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

Kenneth M. Cadigan,1 Ueli Grossniklaus,2 and Walter J. Gehring

Biozentrum, University of Basel, CH-4056 Basel, Switzerland

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.

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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 [Fiore 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 [Hidalgo 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 contrast, 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

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**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 denticle belt). (B) Terminally differentiated embryo homozygous for A34B, 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 2 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 primordia.
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 maintenance 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 \textit{ftz}, \textit{eve}, and \textit{en} expression in \textit{slp} (A34B) mutants. (A) \textit{ftz} protein distribution in a \textit{slp} mutant at stage 8. During germ-band extension, seven additional stripes arise in the posterior part of the even-numbered PSs [arrows]. (B) \textit{slp} mutant stained for \textit{en} [orange] and \textit{ftz} [blue] proteins. The extra stripe in PS2 [arrow] lies just anterior to the PS3 \textit{en} stripe. (C) Stage 8 \textit{slp} mutant stained for \textit{en} [orange] and \textit{eve} protein [blue; overlap is black]. The \textit{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 \textit{eve} and \textit{en} stripes coincide. (E) Late stage 9 wild-type embryo stained for \textit{en} protein [orange] and \textit{gsb} transcript [blue]. At this time, \textit{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 \textit{en} cells [arrow]. (F) \textit{slp} embryo stained as in E. The \textit{en} stripes in the odd-numbered PS are wider, and the anterior border of the \textit{en} and \textit{gsb} stripes now coincide [arrow] or occasionally \textit{en}-staining cells are found anterior of the \textit{gsb} stripes [open arrow]. (G) Stage 8 embryo containing a \textit{ftz-lacZ} construct that expresses \textit{lacZ} in a \textit{ftz}-like pattern. The embryo is stained for \textit{lacZ} protein [orange] and \textit{en} transcript [blue]. The anterior border of the \textit{en} and \textit{lacZ} stripes are identical. (H) Stage 8 \textit{slp} mutant containing the \textit{ftz-lacZ} transgene stained as in G. The anterior-most \textit{en} cells of the even-numbered PS shown [arrows] does not stain for \textit{lacZ}, indicating the expansion of the \textit{en} stripes in \textit{slp} mutants is in the anterior direction. (I) \textit{en} expression in a stage 10 \textit{slp} embryo. The parasegmental grooves are indicated [arrows].

expressing \textit{wg}. There is strong evidence for \textit{ftz} and \textit{eve} being repressors of \textit{wg} transcription \cite{ingham1988,ish-horowicz1989,manoukian1992}, and \textit{en} has been suggested to repress \textit{wg} expression \cite{heemskerk1991}. It may be that \textit{slp} is not a direct activator of \textit{wg} transcription but, rather, activates \textit{wg} expression by repressing a \textit{wg} repressor[s]. This possibility can be tested by analyzing \textit{wg} expression in the appropriate double mutants. If \textit{slp} is activating \textit{wg} by repressing a \textit{wg} repressor, then \textit{wg} expression in \textit{slp} mutants lacking this repressor should persist longer than in \textit{slp} mutants alone.

A dramatic derepression of \textit{wg} transcripts is seen in \textit{ftz} and \textit{eve} mutant embryos, such that all the cells in the even- or odd-numbered PSs express \textit{wg}, respectively \cite{ingham1988}. In \textit{eve} mutants, the PS-wide \textit{wg} stripes fade during germ-band elongation \cite{ingham1993}. \textit{wg} mRNA in \textit{slp}, \textit{eve} double mutants behaves similarly as in \textit{eve} embryos, except that the disappearance of \textit{wg} transcripts occurs slightly earlier [data not shown]. In the crosses performed to create \textit{slp}; \textit{ftz} mutants, only \(\frac{1}{16}\) of the progeny are double mutants. We were not able to unambiguously identify double mutants after staining for \textit{wg} transcripts [data not shown]. We reasoned that we could assay the presence of \textit{wg} in the even-numbered PSs by monitoring the adjacent \textit{en} stripes, which require \textit{wg} expression for proper maintenance \cite{bejsovec1991,heemskerk1991}.
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) ptc1 /slp1 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) nkd7 /slp1 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.

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 contrast 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-
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 [D(2)L)ed221, ptcN] embryos and en protein (right) in slp, nkd [D44B, nkd2] 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.
and largely independent of hh [Ingham et al. 1991; Beisovec 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–slpl], 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 transcription 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
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 [Martínez-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–slp], 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; Martínez-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 (ciD); 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 [Hidalgo 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 maintenance 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 Hidalgo 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. 4E-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. 7E,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 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 determinant, 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-
Both genes encode proteins that are thought to act at the cell surface. The genes fused (fu) and ci are also required for maintenance of wg expression during germ-band extension, and genetic evidence indicates they are required 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 [Praction et al. 1990], and ci 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, 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 comparison 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 are also required for the maintenance of wg and ptc [Forbes et al. 1993]. In contrast, 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 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 contrast 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^{2+1} and CyO Δ34B [hereafter referred to as Δ34B], as well as the enhancer trap insert (CyO Pl[ArZ]A208.1M2) from which Δ34B is derived, are described in Grossniklaus et al. [1992]. CyO Pl[ArB]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^{2+1} homozygotes and Δ34B/Df(2L)ed^{2+1} transheterozygotes all displayed similar misregulation of en and wg expression (Figs. 2, 6, and 7; data not shown), though Df(2L)ed^{2+1} embryos begin to exhibit gross abnormalities not linked to slp during germ-band retraction.

P[ftz-lacZ] contains 6.0 kb of the ftz promoter, enhancer and translation start site sequences fused to lacZ [Hromi et al. 1985]. A first chromosome insertion strain was used for creating a P[ftz-lacZ]; b Adh cn (I2)/Δ34B stock. Other mutant alleles used were eve^{R13} ptc^{IN}, ptc^{278}, nkd^{27}, Df(2R)ren-E, en^{O} and ftz^{2W}, Df(2R)ren-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^{278} [Nakano et al. 1989] and ftz^{2W} [Furukubo-Tokunaga et al. 1992] are nulls, ptc^{IN} gave similar results as ptc^{278}, and the en^{O} and nkd^{27} 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^{2+1} and either ptc^{IN}, eve^{R13}, en^{O}, or Df(2R)ren-E were created by recombination. Complementation tests [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]. ros{slp} embryos were coinfected with the P-element heat shock slp [{P[HS-slp]} constructs and pwt25.7 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^{2+1} 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 dechorionated with bleach and collected on a nylon mesh. This mesh 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 timing 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 rabbit was immunized 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, or alkaline phosphatase conjugated [swine anti-rabbit from Dakopatts and goat anti-rat from Organon Teknika Corp.; both were used at a 1:200 dilution].

Analysis of transcript distribution

In situ hybridization to whole-mount embryos using digoxigenin-labeled probes was performed according to Tautz and Pfeifle (1989), with modifications [a detailed protocol is available on request]. The en cDNA pF7036 [Jöse 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, pC340.6 [Kleiherr et al. 1986] and gsh, pBS962 [Baumgartner et al. 1987] from M. Noll [University of Zurich, Switzerland]; hhh, 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. Higashii and M. Scott [Stanford University, CA]; c2zf, 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-Horovitz et al. 1985] from D. Ish-Horovitz [University of Oxford, UK] and run, pRED-5' [Kania et al. 1990] from M. Klingler and P. Gergen [State University of New York at Stony Brook, NY].

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K M Cadigan, U Grossniklaus and W J Gehring

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