Localized expression of *sloppy paired* protein maintains the polarity of *Drosophila* parasegments

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During germ-band extension in the *Drosophila* embryo, intercellular communication is required to maintain gene expression patterns initiated at cellular blastoderm. For example, the *wingless* (*wg*) single-cell-wide stripe in each parasegment (PS) is dependent on a signal from the adjacent, posterior cells, which express *engrailed* (*en*). This signal is thought to be the *hedgehog* (*hh*) gene product, which antagonizes the activity of *patched* (*ptc*), a repressor of *wg* expression. Genetic evidence indicates that the *hh* signal is bidirectional, but *wg* transcription is only derepressed on the anterior side of the *en*/*hh* stripes. To explain the asymmetric response of the *wg* promoter to the *hh* signal, current models predict that each PS is divided into cells that are competent to express either *wg* or *en*, but not both. The *sloppy paired* (*slp*) locus contains two transcription units, both encoding proteins containing a forkhead domain, a DNA-binding motif. Removal of *slp* gene function causes embryos to exhibit a severe pair–rule/segment polarity phenotype. We show that the *en* stripes expand anteriorly in *slp* mutant embryos and that *slp* activity is an absolute requirement for maintenance of *wg* expression at the same time that *wg* transcription is dependent on *hh*. The *slp* proteins are expressed in broad stripes just anterior of the *en*-positive cells, overlapping the narrow *wg* stripes. We propose that by virtue of their ability to activate *wg* and repress *en* expression, the distribution of the *slp* proteins define the *wg*-competent and *en*-competent groups. Consistent with this hypothesis, ubiquitous expression of *slp* protein throughout the PS abolishes *en* expression and, in *ptc* mutant embryos, results in a near ubiquitous distribution of *wg* transcripts. In addition to demonstrating the role of *slp* in maintaining segment polarity, our results suggest that *slp* works in, or parallel with, the *ptc*/*hh* signal transduction pathway to regulate *wg* transcription.

[*Key Words*: *sloppy paired*; forkhead domain; segment polarity; *wingless*; *engrailed*]

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The establishment of segmentation in the *Drosophila melanogaster* embryo has become a paradigm for the study of pattern formation. Mutant screens have found several classes of zygotic genes specifically affecting this process, which serve to subdivide the embryo into increasingly smaller units. Gap gene mutations result in deletions of several adjacent segments; pair–rule mutants lack alternate segments; and segment polarity mutants lack portions of each segment (Nüsslein-Volhard and Wieschaus 1980). These genes act in a hierarchial manner, with information flowing from the gap genes to the pair–rule, and these in turn regulate the segment polarity genes (Akam 1987; Scott and Carroll 1987; Ingham 1988).

Two important targets of this regulation are the segment polarity genes *wingless* (*wg*) and *engrailed* (*en*). *wg* is required for the formation of naked cuticle in the posterior portion of each segment (Nüsslein-Volhard and Wieschaus 1980; Baker 1988; Bejsovec and Martinez Arias 1991; Noordermeer et al. 1992) and *en* for the generation of segmental borders (Kornberg 1981; DiNardo and O'Farrell 1987; Martinez-Arias and White 1988). The two genes are expressed during embryogenesis in adjacent but nonoverlapping stripes of a single-segment periodicity, with the *wg* stripes located anterior to those of *en*. At full germ-band extension, the parasegmental grooves, the first overt sign of segmentation in *Drosophila* embryos (Martinez-Arias and Lawrence 1985), form where the *wg* and *en* stripes abut (Ingham et al. 1985a; Baker 1987; van den Heuvel et al. 1989).

After establishment as single-cell-wide stripes at cellular blastoderm, *wg* and *en* are required to maintain each other's expression during germ-band elongation (DiNardo et al. 1988; Martinez Arias et al. 1988). Because they are not expressed in the same cells, this regulation

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must involve cell–cell communication. The *wg* gene encodes a cysteine-rich protein (Rijsewijk et al. 1987) that is secreted (van den Heuvel et al. 1989, 1993; Gonzalez et al. 1991) and is thought to be the signal to the *en* cells. The *en* gene contains a homeo box (Fjose et al. 1985; Poole et al. 1985) and encodes a nuclear protein (DiNardo et al.1985), so it is unlikely to be the signal to the *wg* cells. The signal is thought to be the product of the *hedgehog* (*hh*) gene, which is expressed in stripes coincident with those of *en*. *hh* is predicted to encode a cell-surface protein (Lee et al. 1992; Mohler and Vani 1992; Tabata et al. 1992) and appears to be processed to form a secreted protein (Lee et al. 1992; Taylor et al. 1993). The *hh* gene product interferes with the activity of *patched* (*ptc*), a protein with several predicted membrane-spanning domains (Hooper and Scott 1989; Nakano et al. 1989) found at the cell surface (Ingham et al. 1991; Taylor et al. 1993), which functions as a repressor of *wg* expression (Ingham et al. 1991; Bejsovec and Wieschaus 1993). Thus, a model has emerged where *en* protein activates *hh* transcription, and the secreted *hh* protein somehow interferes with the inhibitory signal that the *ptc* protein sends to the nucleus of the *wg*-expressing cells.

During germ-band extension, the regulation of *ptc* expression is in many ways similar to that of *wg*. *ptc* represses its own transcription and *hh* somehow antagonizes this activity (Hildalgo and Ingham 1990; Forbes et al. 1993). This leads to a *ptc* expression pattern of single-cell-wide stripes on either side of the *en*/*hh* stripes (Hooper and Scott 1989; Nakano et al. 1989), presumably because these are the only cells that receive enough *hh* protein to inactivate *ptc* (Ingham 1993). In constrast, *wg* transcription is only derepressed by *hh* in cells on the anterior side of the *en*/*hh* expression domain. This raises the question of how bidirectionally secreted signals, for example, the *hh* protein, can act to generate the polarized responses that are observed in the embryonic segments, for example, expression of *wg* on only one side of the *en* stripes (Hooper and Scott 1992). One possibility is that in addition to *hh*, *wg* transcription at this time depends on preexisting *wg* protein. Thus, *wg* autoactivation would maintain the asymmetric distribution of *wg* transcripts that was originally set up by the pair–rule genes. However, analysis of *wg* expression in pair–rule, *ptc* double mutants indicates that regulation of *wg* expression by *hh* and *ptc* is not coupled to the earlier acting pair–rule genes (Ingham and Hidalgo 1993). Therefore, to explain the polarized response of the *wg* promoter to the *hh* protein, Ingham and co-workers (1991) proposed that each parasegment (PS) is divided into two groups; the cells in the posterior half of the PS are *wg*-competent, and the anterior half are *en*-competent. They suggested that these competency groups are established at cellular blastoderm by the pair–rule genes and provide the foundation on which asymmetry is later maintained.

This report is concerned with the role of the *sloppy paired* (*slp*) locus in segmentation. The locus contains two transcription units, *slp1* and *slp2*, both encoding proteins containing a *forkhead* domain (Grossniklaus et al. 1992), a DNA-binding motif (Weigel and Jäckle 1990; Clark et al. 1993). Deletion of both *slp* genes causes a severe pair–rule/segment polarity defect (Grossniklaus et al. 1992). The results presented here demonstrate that *slp* is an activator of *wg* and a repressor of *en* expression, and through these activities, the distribution of *slp* defines the competency groups described above, that is, cells lacking *slp* protein are *en*-competent and cells containing *slp* protein are *wg*-competent. This hypothesis was confirmed by ubiquitous expression of *slp* (via a heat shock promoter), which abolishes *en* expression and, in *ptc* mutant embryos, where *wg* expression is largely independent of *en* and *hh* (Ingham et al. 1991; Tabata et al. 1992; Bejsovec and Wieschaus 1993), results in a near ubiquitous expression of *wg*. Thus, the localized expression of *slp* is required to maintain the proper anterior border of the *en* stripes and to restrict *wg* expression to one side of the *en*/*hh* domain.

Results

slp mutant embryos have characteristics of pair–rule mutants (i.e., fusions of abdominal segments A1–A2, A3–A4, etc.), as well as features of *wg*-class segment polarity mutants, for example, replacement of naked cuticle by denticles (Fig. 1B). This report is concerned only with the phenotype of embryos lacking both *slp* gene activities. The respective contributions of *slp1* and *slp2*

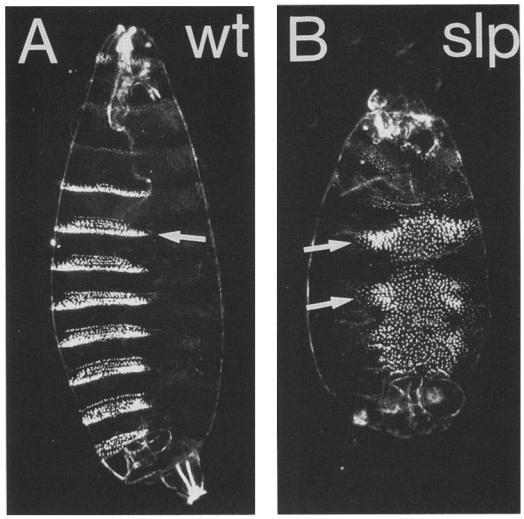

Figure 1. The cuticular phenotype of a *slp* null mutant. (*A*) Ventral view of a wild-type (wt) first instar larvae, the trunk of which is divided into three thoracic and eight abdominal segments (A1–A8). The anterior half of each segment contains a trapezoidal array of hooks known as a denticle belt (the arrow indicates the A2 dentricle belt). (*B*) Terminally differentiated embryo homozygous for Δ34B, a deficiency removing both *slp* transcripts. Notice the pair–rule type fusions of A1–A2 and A3–A4 (see arrows). The naked cuticle normally found in the posterior part of each segment is either missing or replaced by denticles.

to the phenotypes described here are reported elsewhere (Grossniklaus et al. 1992; Cadigan et al. 1994).

Expression of segment polarity and pair–rule genes in slp *mutants*

To understand the *slp* mutant phenotype in more detail, we examined the expression of all pair–rule and zygotic segment polarity genes for which probes were available in *slp* mutants. The most instructive probes in regard to explaining the *slp* mutant cuticle phenotype were those for *wg* and *en* (Fig. 2). *en* expression is an excellent marker for segmentation, for example, pair–rule mutants lacking alternate segments also lack the corresponding *en* stripes (DiNardo and O'Farrell 1987; Martinez-Arias and White 1988). *wg* is believed to specify naked cuticle in each segment and all the zygotic *wg*-class segment polarity genes are thought to exert their effects through *wg* (Peifer and Bejsovec 1992).

In *slp* mutant embryos, *en* expression is initiated normally, except for a wider stripe in the maxillary (Mx) segment primordia (data not shown; DiNardo and O'Farrell 1987). However, during germ-band elongation (stage 8; stages correspond to those described in Campos-Ortega and Hartenstein 1985), all of the *en* stripes widen (Fig. 2, cf. A and B). When germ-band elongation is complete (stage 10), the odd-numbered PS *en* stripes are greatly reduced in intensity, and the even-numbered ones are one to two cells wider than wild type (Fig. 2, cf. C and D).

wg expression is also initiated normally in *slp* mutants, but during early germ-band extension (stage 7), the even-numbered PS stripes begin to fade, so that by stage 9, they are completely gone (Fig. 2F). Shortly thereafter, the remaining stripes rapidly fade and are completely gone at early stage 10 (Fig. 2H). The misexpression of *en* and *wg* in the head and gnathal region (see legend to Fig. 2) will be discussed in more detail elsewhere (U. Grossniklaus, K.M. Cadigan, and W.J. Gehring, in prep.).

Figure 3 shows the expression of some other segment polarity genes in *slp* mutants. In the trunk, *hh* is expressed in the same cells as *en* and is regulated in an identical fashion (Lee et al. 1992; Mohler and Vani 1992; Tabata et al. 1992). Likewise, in *slp* embryos *hh* expression parallels that of *en* (Fig. 3B). The *gooseberry* (*gsb*) gene is expressed in two-cell-wide stripes overlapping the *wg* stripes and the anterior half of the *en* stripes (Gutjahr et al. 1993). In *slp* embryos, *gsb* mRNA decays in a fashion similar to *wg* but with a temporal lag. At early stage 10, the stripes overlapping the even-numbered PS *wg* stripes are completely gone from the epidermis but, unlike *wg*, the remaining *gsb* stripes are still largely intact (Fig. 3D), though they too will fade by stage 11. At stage 11, *ptc* is expressed in single-cell stripes on either side of the *en* domains (Fig. 3E; Hooper and Scott 1989; Nakano et al. 1989). In *slp* mutants, only half of these doublets are found, presumably the ones flanking

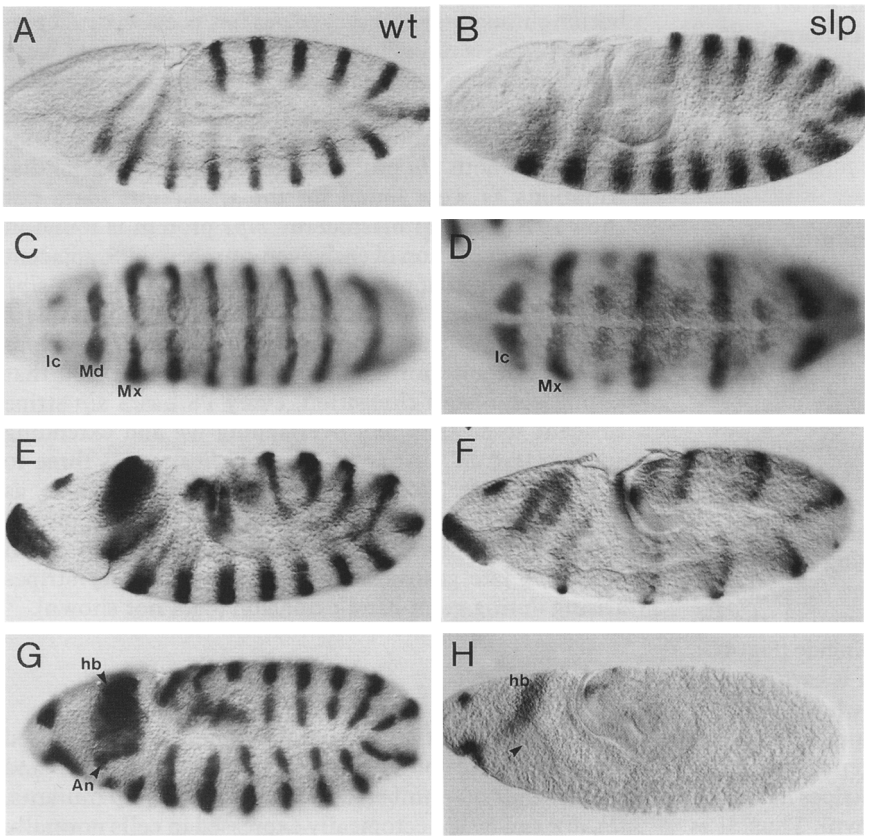

Figure 2. Distribution of *en* (*A–D*) and *wg* transcripts (*E–H*) in wild type (*left*) and *slp* (Δ34B) mutants (*right*). During stage 8, the *en* stripes widen so that by early stage 9, they are significantly wider than wild type (cf. *A* and *B*). Subsequently, the *en* stripes in the odd-numbered PSs begin to fade, as seen in the ventral view in *D*. The mandibular (Md) stripe is completely gone in *slp* mutants, and the intercalary (Ic) domain is greatly expanded. At the beginning of germ-band extension, the *wg* stripes in the even-numbered PSs begin to fade and are completely gone by stage 9 (*F*). The head blob (hb) *wg* domain is greatly reduced and the antennal domain is absent (cf. *G* and *H*; see arrowheads). The remaining stripes fade rapidly and are completely gone by early stage 10 (*H*). (Mx) maxillary segment primodia.

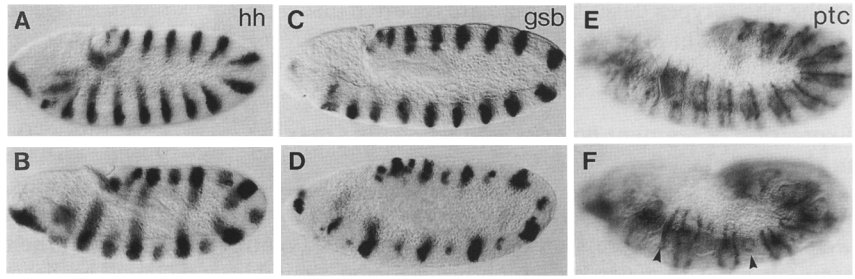

Figure 3. Distribution of *hh* (*A*, *B*), *gsb* (*C*, *D*), and *ptc* transcripts (*E*, *F*) in wild-type (*top*) and *slp* (Δ34B) mutants (*bottom*). The expression of *hh* in the trunk is identical to that of *en*, i.e., a widening of the stripes and the odd-numbered PS stripes fading (*B*). The *gsb* stripes in the even-numbered PSs fade during germ-band extension so that only neuroblast expression is seen at stage 10 (*D*). The remaining stripes fade during stage 11. In early stage 12 wild-type embryo *ptc* is expressed in single-cell stripes on each side of the *en* stripes (*E*; see Hooper and Scott 1989; Nakano et al. 1989). In *slp* mutants at this stage only half of the stripe doublets remain (though some *ptc* expression remains between the doublets; see arrowheads), and the space between each stripe is greater than in wild type (*F*).

the even-numbered PS *en* stripes, although some remnants between them also remain (Fig. 3F).

We have restricted our analysis of pair–rule gene expression in *slp* mutants from syncitial blastoderm to mid-germ-band extension (stage 8), the time when these genes are thought to act in the segmentation hierarchy. *runt* (*run*), *odd-skipped* (*odd*), and *hairy* (*h*) show no detectable misexpression in *slp* embryos (data not shown). *paired* (*prd*) has a slight widening of its first and second stripes, but no other difference (data not shown; Baumgartner and Noll 1991). The pattern of *fushi tarazu* (*ftz*) also begins normally, but during late gastrulation/early germ-band elongation, seven extra stripes arise (Fig. 4A). Double-staining experiments show that these extra stripes are in the posterior-most cells of each even-numbered PS, adjacent to the odd-numbered PS *en* stripes (Fig. 4B). *even-skipped* (*eve*) expression is normal until stage 8, when it broadens, coinciding with the *en* stripe broadening (Fig. 4C, D).

Direction of en *expansion and PS groove formation in* slp *mutants*

To determine the direction of *en* domain broadening in *slp* embryos, two experiments were performed. The first was to examine *gsb* and *en* expression in *slp* embryos. In wild type, as stated above, *gsb* is expressed in two-cell wide-stripes, with the posterior-most cells overlapping with the anterior-most *en* cells (Fig. 4E). In late stage 9 *slp* mutants, the even-numbered PS *en* stripes are widened, and there is a two-cell overlap with the *gsb* stripes (Fig. 4F). The simplest explanation for this is that the *en* stripes have expanded in an anterior direction. To confirm this, we determined *en* and *lacZ* distribution in *slp* mutants containing a chromosome carrying P[ftz–lacC], which puts *lacZ* under the control of the *ftz* promoter (Hiromi et al. 1985). In a wild-type background, the anterior border of *lacZ* and *en* expression coincide (Fig. 4G). In a *slp* mutant background, however, *en* is expressed in a row of cells anterior of *lacZ* (Fig. 4H). We have found no evidence for the anterior border of *ftz* expression changing in *slp* mutants, for example, the anterior borders of *ftz* and *en* coincide (Fig. 4B) until the *ftz* stripes fade prior to the *en* stripes widening (data not shown). Thus, it is extremely unlikely that the *lacZ* stripes have shifted posteriorly in the *slp* mutants, and we conclude that in *slp* mutants, the *en* stripes expand anteriorly.

The first sign of segmentation in *Drosophila* embryos is the parasegmental grooves, which are first apparent at early stage 10 (Campos-Ortega and Hartenstein 1985; Martinez-Arias and Lawrence 1985). *en* is found just posterior of the grooves (Ingham et al. 1985a). In *slp* mutants, the grooves still form to the anterior of *en* (Fig. 4I); therefore, the formation of the PS grooves in *slp* mutants is one or two cells anterior that of wild type.

Expression pattern of slp *protein*

A detailed description of *slp1* and *slp2* transcript distribution throughout embryogenesis has been reported previously (Grossniklaus et al. 1992). *slp* transcripts were found in stripes two cells wide, initially with a double and then a single segment periodicity. The transcripts were localized to the posterior half of each PS. Antibodies against both *slp* proteins show identical spatial distributions as were found for the transcripts (data not shown). At cellular blastoderm, *slp1* protein is found in the posterior portion of each even-numbered PS, adjacent to, but not overlapping, *eve* protein (Fig. 5A). The *slp1* protein is localized to the nucleus as is that of *slp2* (data not shown). During gastrulation, the secondary stripes appear, just anterior of the *ftz* stripes (Fig. 5B), so that during germ-band elongation, every PS has a *slp* stripe adjacent to *en* (Fig. 5E), overlapping *wg* and extending anterior (Fig. 5C). At stage 10, this *slp* stripe is three to four cells wide. The *slp2* protein expression pattern, as was reported previously for that of the transcript, develops later than *slp1*. Protein is first detected in seven stripes at late gastrulation, with the secondary stripes arising during germ-band extension (data not shown).

Is activation of wg *expression by* slp *direct?*

Consistent with its expression in *wg* cells, *slp* is required for maintenence of *wg* expression, first in the even- and then in the odd-numbered PSs. However, in *slp* mutants, *ftz*, *eve* and *en* are ectopically expressed in cells normally

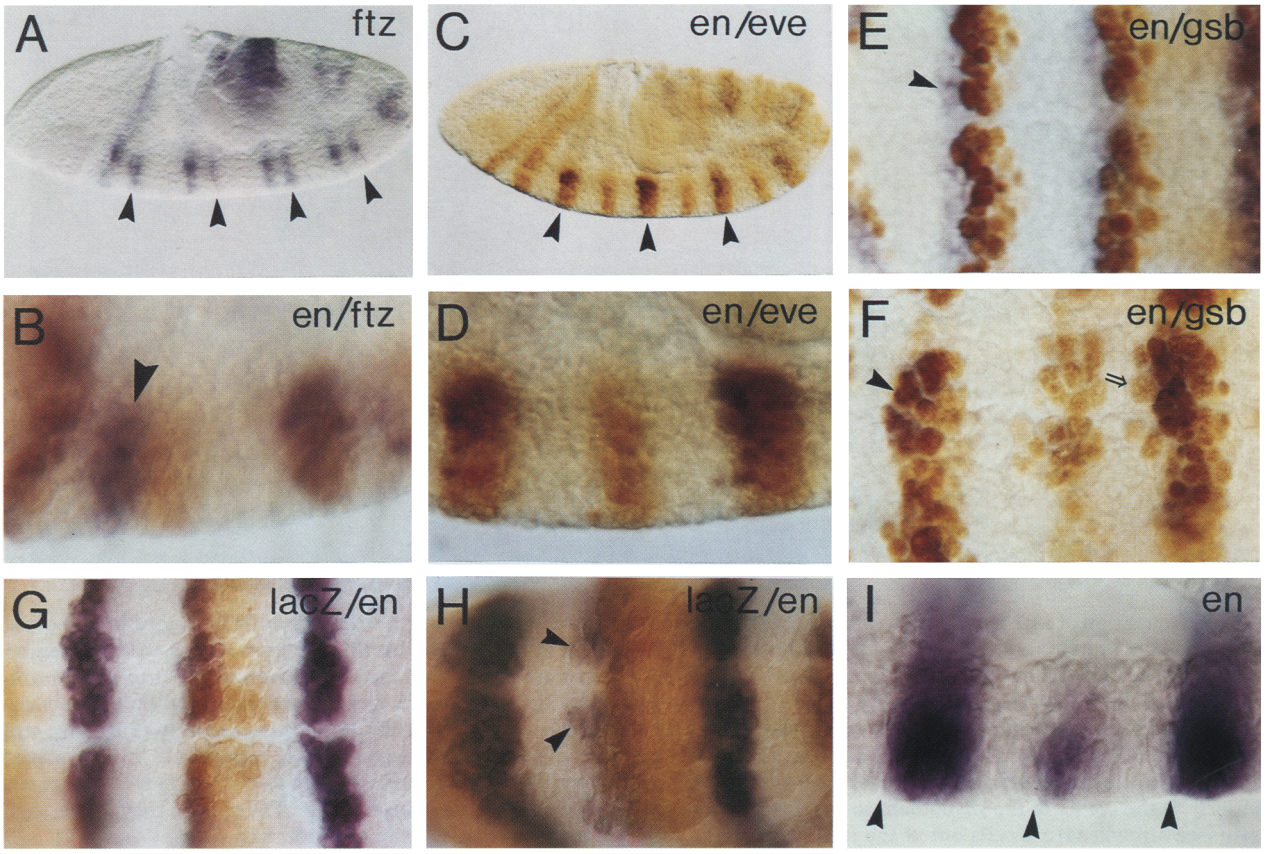

Figure 4. Derepression of *ftz*, *eve*, and *en* expression in *slp* (Δ34B) mutants. (*A*) *ftz* protein distribution in a *slp* mutant at stage 8. During germ-band extension, seven additional stripes arise in the posterior part of the even-numbered PSs (arrows). (*B*) *slp* mutant stained for *en* (orange) and *ftz* (blue) proteins. The extra stripe in PS2 (*arrow*) lies just anterior to the PS3 *en* stripe. (*C*) Stage 8 *slp* mutant stained for *en* (orange) and *eve* protein (blue; overlap is black). The *en* stripes in the odd-numbered PSs (arrows) are wider than the even-numbered ones, which will broaden shortly afterward. (*D*) Higher magnification of *C*. Note that the anterior border of the *eve* and *en* stripes coincide. (*E*) Late stage 9 wild-type embryo stained for *en* protein (orange) and *gsb* transcript (blue). At this time, *gsb* is expressed in a two-cell stripe that straddles the PS border, so that the posterior-most row of cells overlap with the anterior-most row of *en* cells (arrow). (*F*) *slp* embryo stained as in *E*. The *en* stripes in the odd-numbered PS are wider, and the anterior border of the *en* and *gsb* stripes now coincide (arrow) or occasionally *en*-staining cells are found anterior of the *gsb* stripes (open arrow). (*G*) Stage 8 embryo containing a *ftz–lacZ* construct that expresses *lacZ* in a *ftz*-like pattern. The embryo is stained for *lacZ* protein (orange) and *en* transcript (blue). The anterior border of the *en* and *lacZ* stripes are identical. (*H*) Stage 8 *slp* mutant containing the *ftz–lacZ* transgene stained as in *G*. The anterior-most *en* cells of the even-numbered PS shown (arrows) does not stain for *lacZ*, indicating the expansion of the *en* stripes in *slp* mutants is in the anterior direction. (*I*) *en* expression in a stage 10 *slp* embryo. The parasegmental grooves are indicated (arrows).

expressing *wg*. There is strong evidence for *ftz* and *eve* being repressors of *wg* transcription (Ingham et al. 1988; Ish-Horowicz et al. 1989; Manoukian and Krause 1992), and *en* has been suggested to repress *wg* expression (Heemskerk et al. 1991). It may be that *slp* is not a direct activator of *wg* transcription but, rather, activates *wg* expression by repressing a *wg* repressor(s). This possibility can be tested by analyzing *wg* expression in the appropriate double mutants. If *slp* is activating *wg* by repressing a *wg* repressor, then *wg* expression in *slp* mutants lacking this repressor should persist longer than in *slp* mutants alone.

A dramatic derepression of *wg* transcipts is seen in *ftz* and *eve* mutant embryos, such that all the cells in the even- or odd-numbered PSs express *wg*, respectively (Ingham et al. 1988). In *eve* mutants, the PS-wide *wg* stripes fade during germ-band elongation (Ingham and Hidalgo 1993). *wg* mRNA in *slp*, *eve* double mutants behaves similarly as in *eve* embryos, except that the disappearance of *wg* transcripts occurs slightly earlier (data not shown). In the crosses performed to create *slp*; *ftz* mutants, only 1/16 of the progeny are double mutants. We were not able to unambiguously identify double mutants after staining for *wg* transcripts (data not shown). We reasoned that we could assay the presence of *wg* in the even-numbered PSs by monitoring the adjacent *en* stripes, which require *wg* expression for proper maintanence (Bejsovec and Martinez Arias 1991; Heemskerk et

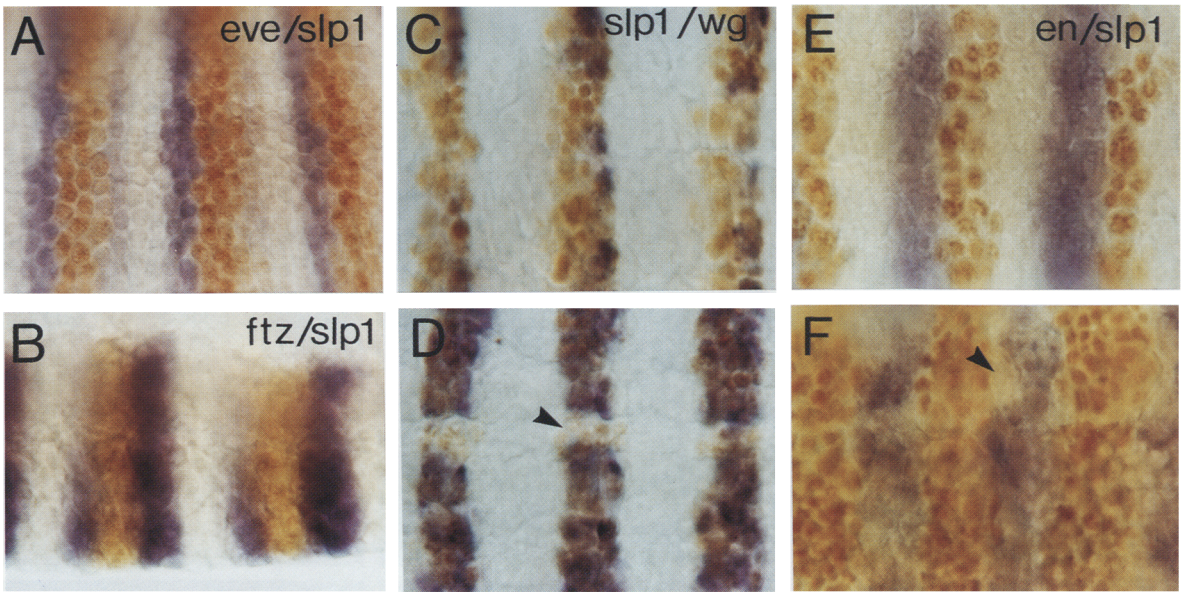

Figure 5. Expression of *slp1* in relation to other segmentation genes. (*A*) Cellular blastoderm wild-type embryo stained for *eve* protein (orange) and *slp1* protein (blue). The primary *slp1* stripes are just anterior of the *eve* stripes. (*B*) Late gastrulating wild-type embryo stained for *ftz* protein (orange) and *slp1* protein (blue). The primary stripes are posterior of *ftz*, and the secondary stripes are forming just anterior of the *ftz* domain. (*C*) Stage 10 wild-type embryo stained for *slp1* protein (orange) and *wg* transcript (blue; the overlap appears black). The *slp1* stripes overlap the *wg* stripes and extend 2 or 3 cells anterior. (*D*) ptc^{IN} mutant stained as in *C*. The *wg* stripe expands anteriorly to fill the *slp* domain (except for the ventral midline where *wg* is not expressed; see arrowhead). (*E*) Late stage 10 wild-type embryo stained with *en* protein (orange) and *slp1* transcript (blue). (*F*) nkd^{7E} mutant stained as in *E*. *en* expression expands posteriorly to the border of the *slp* domain so that every cell in the ventral trunk stains orange or blue. The regions that do not appear to stain (see arrowhead) are cells out of the focal plane, due to the deep grooves that form in *nkd* mutants.

al. 1991). In *slp* mutants, the odd-numbered *en* stripes fade (Fig. 6A) and in *ftz* mutants, the even-numbered ones are missing (Fig. 6B). In the double mutants, the odd-numbered stripes still decay (Fig. 6C). Therefore, in the absence of *ftz* or *eve* activity, *wg* expression is still dependent on functional *slp* protein.

In constrast to the results with *ftz* and *eve* described above, *en* activity was found to be partially responsible for the disappearance of *wg* transcripts in *slp* mutants. At the time when the even-numbered PS *wg* stripes are completely gone in *slp* embryos (Fig. 6D), they are still present in *slp*, *en* double mutants (Fig. 6E). At early stage 10, when the *wg* stripes are completely gone in *slp* mutants, *wg* transcripts are still found in the double mutants (cf. Fig. 2H with Fig. 6F). However, much of the *wg* staining in the *slp*, *en* mutants is not epidermal but, rather, is found in the underlying neuroblasts (Fig. 6F). Thus, *wg* expression in the epidermis is still dependent on *slp* in the absence of functional *en* protein. However, ectopic *en* expression does contribute to the disappear-

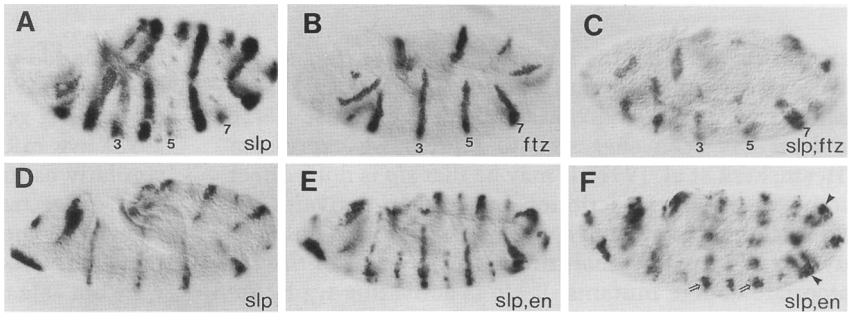

Figure 6. Distribution of *en* transcripts (*A–C*) in *slp*; *ftz* (Δ34B; ftz^{9H}) mutants and *wg* transcripts (*D–F*) in *slp*, *en* ($Df(2L)ed^{SZ1}$, *Df(2R)en-E*) mutants. (*A*) *en* expression in a *slp* mutant, where the odd-numbered PS stripes fade (PS stripes 3, 5, and 7 are indicated). (*B*) *en* expression in a *ftz* embryo. The even-numbered PS stripes never arise. (*C*) *en* expression in a *slp*; *ftz* double mutant. The odd-numbered PS stripes fade as in *slp* mutants. (*D*) *wg* expression in a stage 9 *slp* embryo. The even-numbered stripes have completely faded by this time. (*E*) *wg* transcripts in a late stage 9 *slp*, *en* double mutant. Even though this embryo is older than the one shown in *D*, the even-numbered stripes, though faded, are still readily visible. (*F*) *slp*, *en* stage 10 embryo. In *slp* mutants, *wg* is completely gone from the trunk at this time (Fig. 2H). In the double mutant, staining in the neuroblasts is still strong (*open arrows*), and some epidermal staining also remains (*arrows*). *wg* expression in these regions completely fades by early stage 11.

ance of *wg* transcripts in *slp* mutants, most notably in the neuroblasts, and, to a lesser extent, in the epidermis.

In *ptc* mutants, the single-cell-wide *wg* stripes expand 2–3 cells anteriorly (Martinez-Arias et al. 1988; Ingham et al. 1991). Double staining for *slp1* protein and *wg* transcripts in a *ptc* mutant reveals that *wg* expands only to the border of *slp* expression (Fig. 5D), for example, every cell expressing *wg* in the PS also expresses *slp1* (and, by deduction, *slp2*, because it is expressed in the same cells as *slp1*). The width of the *slp* stripes does not change in *ptc* mutants. To determine whether *slp* is required for the *ptc*-dependent *wg* expansion, we examined *wg* expression in *slp*, *ptc* double mutants. As seen in Figure 7, A and C, *wg* expression fades in the double mutant just as it does in *slp* mutants, demonstrating that the ectopic expression of *wg* in *ptc* mutants requires *slp*.

en *expression is restricted by overlapping* slp *and* naked *activities*

In *naked* (*nkd*) mutants, *en* expression expands posteriorly (DiNardo et al. 1988; Martinez Arias et al. 1988). When *nkd* mutants are stained for *slp1* transcripts and *en* protein (Fig. 5F) it was found that the *en* protein expanded just to the border of *slp* expression (the width of the *slp* stripes were unchanged in *nkd* mutants), such that all cells in the ventral epidermis express either *en* or *slp1* (but never both). This raised the possibility that *slp* limits the posterior expansion of *en* in *nkd* mutants. In *slp*; *nkd* double mutants, *en* protein is found in almost all epidermal cells of the embryonic trunk (Fig. 7E, F). There are cells not expressing *en*, but these gaps are not segmental in periodicity, and several adjacent PSs can be found where all cells are *en*-positive. The simplest model would propose that within each PS, either *slp* or *nkd* can repress *en* and that they have overlapping expression/activity domains (see Fig. 10).

Effect of ectopic slp *protein on* wg *expression*

Experiments of Ingham et al. (1991) have led to a model for the regulation of *wg* transcription during germ-band extension (see Fig. 10, below). The model predicts that the activity of *ptc*, a repressor of *wg*, is antagonized by *hh*, which is expressed in the *en* cells and is predicted to encode a surface protein that is processed into a secreted protein (Lee et al. 1992; Mohler and Vani 1992; Tabata et al. 1992; Taylor et al. 1993). The model, however, cannot explain why *hh* only activates the *wg* promoter on the anterior side of the *en*/*hh* cells. To reconcile this and explain the expression of *wg* and *en* in *ptc* and *nkd* mutants, Ingham et al. (1991) proposed that only the cells in the posterior half of each PS are competent to express *wg*, whereas cells in the anterior half are *en*-competent.

The results presented so far in this paper suggest that the distribution of the *slp* proteins, which function as activators of *wg* and repressors of *en* expression, may define these competence groups. If this were the case, then expression of *slp* in the anterior half of the PS should lead to ectopic *wg* expression there. Constructs were made in which either *slp1*- or *slp2*-coding sequences were placed under the control of a heat shock promoter and transgenic lines were created (see Materials and methods). A summary of the effect of ubiquitous expression of *slp* on *wg* transcript distribution is shown in Figure 8.

The embryo in Figure 8A received its first heat shock at late syncitial blastoderm. This represents the most common class of phenotypes observed after heat shock at this time, with some embryos showing less *wg* transcript expansion and a few expressing *wg* almost everywhere in the trunk of the embryo. The expansion of the *wg* stripes in these embryos is probably attributable to ectopic *slp* protein repressing *eve* and, to a lesser extent, *ftz* (Cadigan et al. 1994). The *wg* expression pattern in

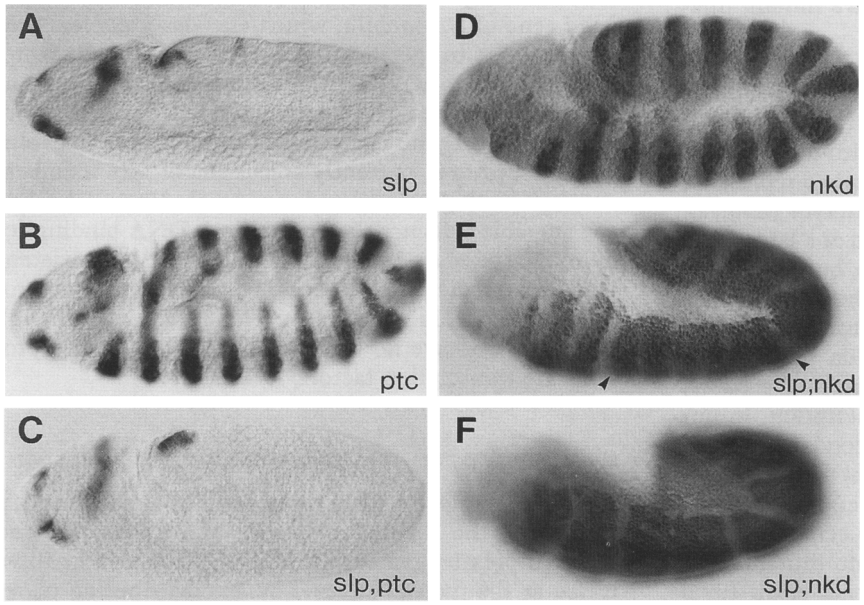

Figure 7. Expression of *wg* transcripts (*left*) in *slp*, *ptc* (*Df(2L)ed*^{SZ1}, *ptc*^{IN}) embryos and *en* protein (*right*) in *slp*; *nkd* (Δ34B; *nkd*^{7E}) embryos. (*A*) *slp* embryo at stage 10. *wg* mRNA has faded from the trunk. (*B*) *ptc* embryo at stage 10. The characteristic wider *wg* stripes are observed. (*C*) *slp*, *ptc* double mutant at stage 10. The expression of *wg* in these mutants is identical to *slp* mutant embryos. (*D*) *nkd* mutant at stage 10 displaying the broader *en* stripes. (*E*) *slp*; *nkd* mutant at stage 10. Although not every cell in the trunk is expressing *en*, the arrows indicate a stretch of 6 PSs where virtually all epidermal cells are *en*-positive. (*F*) *slp*; *nkd* mutant at stage 10. As in *E*, several segments possess ubiquitous *en* expression.

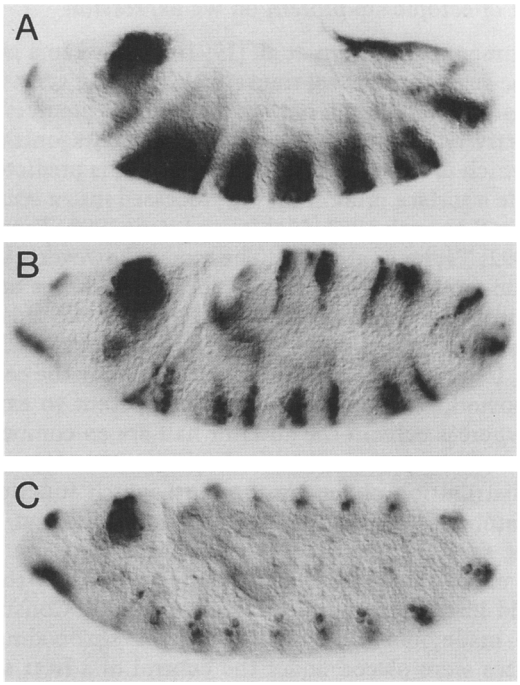

Figure 8. Expression of *wg* transcripts after ubiquitious expression of *slp1*. (*A*, *B*) P[HS–slp1C] embryos received two 5-min heat shocks (at 37°C) separated by 55 min and were fixed 40 min after the second heat shock. The embryo shown in *A* received its first heat shock at approximately late syncitial blastoderm. The *wg* stripes in this embryo are expanded, similar to what occurs in *eve* mutant embryos. The embryo in *B* was first heat-shocked at late cellular blastoderm. Spacing of the stripes is slightly irregular, and some ectopic expression is still observed. (*C*) This embryo was heat-shocked at approximately the same time as that in *B* but was allowed to develop an additional 40 min before fixation. *wg* expression in the epidermis has faded, with only the neuroblast expression remaining.

Figure 8A is very similar to that of an *eve* mutant (Ingham et al. 1988).

Heat shocks first administered after cellularization do not show this *eve* mutant-like expansion (Fig. 8B), and little ectopic *wg* expression is observed. If these embryos are allowed to develop, *wg* mRNA fades from the epidermis (Fig. 8C). This is probably attributable to repression of *hh* and *en* expression in P[HS–slp] embryos (Cadigan et al. 1994). If the heat shocks are applied at a later time, the fading of *wg* transcripts is less severe, but ectopic *wg* expression is never observed (data not shown).

Our hypothesis is that during germ-band extension, *wg* requires two inputs for its expression to be maintained properly: (1) *hh* protein to antagonize *ptc* activity, and (2) *slp* protein to activate *wg* transcription. In our HS–slp experiments, providing the second input (*slp*) ubiquitously removes the first input (*hh*) so that no ectopic *wg* expression can be induced. To circumvent this problem, we examined the effect of *slp* ectopic expression in a *ptc* mutant background. In *ptc* mutants, *wg* maintenance is independent of *en* (Tabata et al. 1992) and largely independent of *hh* (Ingham et al. 1991; Bejsovec and Wieschaus 1993). The results of these experiments are shown in Figure 9.

Induction of *slp* protein in *ptc* mutants caused a dramatic expansion of *wg* transcripts (Fig. 9C–F) compared with non-heat-shocked controls (Fig. 9A, B). The heat shock regime was critical. One heat shock was not sufficient (even for 15 min), and a 5-min heat shock followed by a 10-min one (with 55 min recovery in between) gave the best results. If the first heat shock was administered after gastrulation, the expansion was limited. However, the heat shock could not be too early, or the expansion caused by *eve* repression would occur (see Fig. 8A). Therefore, the first heat shock was given between cellular blastoderm and gastrulation. Under these conditions, no ectopic *wg* expression was seen in P[HS–slp1] embryos. In heat-shocked P[HS–slp1] *ptc* embryos, the expansion did not occur until late stage 9 (data not shown), the same time as ectopic *wg* expression occurs in *ptc* mutants (Ingham and Hidalgo 1993).

As seen in Figure 9, C and D, *wg* mRNA does not always fill the entire PS in P[HS–slp1] *ptc* mutants. To determine which cells are not expressing *wg* in these embryos, we stained P[ftz–lacC]; P[HS–slp1], *ptc* embryos for *lacZ* protein and *wg* transcripts after heat shock. P[ftz–lacC] expresses *lacZ* in a *ftz*-like manner, for example, the anterior border of *lacZ* marks the anterior border of the PS. This experiment demonstrated that the cells not expressing *wg* lie in the anterior-most part of the PS (data not shown), where *en* is normally expressed.

Discussion

The slp *proteins are putative transcription factors*

The DNA sequence of the *slp1* and *slp2* transcription units (Grossniklaus et al. 1992) revealed an extensive region of homology that is now known as the forkhead domain (Weigel and Jäckle 1990), named after the *forkhead* gene of *Drosophila*, which encodes a nuclear protein involved in specification of fore- and hindgut structures (Weigel et al. 1989). This domain is also found in several hepatocyte DNA-binding transcription factors (Lai et al. 1991) and is conserved throughout the evolutionary spectrum, currently consisting of >25 members (Häcker et al. 1992; Clevidence et al. 1993). There is considerable evidence that it encodes a DNA-binding domain (Costa et al. 1989; Clark et al. 1993). Consistent with this predicted DNA-binding activity, the *slp* proteins are found in the nucleus (Fig. 5; data not shown). Although we have no direct evidence that the *slp* proteins are transciption factors, it is likely.

slp *has both a pair–rule and segment polarity function*

As described previously (Grossniklaus et al. 1992) and in this paper, the *slp* mutants have both a pair–rule and segment-polarity phenotype (Fig. 1). This is best illustrated at the molecular level by the distribution of *wg*

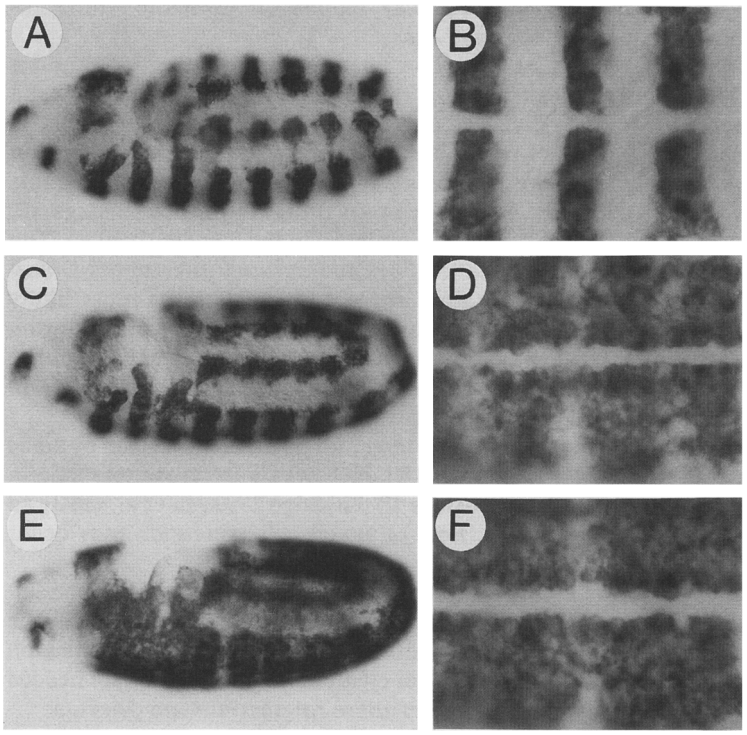

Figure 9. Expression of *wg* transcripts after ubiquitous expression of *slp1* in a *ptc* mutant background. (*A*, *B*) Non-heat-shocked control showing the typical expression pattern of *wg* in *ptc* embryos. (*C–F*) p[HS–slp1C], ptc^{P78} embryos that were heat-shocked for 5 min, allowed to recover for 55 min before a 10-min heat shock, and then fixed after 90 min. The genotype of the embryos was concluded from the fact that p[HS–slp1C] (or any other P[HS–slp] line) never gave a similar phenotype under any conditions. Likewise, ptc^{P78} embryos heat-shocked as described showed no expansion of *wg* expression. The embryo in *C*, which recieved its first heat shock at about the time of gastrulation, displays an intermediate phenotype, with a gap between *wg* domains in each PS. Experiments in which a *ftz–lacZ* chromosome (see Fig. 5G, H) was crossed into a p[HS–slp1C], ptc^{P78} background suggests that the anterior-most part of the PS (where *en* is normally found) is the area not expressing *wg* in these intermediate-type embryos (data not shown). *D* shows a high magnification shot of a similar aged embryo with a stronger phenotype, where each *wg* domain has almost fused with each other. *E* and *F* show embryos slightly younger than those in *C* and *D* (first heat shock occuring at about late cellular blastoderm), both of which show the strongest phenotype observed (seen in more than half the *ptc* embryos at early stage 10). Here, some *wg* domains of adjacent PSs have completely fused.

transcripts in *slp* mutants. The *wg* stripes in the even-numbered PSs fade during germ-band extension (Fig. 2F). This leads to the decay of the adjacent *en* stripes (Fig. 2D), because they require *wg* activity at this time (Bejsovec and Martinez Arias 1991; Heemskerk et al. 1991) so that only the even-numbered PS *en* stripes remain. The number of *en* stripes at this time is an accurate predictor of the number of segments that will form (DiNardo and O'Farrell 1987; Martinez-Arias and White 1988); hence, the fusion of segments seen in *slp* mutants. At full germ-band extension, the remaining *wg* stripes rapidly disappear (Fig. 2G). *wg* is known to be required at this time for the production of naked cuticle (Bejsovec and Martinez-Arias 1991). This correlates well with the lack of naked cuticle in *slp* mutants, similar to *wg*-class segment polarity mutants.

The disappearance of *wg* in *slp* mutants occurs first in the even-numbered PS and then the odd-numbered, so one could imagine *slp* playing consecutive roles, first in one set of PS and then the other. However, we believe it is more accurate to propose that *slp* is required first in the even-numbered PSs and then in every PS for maintenance of *wg* expression. This latter requirement in every PS can be demonstrated by comparing *wg* expression in double mutants of *ptc* and the pair–rule gene *prd* with *slp*, *ptc* double mutants. At early stage 9 both look very similar (the even-numbered PS *wg* stripes are gone). Shortly thereafter, however, the missing stripes arise in *prd*, *ptc* double mutant embryos (Ingham and Hidalgo 1993). This demonstrates that *wg* expression at this time does not require preexisting *wg* and that the derepression seen in a *ptc* mutant background works independently of the earlier pair–rule regulation. In *slp*, *ptc* mutants the missing even-numbered PS *wg* stripes do not return (Fig. 7C), indicating that *slp* is required in these PSs for *wg* expression at stage 9/10. Thus, like other segment polarity genes, *slp* is required in every PS.

Which genes are good candidates for direct targets of slp*?*

A good candidate for direct regulation by *slp* should have an appropriate expression domain, and the misregulation seen in *slp* mutants should not be explainable by misexpression of other suspected regulators of the candidate. For example, even though the odd-numbered *en* stripes fade in *slp* mutants, *en* cannot be a direct target of *slp*, as they are not expressed in the same cells. The effect of *slp* on *en* expression probably occurs through *wg*, which is known to be necessary for *en* maintenance at this time (Bejsovec and Martinez Arias 1991; Heemskerk et al. 1991). Thus, in *slp* embryos, *wg* transcripts in the even-numbered PSs fade, leading to the disappearance of the adjacent (odd-numbered PS) *en* stripes. The even-numbered PS *en* stripes in *slp* mutants remain, because *en* expression no longer requires *wg* activity by the time the odd-numbered *wg* stripes fade (Bejsovec and Martinez Arias 1991; Heemskerk et al. 1991).

Because the *slp* proteins are expressed in the same cells as *wg* mRNA, it is possible that they act directly as positive regulators of *wg* transcription. However, in *slp* mutants, *ftz*, *eve*, *en*, and *hh* are ectopically expressed in

the *wg* cells (Figs. 3 and 5). Three of these genes (*ftz*, *eve*, and *en*) are thought to be repressors of *wg*, based on *wg* distribution in *ftz* and *eve* mutants (Martinez-Arias and Ingham 1988) and heat shock experiments (Ish-Horowicz et al. 1989; Heemskerk et al. 1991; Manoukian and Krause 1992). This raises the possibility that *slp* activates *wg* indirectly by repressing one or more of these *wg* repressors. Analysis of *slp*, *eve* or *slp*; *ftz* double mutants did not reveal any such regulatory circuit (Fig. 6A–C; data not shown). Ectopic *en* expression was found to contribute to *wg* transcript decay in *slp* mutants (Fig. 6D–F), but *wg* expression, especially in the epidermis, is still highly dependent on *slp* in the absence of functional *en*. Perhaps the *ftz*, *eve*, and *en* proteins work together to inhibit *wg* expression in *slp* mutants, so that removing any one will not completely rescue *wg* expression. This may be an explanation for the pair–rule decay of *wg* expression in *slp* mutants during germ-band extension but cannot explain the subsequent decay of the remaining *wg* stripes because (1) *ftz* and *eve* are not present at detectable levels at that time, (2) in some genetic combinations of *slp* alleles that are not null, there is no ectopic expression of *eve* and *en*, but *wg* transcripts still fade (Cadigan et al. 1994), and (3) the expansion of *wg* seen in P[HS–slp1], *ptc* embryos after heat shock cannot be explained by repression of *ftz* or *eve* expression and it can only partially be explained by repression of *en* (see below). Thus, although repression plays a significant role in the effect of *slp* on *wg* expression, an activating function that may be direct must also be postulated.

In *slp* mutant embryos, *ftz*, *eve*, *en*, and *hh* are all ectopically expressed in cells normally containing *slp*. Because none of the other pair–rule genes are affected by *slp* in a segmental fashion, *slp* may regulate *ftz* and *eve* transcription directly. Consistent with this, ectopic expression of either *slp* protein can repress expression of these genes (Cadigan et al. 1994).

At cellular blastoderm, *eve* and *ftz* are both thought to be positive regulators of *en* expression (MacDonald et al. 1986; DiNardo and O'Farrell 1987; Martinez-Arias and White 1988). Does the anterior expansion of *en* stripes in *slp* mutants depend on *ftz* and *eve*? We consider this unlikely for the following reasons. First, when the *en* stripes start to expand (late stage 8), the levels of *eve* and *ftz* proteins are rapidly falling. Embryos can be found in which expanded odd-numbered PS *en* stripes coincide with *eve* protein expression (Fig. 4C, D). However, the time window between onset of *en* stripe widening and *eve* disappearance is extremely brief. The even-numbered PS *en* stripes expand slightly later than the odd-numbered ones (see Fig. 4C), and we were unable to see significant amounts of *ftz* protein by the time the even-numbered *en* stripes were noticeably wider (data not shown). The second line of evidence minimizing the importance of *eve* and *ftz* in the regulation of *en* expression by *slp* is that P[HS–slp] embryos in which the first heat shock is administered at mid-stage 9 (when *ftz* and *eve* have faded) show a complete lack of *en* transcripts (Cadigan et al. 1994). In summary, although we cannot rule out (especially for *eve*) the role of the two pair–rule genes in *en* expansion in *slp* mutants, the evidence seems to indicate that *slp* may repress *en* expression independently of *ftz* and *eve*.

The pattern of *hh* expression in *slp* mutants is similar to that of *en* (Fig. 3B) and P[HS–slp] embryos lack *hh* transcripts after induction (Cadigan et al. 1994). *hh* is regulated by all other segmentation genes in a fashion identical to *en* (Lee et al. 1992; Tabata et al. 1992) but is expressed normally in *en* mutants until late stage 10. Thus, *slp* probably regulates *hh* independently of *en*. The patterns of *ptc* (Fig. 3F) and *cubitus interruptus Dominant* (ci^D; data not shown) in *slp* mutants most likely occur because of altered distribution of the transcription factor *en*, because *en* is known to be a repressor of the expression of these genes (Eaton and Kornberg 1990; Hidalgo and Ingham 1990).

Another possible target for positive regulation by *slp* is the *gsb* gene (Fig. 3D). However, *gsb* transcripts fade in *wg* mutants at stage 10 (Hildalgo 1991; Li et al. 1993), so the positive effects of *slp* on *gsb* expression may occur through *wg*.

In summary, *ftz*, *eve*, *en*, and *hh* are all good candidates for being directly repressed by *slp*, and *wg* is the best candidate for direct activation. Further studies on the *cis*-acting control elements of these genes are needed to ascertain whether these interactions are direct.

slp *maintains the polarity of the PS*

After *wg* transcription is initiated by the pair–rule genes, a signal from the adjacent *en*-expressing cells is needed for maintainence of *wg* expression. This signal is thought to be the *hh* gene product, which is processed to form a secreted protein (Lee et al. 1992; Taylor et al. 1993). In the absence of *hh*, *wg* transcripts are completely gone at early stage 10 (Ingham and Hildalgo 1993), attributable to the repressing activity of *ptc* (Ingham et al. 1991), an integral membrane protein found at the cell surface (Hooper and Scott 1989; Nakano et al. 1989). The *hh* protein is thought to somehow interfere with *ptc* activity, so that *wg* expression is maintained in the cells just anterior of the *en*-expressing cells (see Fig. 10 for diagram). The question remains as to why *hh* does not activate *wg* transcription in the cells posterior of the *en* cells, because *hh* is known to be required for maintenance of *ptc* expression in these cells (Hidalgo and Ingham 1990; Forbes et al. 1993) indicating that *hh* can act symmetrically.

To explain the different responses of the cells on each side of the *en* stripes, Ingham et al. (1991) proposed that PSs are divided into *wg*- and *en*-competent domains. The *wg*-competent group are cells that express *wg* in *ptc* mutants; the *en*-competent group are cells expressing *en* in *nkd* mutants. The *slp* expression domain coincides with the *wg*-competent cells (Fig. 5D). Furthermore, all of the cells expressing *wg* in a *ptc* mutant background require functional *slp* for this expression (Fig. 7A–C). These data are consistent with the idea that the *wg*-competent group is determined by the presence of *slp* protein.

If the presence of *slp* protein does determine a cell to

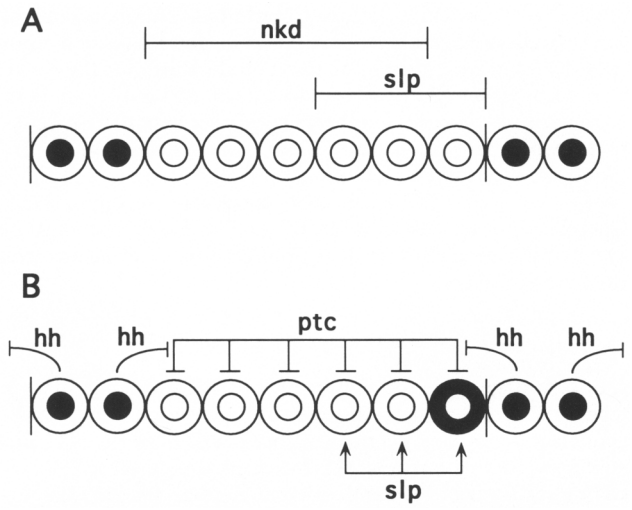

Figure 10. Summary of *slp* action on *en* and *wg* expression. (*A*) PSs are shown eight cells wide (vertical lines denote the location of the parasegmental grooves), with dark nuclei representing the normal expression domain of *en*. We postulate overlapping repressing activities of *slp* and *nkd* on *en* expression. In *slp* mutants, the *en* stripes expand ~1 cell anteriorly (Fig. 4*E–H*). In *nkd* mutants, they expand posteriorly to the anterior border of the *slp* expression domain (Fig. 5F). In *slp*; *nkd* double mutants, virtually every cell in the PS expresses *en* (Fig. 7*E*,*F*). These data are consistent with a model in which either *slp* or *nkd* activity is enough to repress *en* and predicts that *nkd*, which is not yet cloned, will be expressed in the broad domain indicated (or at least have activity in these cells). (*B*) Incorporation of *slp* into the model of Ingham et al. (1991). *en* is represented by the darkened nuclei and *wg* as the darkened cytoplasm. See text for further explanation.

be *wg*-competent, then expression of *slp* on the posterior side of the *en* stripes (via the heat shock promoter) should result in *wg* expression there. However, ectopic *slp* protein abolishes *hh* expression (Cadigan et al. 1994), so that no ectopic *wg* transcripts were observed (Fig. 8). Because *wg* expression is independent (at least in large part) of *hh* in *ptc* mutants (Ingham et al. 1991; Bejsovec and Wieschaus 1993), we examined the distribution of *wg* in P[HS–slp], *ptc* embryos after heat shock (Fig. 9). Consistent with our proposed role for *slp*, we found that *wg* was expressed almost throughout the PS after heat shock. The few cells that do not express *wg* are within the normal *en* domain. The reluctance of these cells to express *wg* may be attributable to the ability of *en* to repress *wg* expression (Fig. 6D–F; Heemskerk et al. 1991), though *en* expression is repressed after heat shock induction of *slp* under the conditions used in Figure 9 (data not shown). It may be that cells previously expressing *en* for a certain time are subsequently not competent for *wg* expression later, despite the absence at that time of *en*. Consistent with this is the observation that the P[HS–slp], *ptc* embryos not expressing *wg* in the *en* domain in general were older at the time of first heat shock than embryos expressing *wg* throughout (see Fig. 9).

Because of its ability to repress *en* (see next section) and activate *wg*, the competence groups proposed by Ingham et al. (1991) can be explained entirely by the distribution of *slp* protein in the PS. *slp* is not required for the initiation of the *wg* and *en* stripes at cellular blastoderm, but localized *slp* expression is needed to maintain the polarized distribution of *wg* and *en* transcripts during germ-band extension. *slp* can be viewed as the benchmark on which segment polarity is maintained.

Repression and regulation of en

Drosophila segments are divided into two compartments, each with a distinct cell lineage (Lawrence 1981). *en* is is expressed in the posterior compartments of embryo and larval segments (Kornberg et al. 1985) and is thought to play an active role in maintaining the lineage boundary between the compartments (Lawrence and Morata 1976; Kornberg 1981). Thus, the regulation of *en* expression has been studied extensively as a model for the establishment of a determined cell state (Heemskerk et al. 1991; Bienz 1992). In embryos mutant for either *slp* or *nkd*, the expression of *en* is initiated normally, but the *en* stripes widen during germ-band elongation, in an anterior direction in *slp* mutants (Fig. 4), and a posterior one in *nkd* mutants (Fig. 5F; DiNardo et al. 1988; Martinez Arias et al. 1988). The amount of *en* expansion in *slp*; *nkd* double mutants is more than the sum of the single phenotypes, that is, almost all the epidermal cells in each segment are *en* positive (Fig. 7D–F), suggesting that *slp* and *nkd* have overlapping repressing activities (Fig. 10). The activity of pair–rule repressors (i.e., *run*, *eve*, and *odd*) is also thought to be important in the initiation of the *en* stripes at cellular blastoderm (Manoukian and Krause 1993) and genes of the *Polycomb* group repress *en* during germ-band shortening (Moazed and O'Farrell 1992). It is interesting to note that in studies on the regulation of another cell fate determinator, *Ultrabithorax*, repression is also thought to play a critical role, first involving the gap gene *hunchback* and then genes of the *Polycomb* class (Bienz 1992).

The elegant study of Vincent and O'Farrell (1992) demonstrated that the expression domain of *en* does not expand clonally in early embryos, that is, some daughters of *en*-positive cells no longer express *en*. However, they did find a strict lineage boundary at the anterior border of *en* stripes, which is where the parasegmental grooves form (Ingham et al. 1985a) and which is thought to mark the anterior extent of the posterior compartment (Lawrence and Martinez-Arias 1985). In *slp* mutants, both the anterior border of *en* and the location of the parasegmental grooves are shifted anteriorly (Fig. 4I). It remains to be seen whether the cells ectopically expressing *en* in *slp* mutants are derived from *en*-positive cells or from cells that have never previously expressed *en*.

A possible role for slp *in the* hh/ptc *signal transduction pathway*

As summarized above, *ptc* activity represses *wg* transcription, and *hh* somehow interferes with this repres-

sion. Both genes encode proteins that are thought to act at the cell surface. The genes *fused* (*fu*) and ci^D also are required for maintenance of *wg* expression during germband extension, and genetic evidence indicates they are required in the *wg*-expressing cell, suggesting that they act in the *hh/ptc* signaling pathway (Forbes et al. 1993; Ingham 1993). *fu* is predicted to encode a serine–threonine kinase expressed throughout the embryo (Preat et al. 1990), and ci^D is a zinc finger protein expressed in all the cells not expressing *en* (Eaton and Kornberg 1990; Orenic et al. 1990). The *slp* proteins are required for *wg* expression at the same time as *ptc*, *hh*, *fus*, and ci^D, raising the possibility that *slp* is a nuclear recipient of the *hh/ptc* signal.

Alternatively, *slp* may not be part of the signaling pathway but may act in parallel with it. This hypothesis is supported by the comparsion of the regulation of *wg* and *ptc* expression. Both are repressed by *ptc* activity which, in turn, is antagonized by *hh*. Moreover, *fu* and ci^D are also required for the maintenance of *wg* and *ptc* (Forbes et al. 1993). In constrast, *slp* is not required for *ptc* maintenance (Fig. 3E, F). Therefore, the simplest model incorporating the available data would predict that *hh*, *ptc*, *fu*, and ci^D are part of a signaling pathway regulating *wg* and *ptc* transcription. This pathway functions both anterior and posterior of the *en/hh* expression domain, but *wg* expression, in constrast to that of *ptc*, is only activated on the anterior side, because it has an absolute requirement for *slp* protein. Thus, the asymmetric distribution of *slp* protein within each PS leads to the polarized response of the *wg* promoter to the *hh/ptc* signal. Further biochemical studies of the *slp* proteins, as well as detailed analysis of the *wg* promoter for *slp*-binding sites, will be necessary to further elucidate the function of *slp* in *wg* regulation and its relationship to the other gene products required for *wg* expression.

Materials and methods

Fly stocks

The *slp* deficiencies $Df(2L)ed^{SZ1}$ and *CyO* Δ34B (hereafter referred to as Δ34B), as well as the enhancer trap insert (*CyO* P[lArB]A208.1M2) from which Δ34B was derived, are described in Grossniklaus et al. (1992). *CyO* P[larB]A208.1M2 homozygotes do not hatch, but their cuticles and the expression of *en* and *wg* were all identical to wild-type embryos (data not shown). Δ34B and $Df(2L)ed^{SZ1}$ homozygotes and Δ34B/$Df(2L)ed^{SZ1}$ transheterozygotes all displayed similar misregulation of *en* and *wg* expression (Figs. 2, 6, and 7; data not shown), though $Df(2L)ed^{SZ1}$ embryos begin to exhibit gross abnormalities not linked to *slp* during germ-band retraction.

P[ftz–lacC] contains 6.0 kb of the *ftz* promoter, enhancer and translation start site sequences fused to *lacZ* (Hiromi et al. 1985). A first chromosome insertion strain was used for creating a P[ftz–lacZ]; *b Adh cn l(2)*/Δ34B stock. Other mutant alleles used were eve^{R13}, ptc^{IN}, ptc^{P78}, nkd^{7E}, *Df(2R)en-E*, en^{IO} and ftz^{9H}. *Df(2R)en-E* is a small deficiency removing both *en* and *invected* (Z. Ali and T. Kornberg, pers. comm.). The eve^{R13} allele is an amorph (Nüsslein-Volhard et al. 1984). ptc^{P78} (Nakano et al. 1989) and ftz^{9H} (Furukubo-Tokunaga et al. 1992) are nulls; ptc^{IN} gave similar results as ptc^{P78}, and the en^{IO} and nkd^{7E} alleles are classified as strong (Tearle and Nüsslein-Volhard 1987). To examine embryos mutant for *slp* (on the second chromosome), and either *ftz* or *nkd* (both on the third), *slp* flies were crossed to either allele (balanced by *TM3*, *Sb*) and *Cy*, non-*Sb* progeny were mated and embryos collected. In both cases, mutant phenotypes segregated in close to the theoretical 9:3:3:1 ratios. Chromosomes containing $Df(2L)ed^{SZ1}$ and either ptc^{IN}, eve^{R13}, en^{IO}, or *Df(2R)en-E* were created by recombination. Complementation tests (and in the case of the deficiencies, in situ hybridization with *slp* or *en* probes) were performed to confirm the genotypes.

Heat shock slp *flies and cuticle preparations*

A brief description of the cloning of heat shock *slp* constructs is given below. A detailed protocol is available upon request. Oligonucleotide primers hybridizing just 5′ and 3′ of the *slp1* and *slp2* open reading frames (ORFs) were used to amplify the *slp*-coding regions from genomic phages (neither *slp* gene has introns; Grossniklaus et al. 1992) with the polymerase chain reaction (PCR) (Saiki et al. 1985) and to introduce restriction sites to facilitate subsequent cloning. Products of two separate reactions for *slp1* and one for *slp2* were cloned into pBluescript. The ORFs were then cloned into pNHT4, a P-element vector containing the 5′ *hsp70* promoter and 3′ termination sequences (Gibson et al. 1990). $rosy^{506}$ embryos were coinjected with the P-element heat shock *slp* (P[HS–slp]) constructs and pπ25.7 as described previously (Rubin and Spradling 1982; Karess and Rubin 1984) and several independent transgenic lines for each construct were established. Examination of cuticle phenotypes after heat shocks during early embryogenesis, which is described in detail elsewhere (Cadigan et al. 1994), showed a qualitatively similar result with all lines, although the strength of the phenotype varied from line to line. The lines selected for further study (ones that produced the strongest and most consistent phenotype) expressed high levels of the respective *slp* transcript and protein after heat shock, as judged by whole mount in situ hybridization or immunostaining. A strong *slp1* heat shock construct on the second chromosome, P[HS–slp1C], was recombined with the ptc^{P78} mutation for the experiments described in Figure 9.

Heat shocks were performed in the following way. Embryos were collected on grape juice plates for a short time (40–60 min) and aged at 25°C. Shortly before heat shock, they were dechlorinated with bleach and collected on a nylon mesh. This mesh was placed on a moist piece of Whatman paper in a petri dish. This dish was sealed by parafilm and submerged in a 37°C bath (usually for 5 min), and the embryos were allowed to recover at 25°C before a second heat shock and subsequent fixation or analysis of cuticle. The time when the first heat shock was administered was approximated by extrapolating back from the age of the individual fixed embryos and a knowledge of the rate of development under our culture conditions.

Cuticles were prepared essentially as described by Nüsslein-Volhard et al. (1984) except that the vitelline membranes were removed by vigorous agitation in methanol/heptane (1:1). The embryos were then transferred to a slide, and the remaining solvent was allowed to evaporate before covering with Hoyer's medium/lactic acid (1:1).

Antibody production and immunostaining

Escherichia coli-expressed protein was the source of *slp1* antigen for immunization. The *slp1* PCR products described above were cloned into pET-3d, an *E. coli* T7 expression vector (Studier et al. 1990). Protein from a SDS gel was transferred to a

nitrocellulose filter. After staining with Ponceau S (0.4% in 3% trichloroacetic acid), the *slp1* band was cut out, the Ponceau S was washed away with PBS containing 0.3% Triton-X100, and the filter dried in an oven overnight. The filter was dissolved in dimethylsulfoxide (DMSO) as described by Knudsen (1985) and mixed with Freund's adjuvant (1:2) before immunization. The *slp2* antigen was a fusion protein consisting of glutathione *S*-transferase linked to the carboxy-terminal third (amino acids 295–445) of *slp2*. The *slp2* DNA fragment was a PCR fragment described in Grossniklaus et al (1992) cloned into pGEX-2T. The fusion protein was produced and purified as described (Smith and Johnson 1988). Rats were immunizied by dermal injection, first with 50 μg antigen emulsified with complete Freund's adjuvant, and then boosted every 4 weeks with antigen/incomplete adjuvant. Animals were terminally bled 10 days after the third or fourth boost.

Antibody stainings were performed essentially as described by Frasch et al. (1987) and Grossniklaus et al. (1992). The rabbit polyclonal anti-*eve* (Frasch et al. 1987) and anti-*ftz* (Krause et al. 1988) antisera were gifts of M. Frasch (Mount Sinai Medical Center, NY) and H. Krause (Toronto University, Canada). The mouse monoclonal anti-*en* antibody (Patel et al. 1989) was a gift from C. Goodman (University of California at Berkeley) and N. Patel (Carnegie Institute, Baltimore, MD). The rabbit anti-*lacZ* polyclonal antisera was from Cappel. The primary antibodies were used at the following dilutions: *en*, 1:1; *slp2* and *ftz*, 1:200; *slp1*, 1:300; *lacZ*, 1:1000 and *eve*, 1:2000. The secondary antibodies were either biotinylated (goat anti-mouse, horse anti-rabbit and rabbit anti-rat; all from the Elite ABC kit, Vectastain, used at a 1:500 dilution) or alkaline phosphatase conjugated (swine anti-rabbit from Dakopatts and goat anti-rat from Orgarion Teknika Corp.; both were used at a 1:200 dilution).

Analysis of transcript distribution

In situ hybridization to whole-mount embryos using digoxygenin-labeled probes was performed according to Tautz and Pfeifle (1989), with modifications (a detailed protocol is available on request). The *en* cDNA pF7036 (Fjose et al. 1985) was used for the *en* probe. The other DNA templates were all cDNAs and were generous gifts from the following sources: *wg*, pBS-CV (Rijsewijk et al. 1987) from M. van den Heuvel and R. Nusse (Stanford University, CA); *prd*, c7340.6 (Kilcherr et al. 1986) and *gsb*, BSH9c2 (Baumgartner et al. 1987) from M. Noll (University of Zurich, Switzerland); *hh*, chh46 (Tabata et al. 1992) from S. Tabata and T. Kornberg (University of California at San Francisco); *ptc*, pGEM7-ptc (Hooper et al. 1989) from Y. Higashi and M. Scott (Stanford University, CA); ci^D, cDNA9-11 (Orenic et al. 1990) from D. Slusarski and R. Holmgren (Northwestern University, IL); *odd* (cDNA clone A; Coulter et al. 1990) from D. Coulter (Washington University, MI); *h* , D2P8 (Ish-Horowitz et al. 1985) from D. Ish-Horowicz (University of Oxford, UK) and *run*, pBED-5′ (Kania et al. 1990) from M. Klingler and P. Gergen (State University of New York at Stony Brook, NY).

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Localized expression of sloppy paired protein maintains the polarity of Drosophila parasegments.

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