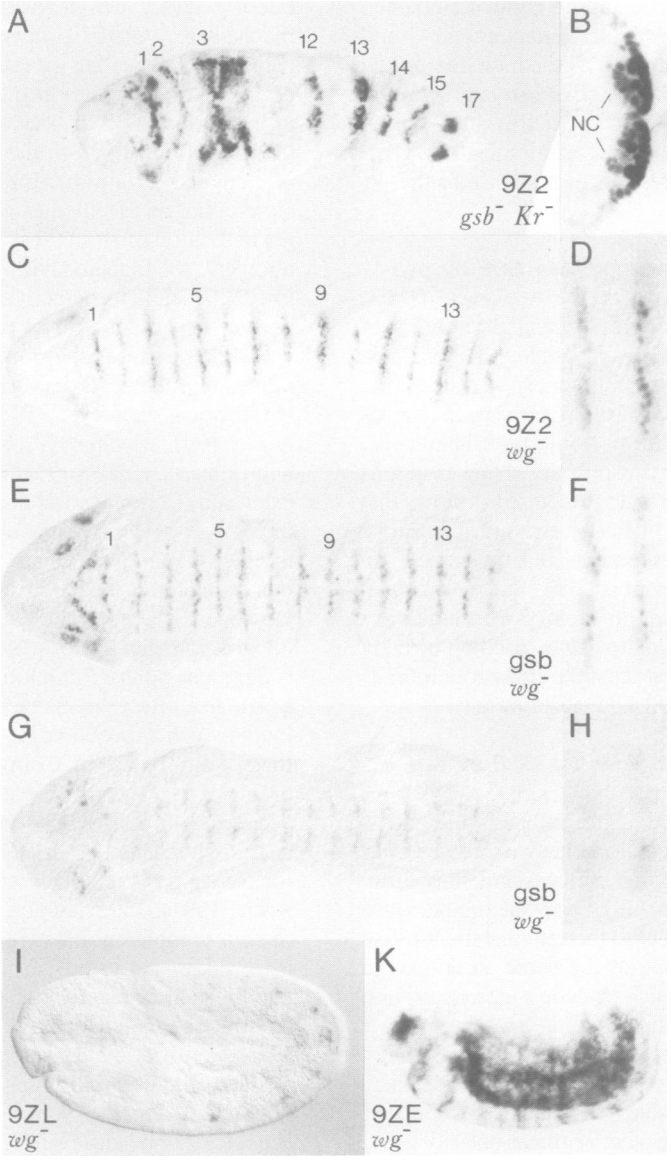

Fig. 7. Expression of *gsb−lacZ* and *gsb* in *gsb* and *wg* mutants. Expression of *gsb−lacZ* is shown in 9Z2 embryos homozygous for *Df(2R)Kr*^{SB1} (**A** and **B**) or *wg*^{IG22} (**C** and **D**), and in 9ZL (**I**) or 9ZE embryos (**K**) homozygous for *wg*^{IG22}. Expression of *gsb* is shown in homozygous *wg*^{IG22} embryos (**E−H**). *wg*^{IG22} is a strong *wg* allele (Tearle and Nüsslein-Volhard, 1987). Panel B is an enlarged optical section of stripe 13 shown in A, focussed to demonstrate that also in neural cells (NC) of *gsb*$^-$ embryos *gsb−lacZ* is expressed as in wild-type embryos (Figure 2N). Panels D, F and H show an enlarged region comprising stripes 4 and 5 (T1 and T2) of the embryos shown in C, E and G, respectively, focussed on the epidermal cells. The remaining *gsb* expression in the embryo of panel G is restricted to the CNS and not affected by *wg*. The bulk of the staining in the embryo of panel K represents ectopic *gsb−lacZ* expression and is not epidermal but rather labels internal tissues. Embryos at late stage 10 (C−F), stage 11 (A, B and I), late stage 11 (G and H), or stage 12 (K) are shown unfolded (A−H) or as lateral views (I and K) with their anterior to the left and their dorsal side up (I and K).

embryos, the expression of both 9Z2 (Figure 7C and D) and *gsb* (Figure 7E and F) begins to decline from the lateral regions during late germ band extension (stage 10), which results in a stripe pattern similar to that activated by GEE (Figure 5B and G). Shortly before germ band retraction, the epidermal expression of *gsb* (Figure 7G and H) and 9Z2 (not shown) is no longer detectable in wg^- embryos. In agreement with these findings, 9ZL fails to be expressed in the epidermis of wg^- embryos (Figure 7I) whereas *wg* has no effect on the low epidermal expression of 9ZE (cf. Figure 7K with Figure 5C). Hence, GLE, but not GEE, responds to *wg*.

Discussion

We have shown here that two separable control elements, GEE and GLE, which respond independently and consecutively to different activators, mediate the initial activation and subsequent maintenance of *gsb* expression in epidermal stripes. While GEE regulates the initial establishment of *gsb* by pair-rule transcription factors, GLE maintains the striped *gsb* expression in response to the *wg* signal.

Establishment of gooseberry stripes via GEE by pair-rule proteins

The striped expression in a single-segment periodicity of segment-polarity genes is initially activated by the pair-rule gene products during late syncytial blastoderm. At this time, the pair-rule genes themselves are expressed in stripes exhibiting a double-segment periodicity. Therefore, regulation of every other segment-polarity stripe responds to the same combination of pair-rule proteins. It follows that a single control element might suffice to activate at least one set of even- or odd-numbered segment-polarity stripes. An example of such a control element is GEE. Since all known pair-rule proteins are transcription factors, it seems very probable that some of them regulate *gsb* by binding directly to GEE. Indeed, we find that at least the *prd* protein binds to GEE *in vitro* (X.Li, unpublished observation).

Maintenance of gooseberry stripes via GLE by the wingless signal

In contrast to the transiently active gap and pair-rule genes, segment-polarity genes remain continuously expressed after activation. This raises the questions of how segment-polarity genes maintain their expression and of how the maintenance phase is coordinated with respect to the establishment phase to ensure continuous expression at the same locations. In the case of the *gsb* gene, which encodes a transcription factor, a simple mechanism would be that its gene product directly activates its own promoter. However, this possibility is in conflict with our observations (Figure 7A). Rather, maintenance of *gsb* activity is achieved by the use of a second control element, GLE, and of another segment-polarity gene, *wg*, producing the activating signal.

Among the segment-polarity genes, *wg* resembles *gsb* in many aspects. For example, *wg* and *gsb* mutants show similar cuticular phenotypes, particularly if *wg* function is lost after 5 h of development at 25°C (Bejsovec and Martinez-Arias, 1991). In addition, *wg* is expressed in a segment-polarity pattern very similar to that of *gsb* after stage 11 (van den Heuvel *et al.*, 1989; González *et al.*, 1991). Double-labeling with antibodies against *wg* and *gsb* proteins indicates that *gsb* is activated in *wg*-expressing cells as well as in an adjacent posterior row of cells (not shown). This row of cells also expresses *engrailed* (*en*) (van den Heuvel *et al.*, 1989; Gutjahr *et al.*, in preparation). Moreover, *wg* is initially activated by pair-rule genes (Ingham *et al.*, 1988) in a similar way to *gsb* (Baumgartner, 1988; X.Li, unpublished observation) such that *gsb* and *wg* are activated in the same cells. Therefore, a mechanism by which *wg* activates *gsb* during the maintenance phase ensures that *gsb* is activated at the same locations as during the early phase. While these observations are consistent with the activation of *gsb* by *wg* after the establishment phase, we have shown here that loss of *wg* function results in the loss of *gsb* expression after late germ band extension as if only GEE, but not GLE, was activated. In agreement with such a mechanism, GLE is never activated in wg^- embryos (Figure 7I). Since the *wg* protein is secreted (van den Heuvel *et al.*, 1989; González *et al.*, 1991), the maintenance of *gsb* in *wg*-expressing cells occurs by an autocrine *wg* signal while the activation of *gsb* in the adjacent posterior row of cells is mediated by a paracrine mechanism. The paracrine *wg* signal (van den Heuvel *et al.*, 1989) is also responsible for the activation of *en* in these cells (DiNardo *et al.*, 1988; for a review, see Ingham and Martinez-Arias, 1992). In contrast to *gsb*, *en* fails to be activated by the autocrine *wg* signal, due to the absence of *en* protein (Siegfried *et al.*, 1992).

On the other hand, *wg* expression itself is maintained by other segment-polarity genes, including *gsb*. In en^- (Martinez-Arias *et al.*, 1988), *armadillo*$^-$ (*arm*$^-$) (Peifer *et al.*, 1991), *hedgehog*$^-$ (*hh*$^-$), or gsb^- embryos (Hidalgo and Ingham, 1990), *wg* expression decays after germ band extension. Complex mechanisms are involved in this process, including cell–cell interactions as most clearly evident from the fact that *en* and *wg* are expressed not in the same cells but in neighboring cells (van den Heuvel *et al.*, 1989). However, it is not yet clear how these genes interact in an integrated pathway to activate *wg*.

Segment-polarity mutations in genes other than *wg*, such as *en*, also affect the maintenance of *gsb*. However, in these mutants *gsb* expression is altered in parallel to that of *wg*. In en^- embryos, for example, *gsb* expression disappears prematurely, and the expression in even-numbered stripes declines before that in odd-numbered stripes (Hidalgo, 1991; X.Li, unpublished observation). The same decay is observed for the *wg* pattern in these embryos (Bejsovec and Martinez-Arias, 1991). Interestingly, in en^- embryos, GLE does not remain completely inactive as in wg^- embryos, but rather is weakly activated in odd-numbered stripes (X.Li, unpublished observation). This argues that *wg* rather than *en* function is indispensable for the activation of GLE. Moreover, the small overlap of *gsb*- and *en*-expressing cells excludes the possibility that *en*, as a target of the *wg* signal (DiNardo *et al.*, 1988; Martinez-Arias *et al.*, 1988), activates *gsb* because such a mechanism fails to explain the activation of *gsb* in cells that do not express *en*. Therefore, we conclude that *wg*, as the target of a signal activated by *en* (Martinez-Arias *et al.*, 1988), regulates *gsb*. In hh^- embryos, *gsb* expression decays as well while in *patched*$^-$ (*ptc*$^-$) embryos *gsb* stripes are expanded anteriorly (Hidalgo, 1991; X.Li., unpublished observation). Again *wg* expression exhibits the same alterations as *gsb* in these mutants (Martinez-Arias *et al.*, 1988; Hidalgo and Ingham,

1990). These observations have been explained by a mechanism in which *ptc* represses *wg* and *hh* inactivates or overrules the repression by *ptc* (Ingham *et al.*, 1991). Hence, *hh* and *ptc* regulate *gsb* by their effect on *wg* expression.

The mechanism by which *wg* activates *gsb* remains unclear. As *wg* encodes a secreted protein, the homolog of the murine *Wnt-1* gene product (Rijsewijk *et al.*, 1987), it could only activate *gsb* indirectly by a signal transduction pathway. In wg^- embryos, the expression of *en* (DiNardo *et al.*, 1988; Martinez-Arias *et al.*, 1988), *hh* (Mohler and Vani, 1992) and *ptc* decays (Hidalgo and Ingham, 1990). Nevertheless, as argued above, it seems improbable that these genes are involved in the transduction of the *wg* signal to activate *gsb*. However, the *arm* protein might be involved in this signal transduction pathway because it accumulates in response to *wg* in a *wg*-like pattern (Riggleman *et al.*, 1990). In addition, *arm* and *wg* mutants display very similar cuticular phenotypes. Consistent with the proposal that *arm* responds to the *wg* signal (Peifer and Wieschaus, 1990; Peifer *et al.*, 1991) which activates *gsb*, *gsb* expression also decays in arm^- embryos (X.Li, unpublished observation). However, since *arm* encodes a homolog of human plakoglobin, a component of desmosomes (Peifer and Wieschaus, 1990), it cannot directly activate *gsb* either.

The continuous expression of gooseberry and the stable cell fate specification

The persistent expression of segment-polarity genes probably reflects their requirement for the specification of stable cell fates. The continuous *gsb* activity is realized by the consecutive action of GEE and GLE. GLE is switched on when GEE activity drops during late stage 10. However, since no temperature-sensitive allele of *gsb* is available, it is difficult to prove the functional necessity for the maintenance phase of *gsb*. Nevertheless, indirect evidence supports its requirement. For example, we note that the cuticular phenotype of temperature-sensitive *wg* embryos that have been shifted to the non-permissive temperature during late stage 10 or stage 11 (Bejsovec and Martinez-Arias, 1991) is very similar to that of gsb^- embryos. This time interval coincides well with the observed late activation of *gsb* in response to *wg*. Therefore, the late *wg* phenotype could be explained by the loss of *gsb* activity. Alternatively, since *gsb* is also required to activate *wg* at this time (Hidalgo, 1991; X.Li, unpublished observation), the close resemblance of the *gsb* and late *wg* phenotypes might result from a failure of *wg* expression in gsb^- embryos after stage 11. In either case, GLE-directed expression of *gsb* is necessary for its function in specifying the cuticular pattern.

Is the regulation of gooseberry a common regulatory mechanism for segment-polarity genes?

In general, the maintenance of segment-polarity gene expression requires cross-regulatory interactions that are not involved in the initial establishment of their expression patterns. In our case, *gsb* is maintained by the *wg* signaling pathway. In this pathway, transcription factor(s) different from pair-rule proteins must finally activate *gsb*. These factors probably require also different sets of binding sites in the *gsb* promoter. Theoretically, the two different sets of binding sites for the two different sets of activators can be either separated or intercalated. For *gsb*, the control regions for maintenance and establishment, GEE and GLE, are separated and function independently. Since the activity of the two phases of *gsb* regulation overlap in time to permit the continued expression of *gsb*, separation rather than interdigitated control regions would avoid interference during the time of transition and hence have a selective advantage during evolution. In addition, separated control elements permit their use as 'domains' that assort independently during evolution as previously proposed (Frigerio *et al.*, 1986).

Therefore, we think that the organization of the *cis*-regulatory region into separable control elements, one responding to pair-rule proteins and the other to segment-polarity gene activities, might be a general feature of segment-polarity genes to achieve independent initiation and maintenance of their activity. Such an idea is also consistent with results obtained from a partial analysis of the *en cis*-regulatory region where various regulatory regions that might control different phases of *en* activity (Heemskerk *et al.*, 1991) have been shown to direct *en* expression in stripes (DiNardo *et al.*, 1988; Hama *et al.*, 1990; Kassis, 1990).

Materials and methods

Plasmid constructions and generation of transgenic flies

The *gsb*–*lacZ* constructs illustrated in Figure 1B–D were prepared by subcloning the genomic *gsb* fragments into Bluescript pKS^+ or its derivatives, pKSpL2 and pKSpL3, with the 5′ upstream *gsb* DNA adjacent to the *Sac*II and *Xba*I site and the transcribed *gsb* sequences next to the *Not*I site of the polylinker. Subsequently, the *gsb* sequences were excised as *Xba*I (or *Sac*II)–*Not*I fragment and ligated into a P-element/*lacZ* expression vector (CZ.1 or pWZ.1) in-frame with *lacZ*. The pKSpL2 vector was derived from Bluescript pKS^+ by destroying the *Not*I site (cleavage and ligation of blunt-ended site), removing a short stretch of the polylinker between *Hin*dIII and *Xho*I (ligation of the filled in sites restores the *Hin*dIII site), and introducing eight base pairs (GCGGCCGC) into the cleaved *Eco*RV site of the polylinker to regenerate a *Not*I site. The *Eco*RI site of pKSpL2 was destroyed (by ligation of the cleaved and filled in site) to generate pKSpL3. The CZ.1 vector was derived from HZ50PL, a P-element/*lacZ* expression vector carrying the *rosy* gene (Hiromi and Gehring, 1987), by removing hsp70 sequences 5′ to the *lacZ* coding region including the hsp70 minimal promoter and leader sequence (but retaining ~1 kb of hsp70 trailer sequences 3′ to the *lacZ* coding region). The pWZ.1 vector was constructed from CZ.1 by replacing the *rosy* gene with the *miniwhite* gene of the pW6 vector (Klemenz *et al.*, 1987).

Plasmid 9Z2 was constructed by subcloning the 8.1 kb *Bam*HI fragment of *gsb* into the *Bam*HI site of pKSpL3 to prepare 9Z2′, from which 9Z2 was obtained by excision of the *Xba*I–*Not*I fragment and ligating it into the corresponding sites of the CZ.1 vector. Plasmids 9Z1, 9Z3, 9Z2-1.6, 9Z2-3.8 and 0.6X were all derived from 9Z2′:9Z1 or 9Z3 by replacing the distal *gsb* fragment *Xba*I (polylinker)–*Xho*I (or –*Nhe*I) with the longer *Bss*HII–*Xho*I or the shorter *Eco*RI–*Nhe*I (reconstituting the *Xba*I and *Eco*RI site) *gsb* upstream fragment, respectively, followed by excision of the *gsb* sequences (*Sac*II or *Xba*I–*Not*I) and insertion into the *Xba*I/*Not*I sites of CZ.1; 9Z2-1.6 and 9Z2-3.8 were constructed by removing the 1.6 or 3.8 kb *Eco*RI fragment (by partial *Eco*RI digestion), religation and subsequent transfer of the *gsb* sequences into the *Xba*I/*Not*I sites of CZ.1; 0.6X was constructed by excising the *Xho*I–*Not*I fragment and ligation into the *Xba*I/*Not*I sites of CZ.1.

To obtain the 2.8S, 1.3N and 0.8R plasmids, the 3.8 kb *Eco*RI *gsb* upstream fragment was subcloned into pKSpL2 and the distal *gsb* fragments *Bam*HI(polylinker)–*Sal*I (–*Nsi*I or –*Rsr*II) removed to obtain, after religation, the constructs 2.8S′, 1.3N′ and 0.8R′. The final plasmids were constructed by replacing the distal *gsb* fragment *Xba*I–*Nhe*I in 9Z3 with the distal *gsb* fragments *Xba*I–*Nhe*I of 2.8S′, 1.3N′ and 0.8R′, respectively. The 0.7N plasmid was obtained from 9Z3 by excising the distal *gsb* fragment *Xba*I–*Nhe*I and religation.

The 9ZB plasmid was prepared from 9Z2 by removing the *gsb* upstream fragment *Xba*I(polylinker)–*Kpn*I (using partial *Kpn*I digestion) and religation. The 9ZL plasmid was constructed as follows. In a pKS^+ subclone of the 3.8 kb *Eco*RI *gsb* upstream fragment first the distal *Pst*I–*Nsi*I and subsequently the proximal *Xho*I fragment were removed to produce 9ZL′. Insertion of the *Kpn*I fragment of 9Z2′ into the *Kpn*I site of 9ZL′ generated 9ZL″, from which 9ZL was obtained by transferring the *gsb*

sequences as *Xba*I–*Not*I fragment into the *Xba*I/*Not*I sites of CZ.1. The 9ZE plasmid was prepared from a subclone of the 3.8 kb *Eco*RI *gsb* upstream fragment in pKSpL2, in which the proximal *Sal*I–*Not*I fragment had been removed by excision and religation to produce 9ZE′. Insertion of the *Kpn*I fragment of 9Z2′ into the *Kpn*I site of 9ZE′ generated 9ZE″, from which 9ZE was obtained by transferring the *gsb* sequences as *Xba*I–*Not*I fragment into the *Xba*I/*Not*I sites of pWZ.1.

P-element-mediated transformation of ry^{506} or w^{1118} flies was performed as described by Rubin and Spradling (1982). The number of independent transgenic lines obtained for each construct varied between 2 and 20, with the exception of 9Z2-1.6 for which only one transgenic line was generated. Different lines from the same construct showed essentially the same expression patterns.

Immunostaining of embryos

Embryos were collected and dechorionated in 50% javel water for 2 min, extensively rinsed with water, fixed for 20 min in a 1:1 mixture of heptane and 80 mM PIPES, pH 7.5, 0.8 mM $MgSO_4$, 1.6 mM EGTA, 7.4% formaldehyde, devitellinized by vortexing for a few seconds in a 1:1 mixture of methanol and heptane, and rinsed 3 or 4 times in methanol. Embryos were then rehydrated by 3 or 4 rinses in PBS containing 0.05% Tween-20 (PBST) and incubated overnight in 0.5 ml of a rabbit anti-β-galactosidase antiserum (Cappel) at a 1:2000 dilution or in 0.5 ml of an affinity-purified rabbit anti-*gsb* antiserum at a 1:100 dilution (Gutjahr *et al.*, in preparation). The embryos were rinsed briefly three times and washed three times for 20 min in PBST, and incubated for 2 h in 0.5 ml with the secondary antibody, a biotinylated goat anti-rabbit IgG (Vectastain), at a dilution of 1:500. After the embryos had been rinsed and washed as before, 0.3 ml of the preformed AB complex (Vectastain) was added and allowed to react for 1 h. After another cycle of rinses and washes, the color reaction was started by suspending the embryos in 0.5 ml of 0.1 M Tris–HCl, pH 7.0, DAB (1 mg/ml), 0.1% $NiCl_2$, 0.03% H_2O_2. The stained embryos were rinsed and mounted in 90% glycerol. Before use, all antisera were preabsorbed with 0–22 h embryos as described by Gutjahr *et al.* (1993). Double-stainings were carried out according to Lawence *et al.* (1987).

Determination of gsb transcriptional start site

The transcriptional start site of *gsb* was determined by primer extension (Kingston, 1989), using the sequence 5′-TTGGAGTGTGTATCTGCTG-TTGCGAGTGCG-3′ (complementary to that of nucleotides 44–73 in Figure 1E) as primer.

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