Molecular Cell, Vol. 6, 203–209, July, 2000, Copyright ©2000 by Cell Press

Drosophila Segment Borders Result from Unilateral Repression of Hedgehog Activity by Wingless Signaling

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Summary

Body structures of *Drosophila* develop through transient developmental units, termed parasegments, with boundaries lying between the adjacent expression domains of *wingless* and *engrailed*. Parasegments are transformed into the morphologically distinct segments that remain fixed. Segment borders are established adjacent and posterior to each *engrailed* domain. They are marked by single rows of *stripe* expressing cells that develop into epidermal muscle attachment sites. We show that the positioning of these cells is achieved through repression of Hedgehog signal transduction by Wingless signaling at the parasegment boundary. The nuclear mediators of the two signaling pathways, Cubitus interruptus and Pangolin, function as activator and symmetry-breaking repressor of *stripe* expression, respectively.

Introduction

In *Drosophila*, the process of how parasegments (PS) (Martinez Arias and Lawrence, 1985; Martinez Arias, 1993) are established has been studied in great detail (Lawrence et al., 1987; Lawrence and Johnston, 1989; Cadigan et al., 1994; Mullen and DiNardo, 1995). PS are transient developmental units in which homeotic gene expression and cell lineage restriction occur (Martinez Arias and Lawrence, 1985; Martinez Arias, 1993). Their formation can be traced back to the initial coordinate system of the oocyte (Driever, 1993; St. Johnston, 1993), followed by the activity of the zygotic segmentation gene cascade during the blastoderm stage (Martinez Arias, 1993; Pankratz and Jäckle, 1993). At the endpoint of this cascade, the activity of the pair-rule genes establish a series of adjacent domains of *wingless* (*wg*) and *engrailed* (*en*) expression, defining the PS boundary between them (Morata and Lawrence, 1975; Martinez Arias, 1993). Subsequently, PS boundaries are stabilized by a paracrine autoregulatory loop that involves signaling by Hedgehog (Hh), a secreted factor that emanates from the *en* expression domain, and Wingless (Wg), a secreted factor as well.

The cells flanking the PS boundaries provide spatial signals needed to establish cell identity between the boundaries (DiNardo et al., 1988; Martinez Arias et al., 1988). At the end of embryogenesis, different cell identities can be observed in the epidermis, which becomes decorated by segmentally arranged alternating bands of naked cuticle and characteristic belts of denticles (Martinez Arias et al., 1988). Wg has been shown to have two distinct functions in cuticle patterning. Its early activity is to maintain *en* expression, which, in turn, has indirect consequences since *en* controls the activity of *hedgehog* (*hh*), which affects cuticular patterning (Pfeiffer and Vincent, 1999). Wg has also a more direct and late effect on the final cuticular pattern and is sufficient to form uniform naked cuticle when expressed in all epidermal cells (Sanson et al., 1999). Recent results have shown that Wg specifies naked cuticle cell fate over a range of up to five cells in anterior direction, whereas its posterior range of activity covers only the row of adjoining cells (Sanson et al., 1999).

Most accounts of Wg signaling emphasize an activating function that is mediated by the TCF/Lef1 homolog Pangolin (Brunner et al., 1997; van de Wetering et al., 1997), and there is one instance of possible repression by Wg signaling. This example of *wingless*-dependent repression is based on genetic analysis of the expression of *shavenbaby* (*svb*), which is the result of integrated Wg/DER signaling to control epidermis differentiation (Payre et al., 1999). Thus, whereas the molecular patterning process leading to the formation of PSs, the maintenance of the PS boundaries, and patterning processes within the PSs are quite well understood, the genetic input that establishes the segment borders, meaning the molecular basis underlying the subsequent transition of PS into segment structures (Martinez Arias, 1993) that remain fixed during the life cycle of the fly, is unknown.

Here we show an analysis of the regulatory input that controls gene expression in precursors of the epidermal muscle attachment sites. These cells represent the prospective segment borders (Martinez Arias, 1993) and were shown to differentiate in response to the activity of the gene *stripe* (*sr*) (Frommer et al., 1996). We identified a minimal enhancer element of the *sr* gene that is necessary and sufficient to drive gene expression in a single cell wide stripe per segment, just behind and adjoining the *en* expression domain of each PS. Analysis of the *cis*-acting requirement and transregulatory inputs reveals that the Hh and Wg signaling pathways converge on the enhancer and mediate the spatial activation of *sr* expression in the prospective segment border cells. Our results make it possible to trace back the molecular patterning process all the way from the initial coordinate system of the oocyte to the precise positioning of the segment border cells and they explain how the asymmetric readout of Hedgehog signaling is achieved.

Results and Discussion

sr is expressed in all precursors of the epidermal muscle attachment sites, including those marking the segment border in the *Drosophila* larvae (Frommer et al., 1996)

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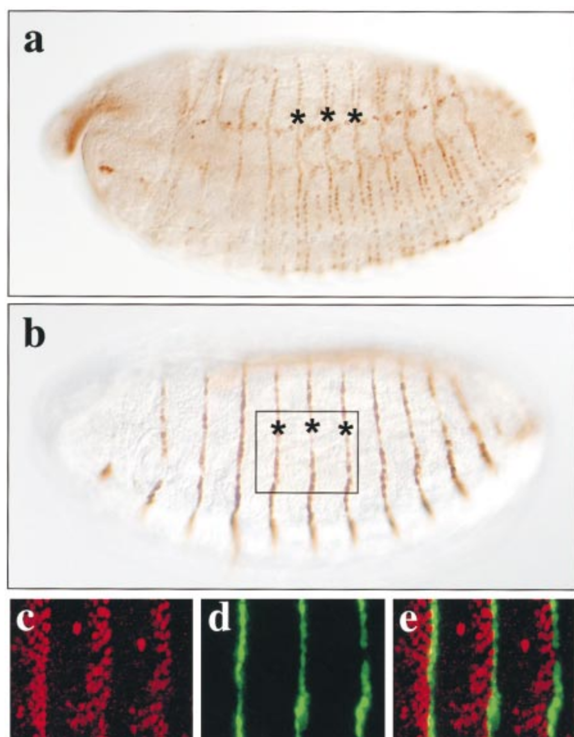

Figure 1. *sr* and sr1.9 Reporter Gene Expression in Stage 15 Embryos

(a) Anti-Sr antibody staining showing that Sr is expressed in all muscle attachment sites. Note the row of cells (asterisks) that mark the segment border cells.

(b) Anti-β-galactosidase antibody staining showing that the sr1.9 element mediates gene expression in segment border cells only. Inset shows the position enlarged in (c)–(e); asterisks (a and b) mark corresponding positions.

(c–e) Enlargements showing double staining with anti-En ([c and e], red) and anti-β-galactosidase ([d and e], green) antibodies. The merged view (e) shows that sr1.9-mediated gene expression occurs in a row of cells posteriorly adjacent to the *en* domain. Orientation of embryos: anterior is left, dorsal side up.

(Figure 1a). To obtain an early molecular marker for the segment border corresponding to the row of cells posteriorly adjacent to the *en* expression domain (Martinez Arias, 1993), we isolated a 1.9 kb enhancer element of the *sr* gene (sr1.9) (see Experimental Procedures) that is both necessary and sufficient to direct transgene-dependent *lacZ* expression (Figure 1b) in segment border precursor cells in a dorsal and lateral position of the embryo (Figures 1c–1e). Expression of the reporter gene is activated in parallel with *sr*, which is first expressed during late stage 10 (stages according to Campos-Ortega and Hartenstein, 1997). At this time, the initial equal distribution of Wg has already become asymmetric, meaning that the protein spreads anteriorly over a range of maximally five cells but is restricted to only one row of cells directly adjoining the posterior margin of the expression domain (Pfeiffer and Vincent, 1999). *sr* acts as a transcription factor required for setting up the cell fate of the muscle attachment sites which mark the segment border of the fly (Vorbrüggen and Jäckle, 1997). Thus, sr1.9-dependent reporter gene expression can be employed to study the transregulatory requirement for positioning the segment border cells within the PS.

To explore the regulatory circuitry that defines the position of segment border cells, we asked whether sr1.9-dependent gene expression is controlled by signals emanating from cells around the PS boundary. Cells at this boundary, which is defined by the interface between the *wg* and *en* expressing cells (Morata and Lawrence, 1975; Martinez Arias, 1993), secrete Hh and Wg (DiNardo et al., 1988; Martinez Arias et al., 1988) that function as ligands that activate the correponding signaling cascades (Figure 2a). In order to examine a possible direct or indirect regulatory effect of these organizer activities on sr1.9-dependent gene expression in segment border cells, we crossed the sr1.9-driven reporter gene with various mutants in a way that allowed us to monitor the β-galactosidase expression pattern in homozygous mutant embryos and in embryos expressing Hh or Wg signaling components in ectopic positions (see Experimental Procedures). We examined sr1.9-dependent gene expression in stage 11 and stage 15 embryos (Campos-Ortega and Hartenstein, 1997), and found no difference with respect to the spatial patterns observed, whereas the level of expression was weaker in the younger embryos. Thus, the results were documented at stage 15 where the staining patterns were most intense.

Hh Activates sr1.9-Dependent Transcription Posterior but Not Anterior to the *en* Domain

Hh emanates from the *en* domain (Lee et al., 1992; Tabata et al., 1992) and acts via the transcription factor Cubitus interruptus (Ci) (Alexandre et al., 1996; Von Ohlen et al., 1997) (reviewed in Aza-Blanc and Kornberg, 1999). Ci is expressed in a pattern complementary to *en*, showing increased levels of expression in cells adjacent to the *en* domain (Eaton and Kornberg, 1990; Orenic et al., 1990) and is needed to maintain *wg* expression anterior to the *en* domain (DiNardo et al., 1994). The absence of *sr* and sr1.9-dependent gene expression in *hh* mutant embryos (data not shown) suggested to us that the selection of segment border precursor cells depends on Hh activity. To test for Hh-dependent *sr* gene activation more directly, we used the Gal4::UAS system (Brand and Perrimon, 1993) to ectopically express Hh within the *patched* (*ptc*) expression domain (Hooper and Scott, 1989; Nakano et al., 1989) (see Figure 2a). Whereas in wild-type embryos, sr1.9-dependent gene expression is restricted to a single row of cells (Figures 1c–1e), it covers the *ptc* domain posterior to *en* in embryos bearing the *ptc*-Gal4::UAS-*hh* transgene combination (Figures 2b–2d). The same expression pattern was observed in *ptc* mutant embryos (Figures 2e–2g) where Ci is constitutively activated outside the *en* domain (Forbes et al., 1993; Ingham and Hidalgo, 1993). These findings suggest that *sr* expression at the segment border is activated by Ci-mediated Hh signaling.

Ectopic expression of a dominant active form of Ci, termed CiZn/C (Hepker et al., 1997), caused sr1.9-dependent gene activation in the *ptc* domain (Figures 2h–2j). Furthermore, the expression of a dominant repressor variant of Ci (Methot and Basler, 1999), termed

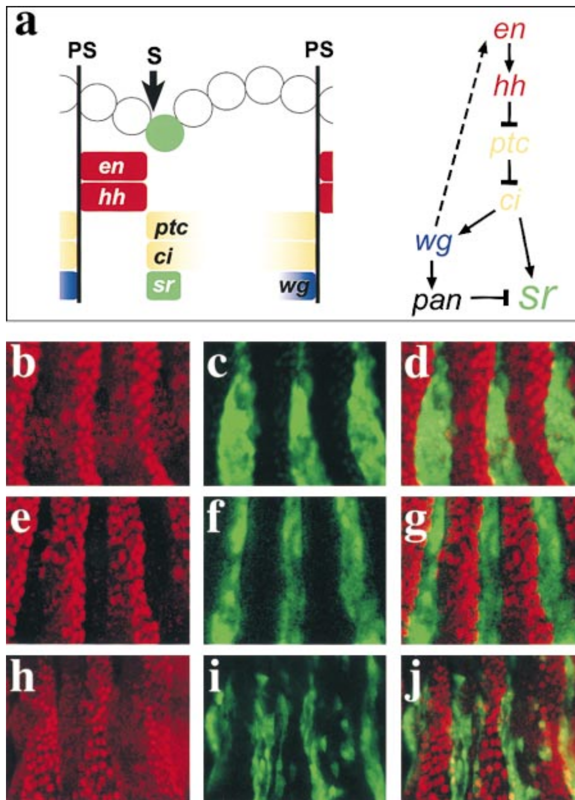

Figure 2. Hyperactivation of the Hh Signaling Pathway Causes Expansion of sr1.9-Mediated Gene Expression in Stage 15 Embryos

(a) Schematic representation of gene expression patterns (boxes) within a parasegment that are relevant to this work (left side). Epistatic interactions of gene activities leading to *wg* and *sr* expression (right side). PS, parasegmental boundary; S, segmental border.

(b–j) Confocal images showing parts of three abdominal parasegments (for position and orientation, see Figure 1) labeled with anti-En ([b, e, and h], red) or anti β-galactosidase antibodies ([c, f, and i], green) and merged views (d, g, and j). Corresponding expression patterns in wild-type embryos are shown in Figures 1c–1e. Note the posterior expansion of the sr1.9-mediated expression domain in embryos that misexpress Hh in the *ptc* domain (mediated by *ptc*-Gal4::UAS-*hh*) (31, 33) (c and d), in ptc^{in} mutant embryos (f and g), and in embryos that misexpress dominant active Ci in the *ptc* domain (mediated by *ptc*-Gal4::UAS-*ci*ZN/C) (i and j). For details, see text.

Ci^{cell}, caused the opposite effect, that is, repression of reporter gene activity (data not shown). These results imply that the sr1.9 element mediates gene activation in direct response to the transcription factor Ci. We therefore examined by DNaseI footprinting whether the sr1.9 DNA fragment contains Ci binding sites. We found two Ci in vitro binding sites (Kinzler and Vogelstein, 1990) within a 239 bp subfragment (see Experimental Procedures) of the sr1.9 element (sr239; Figures 3a and 3b). In order to show that the Ci binding sites are functional in the context of sr1.9-dependent gene expression, we performed expression studies in which the *lacZ* reporter gene was driven by subfragments of the sr1.9 element serving as *cis*-acting control elements.

Subfragments of the sr1.9 element lacking sr239 failed to activate discernible gene expression (data not shown), whereas sr239 directs sr1.9-like gene expression in the row of cells posterior to the *en* domain (Figure 3b). Moreover, sr239-dependent gene activation could be achieved upon ectopic CiZn/C (Hepker et al., 1997) expression within the *en* domain. Thus, the Ci binding site–containing sr239 element is both necessary and sufficient to activate gene expression in segment border precursor cells, and it is sufficient to mediate gene activation in response to dominant active Ci. We next asked whether the two Ci binding sites within the sr239 element are necessary to mediate Hh-dependent gene activation. For this experiment, we generated sr239 variants lacking either one or both Ci binding sites. sr239 variants lacking only one functional Ci binding site can mediate gene expression in the correct spatial pattern, but the level of expression was strongly reduced (Figure 3c). In contrast, lack of both Ci binding sites abolished Hh/Ci-dependent gene activation completely (Figure 3d). In summary, these findings establish that Ci activates gene expression in segment border cells. They confirm, by direct means, that Hh signaling acts not only anteriorly and across the PS boundary to maintain Wg activity (DiNardo et al., 1994), but functions in a symmetric fashion and thereby determines the position of the segment border within the PS.

Wg Represses sr1.9-Dependent Transcription Anterior but Not Posterior to the *en* Domain

Since Hh signaling appears to be symmetric, we wanted to know why the sr1.9 element fails to mediate gene activation in the row of cells anterior to the *en* domain. An explanation for this phenomenon would be that signaling by Wg causes region-specific repression, preventing gene activation by Hh-dependent Ci. To test this proposal, we ectopically expressed Wg in the *ptc* domain and examined the change of sr1.9-driven gene expression in such embryos. Furthermore, we analyzed the sr1.9-mediated gene activation in embryos mutant for *lines* (*lin*), *sloppy paired* (*slp*), and *naked* (*nkd*) (Tearle and Nüsslein-Volhard, 1987; Grossniklaus et al., 1992). Each of these mutant embryos express *en*, but the *wg* pattern is altered (Tearle and Nüsslein-Volhard, 1987; Grossniklaus et al., 1992).

sr1.9-mediated gene expression was abolished in response to ectopic Wg activity (Figures 4a–4c). The same effect was observed in *nkd* mutant embryos (Figures 4d–4f) where Wg is expressed at each side of an enlarged *en* domain (Martinez Arias et al., 1988; Dougan and DiNardo, 1992). Conversely, in *slp* (Figures 4g–4i) and *lin* (Figures 4j–4l) mutants where Wg activity is not maintained (van den Heuvel et al., 1993; Cadigan et al., 1994; Bokor and DiNardo, 1996), sr1.9-mediated gene expression is found in two rows of cells, one on each side of the *en* domain. Furthermore, sr1.9-mediated gene expression was also observed in cells anterior to the region of *en* expression in embryos in which late Wg activity was abolished due to a temperature-sensitive *wg* mutation (Tearle and Nüsslein-Volhard, 1987) (data not shown). This establishes that the repression of Hh action in the cells anterior to the wild-type *en* domain is dependent on Wg activity.

To explore how Wg exerts its repressing function, we examined whether the *Drosophila* TCF/Lef1 homolog Pangolin (Pan), the nuclear mediator of Wg activity (Brunner et al., 1997; van de Wetering et al., 1997), can

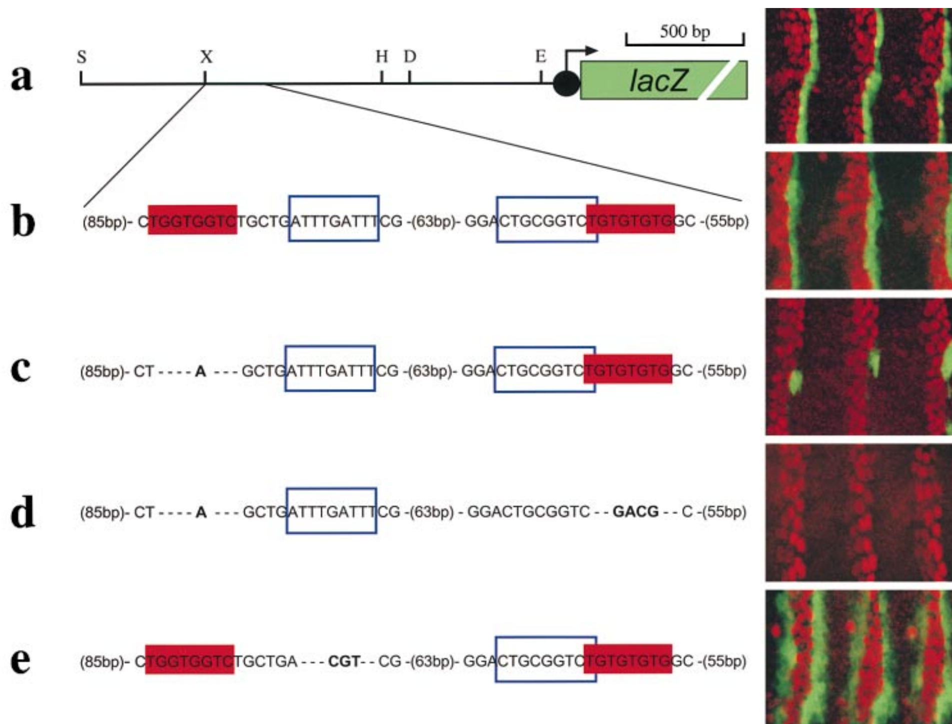

Figure 3. sr1.9-Mediated Gene Expression Depends on Ci and Pan Binding Sites

(a) Schematic representation of the sr1.9-dependent reporter gene containing the sr1.9 element (SpeI/EcoRI fragment of the *sr cis*-acting element [Frommer et al., 1996]; diagnostic restriction sites are S, SpeI; X, XbaI; H, HincII; D, DraII; E, EcoRI) fused to the hsp43 minimal promoter (Thummel and Pirrotta, 1992) (closed circle) in front of the *lacZ* reporter. Reporter gene expression (green) in cells posterior to the *en* domain (red [see also Figures 1b and 1e]) is shown in enlarged images on the right side (for position and orientation, see Figures 1b–1e).
(b) The sr239 element (see text), which contains two Ci (red boxes) and Pan (blue frames) binding sites, is sufficient to activate sr1.9-like gene expression specifically in segment border precursor cells.
(c) Deletion of one Ci binding site of the sr239 element causes a strong reduction of reporter gene expression.
(d) No sr239-mediated gene expression was observed after deletion of both Ci sites.
(e) Deletion of one Pan binding site (sr239ΔPan) causes ectopic activation of sr239-mediated gene expression anterior to the *en* domain (see also Figure 4 and details in the text).
Bold letters (c–e) refer to replacements, dashes to nucleotide deletions in Ci and Pan binding sites, respectively. Expression of the reporter genes are shown in stage 15 embryos.

directly interact with the sr239 element. We found Pan in vitro binding sites next to and partially overlapping the Ci binding sites (Figure 3e). Deletion of one Pan binding site (see Experimental Procedures) that leaves the Ci binding sites intact (sr239ΔPan; Figure 3e), resulted in gene activation anterior to the *en* domain (Figures 4m–4o). In contrast to sr239-mediated gene expression that can be suppressed by *ptc*-Gal4-driven Wg activation, sr239ΔPan-mediated gene expression is not abolished in response to ectopic Wg activity (data not shown).

Unilateral Repression of Hedgehog by Wg Signaling Positions Segment Border Cells

The results presented here provide evidence that the *sr*-dependent determination of segment border-marking muscle attachment sites (Frommer et al., 1996) results from Wg signaling at the PS boundary, which restricts gene activation by Hh to only one side of the *en* domain. Both signaling molecules act through their canonical nuclear mediators Pan (Brunner et al., 1997) and Ci (Alexandre et al., 1996), respectively. Pan has previously been shown to act as a repressor or activator of transcription. In cells that have not received the Wg signal, Pan can associate with corepressors such as dCBP (Waltzer and Bienz, 1998) or Groucho (Cavallo et al., 1998). Upon reception of the Wg signal, Pan is switched into an activator of transcription (van de Wetering et al., 1997) by association with Armadillo, a coactivator of Wg target genes (Riese et al., 1997). Our findings suggest an alternative mechanism since the Pan binding sites of the sr1.9 and sr239 elements mediate Pan-dependent repression in cells with high Wg activity. This repression is necessary and sufficient to antagonize Ci-dependent transcriptional activation. Pan could thereby exert this function by competing sterically for Ci binding, by short-range quenching of Ci-mediated gene activation, or by active repression (Mannervik et al., 1999). Each way, Pan would allow for the formation of only one row of segment border cells within each PS by repressing the Hh-dependent *sr* activation in the *wg* domain. It has been previously reported that the requirement for Fused, a component for Hh signaling, is asymmetric (Therond et al., 1999). This finding implies that the readout of Hh signaling may differ in areas anterior and posterior to the Hh source. Our results show that the qualitative

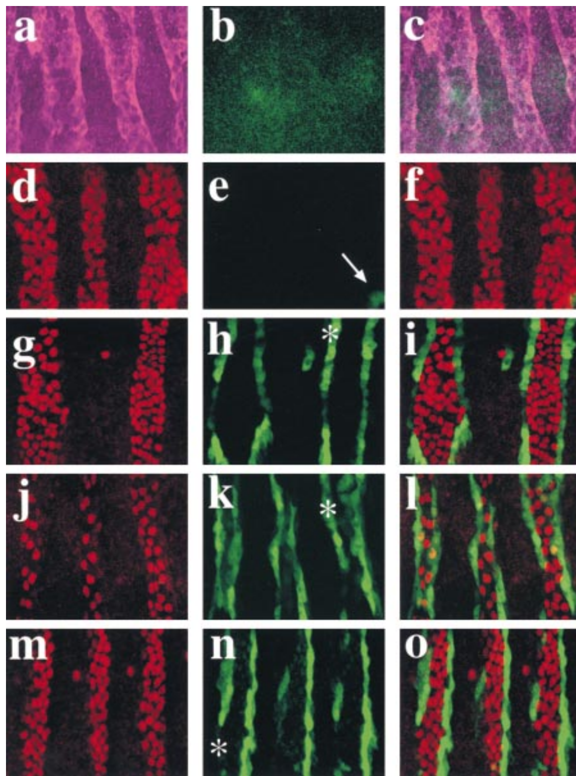

Figure 4. sr1.9-Mediated Gene Activation Is Repressed by Wg Signaling Anterior to the *en* Domain of Stage 15 Embryos

Confocal images showing parts of three abdominal parasegments (for position and orientation, see Figure 1).

(a–c) Ectopic expression of Wg in the *ptc* domain ([a], blue) represses sr1.9-dependent activation ([b]; merged view in [c]).

(d–l) En ([d, g, and j], red), sr1.9-mediated gene expression ([e, h, and k], green), and merged view (f, g, and l) in mutant embryos.

(d–f) sr1.9-mediated gene expression is absent in nkd^{7E} mutants (except in few cells that appeared rarely; see arrow in [e]). Note a slight broadening of the *en* domain (d and f).

(g–i) $slp^{\Delta 34B}$ mutants, which lack the odd-numbered *en* stripes (see [g]), show sr1.9-mediated gene expression in a row of cells anterior and posterior to the *en* domain ([h], asterisks marks an anterior row of cells).

(j–l) lin^{2} mutant embryos (note the slightly reduced *en* stripes in [j]) show sr1.9-mediated gene expression in a row of cells anterior and posterior to the *en* domain ([k], asterisks marks an anterior row of cells).

(m–o) En ([m], red) and sr239ΔPan-mediated gene expression ([n], green; merged view in [o]) in rows of cells anterior and posterior to the *en* domain. Note that due to the focal plane, the stripes of sr1.9- and sr239ΔPan-mediated gene expression are not always in focus.

aspects of *sr* expression does not involve this mechanism. However, it may contribute in a quantitative manner to the asymmetric activation of *sr*, a parameter not addressed by our study.

sr encodes a transcription factor that is necessary to establish segment border cell identity (Vorbrüggen and Jäckle, 1997). Accordingly, *lin* mutant embryos that express *sr* and sr1.9-driven reporter gene in a row of cells on each side of the *en* expression domain (Figures 4j–4l) develop a second set of functional muscle attachment sites, indicating that a second segment border has been formed within the PS (Volk and VijayRaghavan, 1994). Our results are therefore consistent with a model describing that positioning of segment border cells is achieved solely in response to symmetric Hh signaling from the *en* domain, which is caused by symmetry-breaking repression by Wg activity anterior to the PS boundary. Our observations confirm the asymmetric range of Wg signaling within the embryonic epidermis (Sanson et al., 1999), and they allow us to trace the molecular patterning process all the way from the initial anterior–posterior coodinate system of the oocyte via the segmentation genes and PS boundaries to the precise positioning of permanent segment borders.

Experimental Procedures

Fly Stocks and Embryos

ptc^{In}, lin^{2}, nkd^{7E} (Bloomington and Umea stock centres), and $slp^{\Delta 34B}$ (gift of W. J. Gehring) mutant alleles were used. Ectopic expression experiments were performed with the Gal4::UAS system (Brand and Perrimon, 1993) using the *ptc*-Gal4 driver in combination with UAS-*hh*, UAS-*wg*, and UAS-*ciZN/C*, as described (Vorbrüggen and Jäckle, 1997). Eggs were collected at 25°C, except for the *ptc*::Wg experiments where collection was at 29°C. Staged embryos (Campos-Ortega and Hartenstein, 1997) were fixed and stained with anti-Sr (Frommer et al., 1996), anti-En (4D9; DSHB [Iowa City, IA]), anti-β-galactosidase, anti-HA.11 (BAbCO [Richmond, CA]), and/or anti-Wg (4D4, DSHB) antibodies as described in Vorbrüggen and Jäckle (1997) employing fluorescent secondary antibodies (Alexa488 and Alexa546; Molecular Probes [Eugene, OR]) or the Vectastain Elite ABC kit (Vector [Burlingame, CA]) for visualization in the Zeiss LSM 410 confocal microscope and under a Zeiss axiophot, respectively.

Generation of Constructs and Transformation

Reporter constructs were generated by cloning genomic sequences of the *sr* locus into the pCaSpeR-AUG-β-GAL vector (Thummel and Pirrotta, 1992). Flanking sequences of sr1.9 within the genomic phage λsr1 (Frommer et al., 1996) are ACTAGTGAATGCAGGCAT GATTTAA and AAGCATTTTGTGAATGAATGAATTC. Primers used to generate subfragment sr239 were TCTAGACCCAATAGAGTTTGA and AATGAACTAAGGCATACAATTAC. Mutagenesis was carried out with the Quickchange kit (Stratagene [La Jolla, CA]) and appropriate primers. P element–mediated transformation of flies was according to standard procedures (Ashburner, 1989). Transgenic lines were assayed for β-galactosidase expression by antibody staining. At least three independent lines were analyzed for each construct.

Acknowledgments

We thank our colleagues in the lab for various important contributions, T. Kornberg and P. Aza-Blanc for critical discussions, G. Dowe for sequencing, K. Basler, W. J. Gehring, R. Holmgren, S. Cohen, and T. Orenic for mutants, T. Volk for antibodies, and H. Taubert for injections. Work was supported by the Max Planck Society and by the SFB 271 (H. J. and G. V.).

Received March 2, 2000; revised May 25, 2000.

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