An anteroposterior Dorsal gradient in the *Drosophila* embryo

Audrey M. Huang, Jannette Rusch,¹ and Michael Levine²

Department of Molecular and Cell Biology, Division of Genetics, University of California, Berkeley, California 94720 USA

Dorsoventral (DV) patterning of the *Drosophila* embryo is initiated by a broad Dorsal (Dl) nuclear gradient, which is regulated by a conserved signaling pathway that includes the Toll receptor and Pelle kinase. We investigate the consequences of expressing a constitutively activated form of the Toll receptor, Toll^{10b}, in anterior regions of the early embryo using the *bicoid* 3' UTR. Localized Toll^{10b} products result in the formation of an ectopic, anteroposterior (AP) Dl nuclear gradient along the length of the embryo. The analysis of both authentic dorsal target genes and defined synthetic promoters suggests that the ectopic gradient is sufficient to generate the full repertoire of DV patterning responses along the AP axis of the embryo. For example, mesoderm determinants are activated in the anterior third of the embryo, whereas neurogenic genes are expressed in central regions. These results raise the possibility that Toll signaling components diffuse in the plasma membrane or syncytial cytoplasm of the early embryo. This study also provides evidence that neurogenic repressors may be important for the establishment of the sharp mesoderm/neuroectoderm boundary in the early embryo.

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The Dorsal (Dl) nuclear gradient is established by a highly conserved signaling pathway that is related to the mammalian IL-1 pathway (for review, see Steward and Govind 1993; Wasserman 1993; Belvin and Anderson 1996). A variety of studies suggest that the Toll receptor is activated in ventral regions by a localized source of processed Spätzle (Spz) ligand (Morisato and Anderson 1994; Schneider et al. 1994). The activation of Toll leads to the induction of the Pelle kinase (Shelton and Wasserman 1993) and ultimately to the phosphorylation and degradation of the cactus inhibitor (Bergmann et al. 1996; Reach et al. 1996), thereby permitting translocation of Dl from the cytoplasm to the nucleus (Roth et al. 1989; Rushlow et al. 1989; Steward 1989). Embryos derived from *Toll*⁻ females do not exhibit nuclear transport of the Dl protein, and display a "dorsalized" mutant phenotype, whereby all tissues form derivatives of the dorsal ectoderm (Roth et al. 1989). In contrast, a constitutively activated form of the Toll receptor, Toll^{10b}, results in promiscuous nuclear transport of the Dl protein so that all cells undergo the initial phases of mesoderm differentiation (Erdelyi and Szabad 1989; Schneider et al. 1991).

The Dl gradient specifies five distinct thresholds of

gene activity (summarized in Fig. 1). Threshold I is defined by peak levels of Dl in the ventral-most regions of early embryos. *folded gastrulation (fog)* may be an example of a type I target gene because it is expressed only in a subdomain of the ventral mesoderm (Costa et al. 1994). Threshold II is exemplified by *snail*, which is activated by peak and high levels of the Dl gradient throughout the presumptive mesoderm (Ip et al. 1992a). The lateral limits of the *snail* expression pattern establish the boundary between the presumptive mesoderm and neuroectoderm (Alberga et al. 1991; Kosman et al. 1991; Leptin 1991). Threshold III is established by intermediate levels of the Dl gradient in the ventral-most regions of the neurogenic ectoderm, which correspond to the presumptive mesectoderm. Several type III target genes have been identified, including *single minded (sim)* and the *m7* and *m8* genes of the Enhancer of split [E(spl)] complex (Kasai et al. 1992; Martin-Bermudo et al. 1995). Threshold IV is represented by the *rhomboid (rho)* gene, which is expressed in the ventral half of the lateral neuroectoderm (Bier et al. 1990; Ip et al. 1992b). Finally, threshold V is defined by the lowest levels of the Dl gradient, which regulate *short gastrulation (sog)* (Francois et al. 1994; Holley et al. 1995), and *zerknüllt (zen)* (Jiang et al. 1993). *sog* is activated by the lowest levels of the Dl gradient present at the junction between the presumptive dorsal ectoderm and neuroectoderm (Fig. 1). These same low levels of Dl are also sufficient to repress genes such as *decapentaplegic (dpp)* and *zen* (Huang et

¹Current address: Department of Biology, University of California San Diego, La Jolla, California 92093-0347 USA.

²Corresponding author.

E-MAIL mlevine@mendel.berkeley.edu; FAX (510) 642-7000.

Figure 1. Summary of gradient thresholds. Previous studies have identified at least five distinct thresholds of gene activity in response to the DL nuclear gradient (e.g., Jiang and Levine 1993; Rusch and Levine 1996). The type I target gene *fog* is activated only in response to peak levels of the DL gradient (Costa et al. 1994), so that expression is restricted to a subdomain of the presumptive mesoderm. The PE enhancer from the *twist* promoter region exhibits a similar pattern of expression (Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991). This enhancer contains a cluster of low-affinity DL binding sites (open circles), which restrict expression to the ventral-most regions of early embryos. The type II target gene *snail* contains a series of low-affinity DL-binding sites, as well as binding sites for the bHLH activator, Twist (Ip et al. 1992a). The DL and Twist proteins appear to make synergistic contact with the basal transcription complex, so that *snail* is activated throughout the presumptive mesoderm in response to both peak and high levels of the DL gradient (Szymanski and Levine 1995). The ventral midline arises from the mesectoderm, which is derived from the ventral-most regions of the neuroectoderm. Mesectoderm differentiation is controlled by the bHLH-PAS gene, *sim* (for review, see Crews et al. 1992). Some of the genes of the E(spl) complex also exhibit early expression in the presumptive mesectoderm (Martin-Bermudo et al. 1995). A synthetic enhancer containing high-affinity DL-binding sites (solid circles) and twist binding sites exhibits expression in this region (Gray and Levine 1996a). The type IV target gene *rho* is expressed in lateral stripes that encompass the ventral half of the presumptive neuroectoderm (Bier et al. 1990). These stripes are regulated by a 300-bp enhancer (NEE) that contains high-affinity DL-binding sites (solid circles), Twist-binding sites, and "generic" E-box sequences that appear to bind ubiquitously distributed bHLH activators [Daughterless (Da) and scute (T4)] that are present in the unfertilized egg (Ip et al. 1992b; Gonzalez-Crespo 1993). The fifth and final threshold response is defined by the lowest levels of the DL gradient. The *zen* target gene is repressed by high and low levels of the gradient, so that expression is restricted to the presumptive dorsal ectoderm. Previous studies have shown that the *zen* promoter region contains high-affinity DL-binding sites and closely linked "corepressor" sites (Lehming et al. 1994; Cai et al. 1996). Efficient occupancy of the DL sites appears to depend on strong, cooperative DNA-binding interactions between DL and the corepressors. The same low levels of DL that repress *zen* also activate *sog*; the *sog* promoter region has not been characterized (Francois et al. 1994; Holley et al. 1995). The *sim*, *E(spl)*, *rho*, and *sog* expression patterns are restricted to the neurogenic ectoderm and excluded from the ventral mesoderm by Snail, which encodes a zinc finger repressor (e.g., Ip et al. 1992b).

al. 1993; Jiang et al. 1993). In principle, these latter genes can be activated throughout the early embryo but are repressed in ventral and lateral regions by the DL gradient.

These different thresholds of gene activity do not appear to be specified by a continuum of DL operator sites with distinct binding affinities. Instead, there appear to be just two types of DL-binding sites, high affinity and low affinity (Jiang and Levine 1993). The three thresholds generated by high-affinity sites (see Fig. 1 for summary) depend on combinatorial interactions between DL and other transcription factors. For example, the activation of the *rho* lateral stripe enhancer by low levels of the DL gradient depend on cooperative DNA-binding interactions between DL and closely linked basic helix-loop-helix (bHLH) activators (Ip et al. 1992b; Jiang and Levine 1993).

DL gradient thresholds represent the clearest manifestation of the Wolpert French flag model, whereby a morphogen gradient establishes different territories of gene activity and tissue differentiation (e.g., Wolpert 1996).

Recent studies suggest that similar principles might apply to extracellular gradients of the Dpp and Wingless (Wg) ligands in the wing imaginal disk (Lecuit et al. 1996; Nellen et al. 1996; Zecca et al. 1996; Neumann and Cohen 1997), although the analysis of threshold responses in these gradient systems is complicated by the occurrence of numerous rounds of cell proliferation before the detection of target gene activity (Lecuit et al. 1996). In contrast, the syncytial blastoderm provides a simple "readout" system as there is only a 30-min lag between the formation of the DL gradient and the transcription of target genes.

In this study we describe the consequences of creating an ectopic DL gradient along the anteroposterior (AP) axis of the early embryo. The constitutively activated Toll^{10b} receptor was expressed in anterior regions of transgenic embryos using the Bicoid (Bcd) 3'-untranslated region (UTR) localization sequence (Macdonald and Struhl 1988). The ensuing ectopic DL gradient mediates a dramatic transformation of the AP pattern into a series of dorsoventral (DV) patterning thresholds along the length

of the embryo. All five thresholds of gene activity are established along the AP axis, even though the slope of the ectopic DI gradient is distinct from the endogenous gradient. These results demonstrate that a robust DV patterning response can be obtained in the absence of Spz and other potential polarizing factors in the perivitelline fluid (Stein and Nüsslein-Volhard 1992; Roth 1993; Morisato and Anderson 1994). We propose that the detailed slope of the DI gradient might involve the diffusion of Spz-Toll complexes in the plasma membrane, or the diffusion of Toll target proteins in the cytoplasm. Such diffusion might be particularly important for differential patterns of gene expression in the lateral neuroectoderm, where there are three distinct thresholds of gene activity over a distance of just ~10–12 cell diameters.

Results

The dominant *Toll^{10b}* mutation is caused by a single amino acid substitution at residue 781 (C781Y), which is located on the external surface near the transmembrane spanning domain (Schneider et al. 1991). A *Toll^{10b}* cDNA was inserted into a P-element transformation vector that contains either the *Hsp83* or *bcd* promoter, and the *bcd* 3' UTR (see diagram above Fig. 2A), which localizes maternal transcripts to the anterior pole of oocytes and embryos (Macdonald and Struhl 1988; Ephrussi and Lehmann 1992; Rusch and Levine 1997).

Ectopic activation of the Toll signaling pathway

Embryos derived from transgenic females were hybridized with a digoxigenin-labeled Toll antisense RNA probe (Fig. 2A). Ectopic Toll transcripts are detected at the anterior pole, along with ubiquitously distributed endogenous RNAs (Fig. 2, cf. A with B). *Toll^{10b}* RNAs can be distinguished from the endogenous RNAs because the former are localized within the apical cytoplasm by the *bcd* 3' UTR (arrow, Fig. 2A; Gertulla et al. 1988; Davis and Ish-Horowicz 1991). Despite the apparently tight localization of *Toll^{10b}* transcripts, transgenic embryos exhibit a broad DV patterning response along the length of the AP axis (see below).

Immunolocalization assays were done to visualize the distribution of the *Toll^{10b}* protein in transgenic embryos. The endogenous protein is present at low levels, and weak staining can be detected throughout the plasma membrane of early embryos (data not shown; Hashimoto et al. 1991). *Toll^{10b}* products are first visualized in early cleavage embryos, before nuclear cycle 8, in anterior regions that correspond to sites of RNA accumulation (Fig. 3A). By the onset of nuclear cleavage cycle 14 there is a rather broad distribution of *Toll^{10b}* protein (Fig. 3B,C), with peak levels present in the anterior-most regions.

The subcellular localization of the *Toll^{10b}* protein is distinct from the endogenous, wild-type protein. *Toll^{10b}* accumulates in dense rings that are detected at or near the cell surface (Fig. 3D). Such punctate accumulations

Figure 2. Localized *Toll^{10b}* transcripts lead to the formation of an AP DI gradient. Embryos were derived from heterozygous females carrying one copy of the *Toll^{10b}* P-element transposon shown above panel A. The embryos are oriented with anterior to the left and dorsal up. (A) Nuclear cleavage cycle-14 embryo undergoing the initial phases of cellularization. The embryo was hybridized with a digoxigenin-labeled *Toll* antisense RNA probe and stained with an anti-digoxigenin antibody. Endogenous *Toll* transcripts are distributed throughout the basal cytoplasm (Gertulla et al. 1988), whereas the transgenic *Toll^{10b}* transcripts are mainly restricted to the apical cytoplasm in anterior regions. (B) Same as A, except that the embryo lacks the *Toll^{10b}* transgene. Endogenous *Toll* transcripts are detected throughout the basal cytoplasm. (C) Dorsolateral view of a nuclear cleavage cycle-13 embryo stained with anti-DI antibodies and visualized by indirect immunofluorescence. Intense nuclear staining is detected in anterodorsal regions, which corresponds to sites of peak *Toll^{10b}* mRNA accumulation in early embryos. Staining is restricted primarily to nuclei in anterior regions, and is partitioned between nuclei and cytoplasm in central regions. DI protein is restricted to the cytoplasm in posterior regions. The endogenous DV DI nuclear gradient cannot be seen in this orientation.

are not observed for the wild-type protein (data not shown). Perhaps these rings represent sites of active Toll signaling, either attributable to the aggregation of the receptor at the cell surface or the endocytosis of active receptor. *Toll^{10b}* rings are more pronounced in dorsal regions of the embryo. In ventral regions, activation of the endogenous receptor by Spz might somehow inhibit *Toll^{10b}* aggregation.

Ectopic expression of *Toll^{10b}* results in the formation

Figure 3. Distribution of the *Toll*^{10b} protein. Transgenic embryos are oriented with dorsal up and anterior to the left. Embryos were incubated with an anti-Toll antibody and visualized by histochemical staining with horseradish peroxidase. (A) Nuclear cleavage cycle-8 embryo. *Toll*^{10b} proteins are restricted to the anterior pole. The diffuse, general background staining is probably attributable to the endogenous Toll protein. (B) Cleavage cycle-13 embryo. Staining is no longer restricted to the anterior pole, but extends to more posterior regions. Higher levels of *Toll*^{10b} are detected in dorsal vs. ventral regions. This DV asymmetry is also observed for the *Toll*^{10b} RNAs (see Fig. 2A), and is probably attributable to the *bcd* 3' UTR. (C,D) Higher magnification views of the embryo shown in B. *Toll*^{10b} proteins appear to aggregate in rings at or near the cell surface. This type of punctate staining is not observed for the normal, endogenous protein.

of a broad AP Dl nuclear gradient (Fig. 2C). In the anterior third of the embryo, the Dl protein is mainly localized within nuclei, whereas in middle regions, the protein is present in both nuclei and the cytoplasm; it is strictly cytoplasmic in posterior regions. The slope of the ectopic AP gradient appears to be distinct from the endogenous DV gradient. For example, peak nuclear staining is normally detected in just 10 ventrolateral nuclei on either side of the ventral midline (Roth et al. 1989; Rushlow et al. 1989; Steward 1989), whereas the ectopic gradient encompasses at least twice as many nuclei in anterior regions (Fig. 2C). Despite this difference, the ratio of the zones exhibiting strictly nuclear staining and mixed staining in both nuclei and cytoplasm is approximately equivalent (roughly 1:1) for both the AP and DV gradients (see below).

Multiple DV thresholds along the AP axis

Toll^{10b} transgenic embryos were hybridized with a number of different RNA probes to examine the impact of the AP Dl gradient on development (Fig. 4). The type II target gene, *snail*, is activated in the ventral mesoderm (Fig. 4A; Ip et al. 1992a) but is also expressed in the anterior third of transgenic embryos (Fig. 4D). The type V target gene *sog* is expressed in broad lateral stripes (Fig. 4B) and in the central two-thirds of transgenic embryos (Fig. 4E; Francois et al. 1994; Holley et al. 1995). Both the ectopic and endogenous *sog* patterns are excluded from anterior regions by Snail, which functions as a transcriptional repressor of type III, IV, and V genes (Gray et al. 1994; Gray and Levine 1996b). Another type V gene, *zen* (see Fig. 1 for summary), is repressed by the AP and DV Dl gradients in the same regions that activate *sog* (Fig. 4C,F). Staining at the anterior pole results from a Dl-Torso interaction, whereby the Torso (Tor) receptor tyrosine kinase (RTK) masks the ability of Dl to function as a transcriptional repressor (Rusch and Levine 1994).

Additional expression assays were conducted in mutant embryos lacking the endogenous Toll pathway and DV dorsal nuclear gradient. This was achieved by crossing the *Toll*^{10b} P-element transposon into a strain carrying a null mutation in *gastrulation defective* (*gd*), one of the putative serine proteases required for the processing of the Spz ligand (Konrad et al. 1988). Such embryos lack an intrinsic DV polarity, so that the expression of Dl target genes depends solely on the ectopic AP Dl gradient generated by the localized *Toll*^{10b} mRNA. Embryos derived from transgenic females were hybridized with a mixture of *snail* and *zen* probes (Fig. 4G). Staining is restricted to anterior and posterior regions that correspond to sites of ectopic *snail* and *zen* expression, respectively.

sog is expressed broadly in central regions of these embryos (Fig. 4H). A similar pattern is observed for *rho*, which is a type IV target gene (Fig. 4I). The posterior limit of the pattern does not extend as far as *sog* (Fig. 4H), as the two genes exhibit differential threshold responses to diminishing amounts of the Dl gradient (see Fig. 1 for summary). Both patterns are excluded from the anterior third of the embryo by the ectopic Snail repressor. These results suggest that the localized *Toll*^{10b} receptor can specify multiple DV patterning responses in the absence of processed Spz ligand.

Analysis of synthetic promoters

Further evidence that the AP Dl gradient specifies the full complement of DV thresholds was obtained by analyzing derivatives of a 260-bp enhancer (PE) from the promoter region of the type II target gene, *twist* (Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991). The PE is activated only by peak levels of the Dl gradient, similar to the pattern observed for the type I target gene, *fog* (see Fig. 1). A PE-*lacZ* fusion gene is activated in the anterior fourth of transgenic embryos (Fig. 5A). Two tandem copies of a slightly modified version of this fusion gene

Figure 4. Misexpression of Dl target genes along the AP axis. *Toll^{10b}* transgenic embryos are undergoing cellularization, and are oriented with dorsal *up* and anterior to the *left*. The embryos were hybridized with various digoxigenin-labeled antisense RNA probes. (A) *snail* expression pattern in a wild-type embryo. Staining is restricted to ventral regions that will form the mesoderm. (B) *sog* expression pattern in a wild-type embryo. Staining is detected in two broad lateral stripes that encompass the entire presumptive neurogenic ectoderm. Only one of the stripes can be seen in this ventrolateral view. The other stripe is out of the plane of focus. (C) *zen* expression pattern in a wild-type embryo. Staining is detected in dorsal regions, and at the anterior and posterior poles. The endogenous Dl nuclear gradient represses *zen* expression in ventral and lateral regions. The Tor RTK pathway blocks the ability of Dl to function as a repressor at the poles (Rusch and Levine 1994). (D) *snail* expression pattern in an embryo derived from a transgenic female. Ectopic staining is observed in the anterior third of the embryo. Because the *Toll^{10b}* transgene is expressed in a wild-type strain, the normal, endogenous staining pattern is also observed in the ventral mesoderm. (E) *sog* expression pattern. Ectopic staining is detected in a broad domain in central regions of the embryo. The endogenous lateral stripes are also observed. Both the ectopic and endogenous staining patterns are excluded from the anterior third of the embryo by the Snail repressor (see A). (F) *zen* expression pattern. Staining is detected at the anterior and posterior poles, and in a dorsal patch in the presumptive abdomen. The latter site of expression corresponds to the only region that lacks both the endogenous and ectopic dorsal nuclear gradients (Fig. 2C; data not shown). Staining at the poles coincides with regions where the Dl repressor is masked by the Tor RTK pathway. (G) Embryo derived from a female carrying a copy of the *Toll^{10b}* transgene and homozygous for a null mutation in *gd*. This latter mutation abolishes the activity of the endogenous Toll signaling pathway (Konrad et al. 1988) so that Dl target genes are regulated solely by the ectopic AP dorsal gradient. The embryo was stained with a mixture of *snail* and *zen* hybridization probes. Staining in the anterior third of the embryo corresponds to sites of *snail* expression, whereas posterior staining corresponds to *zen*. (H) Same as G except that the embryo was stained with a *sog* hybridization probe. A broad band of expression is detected in central regions of the embryo, extending from ~70%–20% egg length. The anterior border is probably specified by the Snail repressor, whereas the posterior border may be formed by limiting amounts of the AP Dl nuclear gradient. (I) Same as G and H except that the embryo was stained with a *rho* hybridization probe. The staining pattern is narrower than the *sog* pattern (H) because *sog* is activated by lower threshold levels of the Dl gradient (see Fig. 1 for summary).

(2xPEe), which contains nucleotide substitutions in the Dl operator sites that increase their binding affinities (Jiang and Levine 1993), mediate *lacZ* reporter gene expression in the anterior third of the embryo (Fig. 5B). A modified form of 2xPEe that contains Twist protein binding sites (2xPEe-Et) is activated by both high and intermediate levels of the Dl gradient (Jiang and Levine 1993) so that staining includes nearly the entire anterior half of the embryo (Fig. 5C). These results, together with the analysis of endogenous target genes (Fig. 4), indicate that the AP Dl gradient can direct the full repertoire of patterning thresholds, including the type III *sim* pattern (data not shown).

PE-*lacZ* fusion genes are not expressed at the anterior poles of either wild-type or transgenic embryos (e.g., Fig. 5A–C). There are at least two possible explanations for this observation. First, previous studies have shown that

the Tor RTK pathway influences Dl activity at both embryonic poles (Rusch and Levine 1994). This Dl-Tor interaction somehow masks the ability of Dl to function as a transcriptional repressor. It is conceivable that Tor also attenuates Dl-mediated activation. Alternatively, it is possible that the PE regulatory sequence contains repressor sites for *tor* target genes, such as Tailless, which represses gene expression at the poles (Pignoni et al. 1990).

The endogenous *snail* and *twist* expression patterns exhibit abnormal gaps in the ventral mesoderm (data not shown). About half of these embryos display a similar gap in the endogenous *snail* pattern (Fig. 5D). The simplest explanation for the gap is that one or more neurogenic repressors are activated in lateral regions of wild-type embryos and help sharpen the type II *snail* pattern (see Fig. 4A). The ectopic AP Dl gradient might activate these putative repressors in central regions of transgenic

Figure 5. Transcription thresholds and ectopic expression of putative repressors. Embryos were hybridized with a *lacZ* antisense RNA probe (A–C), or with various probes for monitoring the expression of different endogenous genes (D–I). Embryos are oriented with anterior to the left. (A) Horizontal view of a *Toll*^{10b} transgenic embryo that contains the PE-*lacZ* fusion gene. PE is derived from the proximal region of the *twist* promoter, and contains two low-affinity D1-binding sites (see Fig. 1). Expression is restricted to the anterior fourth of the embryo where there are peak levels of the ectopic AP D1 gradient. Staining in the presumptive mesoderm is not seen in this plane of focus. (B) Horizontal view of an embryo carrying the 2xPE-*lacZ* fusion gene, which contains two tandem copies of a modified PE sequence whereby the low-affinity dorsal sites were converted into high-affinity sites (Jiang and Levine 1993). Staining is detected in the anterior third of the embryo in response to both high and intermediate levels of the D1 gradient. The endogenous pattern can be seen as a weak band of staining that runs along the center of the embryo. (C) Same as B except that the embryo contains the 2xPE-*Et* fusion gene, which contains both high-affinity D1-binding sites and closely linked *Twist* sites (Jiang and Levine 1993). The fusion gene is activated in nearly the entire anterior half of the embryo (arrow), in response to both high and intermediate levels of the AP D1 gradient. Strong staining is also detected in ventral and ventrolateral regions, attributable to the endogenous D1 gradient. (D) Lateral view of an embryo stained with a *snail* antisense RNA probe. A gap is observed in the endogenous expression pattern just behind the ectopic staining pattern. About half of the embryos derived from *Toll*^{10b} transgenic females exhibit this gap, whereas the other half show an essentially normal endogenous pattern (e.g., Fig. 4D). (E) *rho* staining pattern in an embryo derived from transgenic females in a wild-type background at the early phase of nuclear cleavage cycle 14. Staining is detected in broad lateral stripes (arrow) attributable to the endogenous DV D1 gradient. In addition, expression is also observed in a broad central band. (F) Same as E except that the embryo is older, and undergoing gastrulation. Both the endogenous lateral stripes (arrow) and the ectopic central band are refined into sharper patterns (cf. with E). (G) *tinman* (*tin*) staining pattern in an embryo derived from transgenic females in a wild-type background undergoing gastrulation. *tin* is activated in the presumptive mesoderm during invagination (Apiazu and Frasch 1993; Bodmer 1993). Both the endogenous ventral furrow (arrowhead), and an ectopic site of mesoderm invagination (arrow) can be seen. (H) *rho* staining pattern in an embryo derived from transgenic females in a wild-type background lacking the endogenous DV gradient. At this stage, early nuclear cleavage cycle 14, the *rho* staining pattern includes a broad, central band. (I) Same as H except that the embryo is older, and undergoing gastrulation. By this stage the ectopic *rho* staining pattern has refined into a sharp stripe.

embryos, and create the observed gap in the endogenous *snail* pattern.

Additional evidence for neurogenic *snail* repressors stems from the analysis of transgenic lines that express the *bcd-Toll*^{10b} transgene. The *bcd* promoter mediates relatively weak expression of *Toll*^{10b}, and consequently, the resulting AP D1 gradient is distinct from that generated by the Hsp83 promoter (Fig. 6; data not shown). Despite this variation, there is always a gap in the endogenous *snail* expression pattern just posterior to the ectopic staining (Fig. 6A,B). These results are consistent with the notion that low levels of the AP D1 gradient activate a repressor which helps refine the sharp *snail* border.

Subdivision of embryonic tissues

The misregulation of D1 target genes along the AP axis

results in the formation of ectopic DV tissues. For example, the expression of type II target genes in anterior regions results in the invagination of ectopic mesoderm (Fig. 5G). The *rho* expression pattern suggests that the ectopic neurogenic ectoderm is subdivided into multiple cell types. *rho* is activated initially in broad lateral stripes that encompass 8–10 cells (arrow, Fig. 5E). During gastrulation, these stripes are refined progressively to the ventral-most regions of the neurogenic ectoderm, which includes the presumptive mesectoderm (arrow, Fig. 5F). It has been suggested that this refinement is a manifestation of the subdivision of the neurogenic ectoderm into ventral and lateral cell types (Rusch and Levine 1996). A similar refinement of the ectopic *rho* pattern is observed in mutant embryos lacking the endogenous DV gradient (Fig. 5H,I). Initially, *rho* is activated in a broad central domain (Fig. 5H), but is refined into a sharp stripe by the

Figure 6. Differential AP Dl gradients. *Toll*^{10b} transgenic embryos are oriented with anterior to the *left* and dorsal *up*. (A) *snail* expression pattern in a transgenic embryo carrying the *bcd-Toll*^{10b} transgene. Ectopic staining is restricted to the anterior pole because the transgene expresses only low levels of the *Toll*^{10b} receptor. There is a gap in the endogenous *snail* pattern just posterior to the ectopic site of expression. (B) Same as A except that the embryo was stained to reveal the *sog* expression pattern. An ectopic band of staining is observed in anterior regions, just behind the ectopic *snail* pattern (cf. with A). The ectopic *sog* band is somewhat broader than the ectopic presumptive mesoderm at the anterior pole.

onset of gastrulation (Fig. 5I). This refinement process is reminiscent of the regulation of *even-skipped* (*eve*) stripe 2, whereby spatially localized repressors define both the anterior and posterior stripe borders (e.g., Arnosti et al. 1996). As discussed earlier, the Snail repressor establishes the anterior border of the ectopic *rho* stripe (Fig. 5I); it is conceivable that additional repressors localized in more posterior regions help define the posterior border. These results reinforce the view that the *bcd* and Dl regulatory gradients employ a similar strategy to specify stripes of gene expression in the early embryo (e.g., Ip et al. 1992b).

Discussion

We have shown that a constitutively activated Toll receptor can specify multiple “French flag” transcription thresholds across the AP axis of early embryos. These results extend previous evidence that the early embryo is inherently “naïve” with respect to DV patterning, and that cell fate is dictated by the intranuclear concentration of the Dl regulatory protein (e.g., Anderson et al. 1985; Roth 1993). It is conceivable that the normal DV Dl nuclear gradient depends on the diffusion of Toll-signaling components in the plasma membrane or cytoplasm. Another implication of this study is that the specification of multiple cell fates might be dictated by the total number of fully activated Toll receptor molecules. The analysis of the *snail* and *rho* expression patterns suggests that one or more neurogenic repressors might be important for the sharpening of the mesoderm-neuroectoderm boundary and the subdivision of the neurogenic ectoderm.

Diffusion in multiple compartments

It has been proposed that the normal, DV Dl nuclear gradient is established by the differential activation of the Toll receptor in ventral and ventrolateral regions by an extracellular Spz gradient in the perivitelline fluid (Stein and Nüsslein-Volhard 1992; Roth 1993; Morisato and Anderson 1994; Schneider et al. 1994). However, we have shown that a full DV patterning response can be obtained in the absence of Spz, thereby raising the possibility that diffusion of Toll-signaling components in the plasma membrane or cytoplasm might contribute to this process (summarized in Fig. 7). It is possible that Spz-Toll complexes formed in ventral regions diffuse within the plasma membrane and assist in the nuclear transport of low levels of Dl protein in lateral regions. A nonexclusive alternative is that cytoplasmic targets of the Spz-Toll complex, such as the Pelle kinase (Shelton and Wasserman 1993), can diffuse after being recruited to the cell surface in ventral regions. Therefore, the final Dl nuclear gradient might depend on the diffusion of Toll-signaling components in all three cellular compartments—the perivitelline fluid, plasma membrane, and cytoplasm.

The demonstration that *Toll*^{10b} can reconstruct multiple DV patterning responses does not reject current models regarding an extracellular Spz gradient in the perivitelline fluid. Cytoplasmic injection experiments suggest that the normal Dl gradient is not formed in the absence of an extracellular patterning asymmetry in the perivitelline fluid (Roth 1993). Specifically, a steep Dl gradient is observed when *Toll*⁺ cytoplasm is injected

Figure 7. Formation of the Dl nuclear gradient. The gradient might depend on diffusion in all three cellular compartments. It has been proposed previously that the Toll receptor is differentially activated by an extracellular Spz gradient (indicated by the blue diamond and arrows) in the perivitelline fluid (Stein and Nüsslein-Volhard 1992; Morisato & Anderson 1994; Schneider et al. 1994). The present study raises the possibility that Spz-Toll (red Y and arrows) complexes formed in ventral regions might diffuse into ventrolateral regions within the plasma membrane (indicated by the two parallel black arcs). Finally, activation of the Toll receptor might induce the recruitment of the pelle kinase to the ventral surface. The activated kinase (green oval and arrows) might diffuse into lateral regions within the cytoplasm.

within a narrow stripe along the dorsal surface of *Toll* embryos. This gradient appears to be much steeper than the endogenous DV gradient present in wild-type embryos. The Roth study is somewhat constrained by the number of Dl target genes that were monitored and by potential alterations in the timing of DV signaling events. Nonetheless, it provides substantial evidence that diffusion in the plasma membrane or cytoplasm could not be the sole basis for the establishment of the Dl gradient. A prudent interpretation of the current study is that limited diffusion in these cellular compartments might contribute to the overall slope of the gradient, particularly in the presumptive neurogenic ectoderm where only a small fraction of the Dl protein is liberated from the cytoplasm.

Cell fate is dictated by the number of fully activated receptors

The full range of DV patterning thresholds was obtained with a single, constitutively activated form of the Toll receptor. Low levels of Toll^{10b} result in the activation of type III, IV, and V target genes and the specification of neuroectoderm. In contrast, high levels of the receptor lead to the activation of type I and II target genes and the initiation of mesoderm differentiation. A general implication of these studies is that Spz and other putative long-range signaling molecules specify multiple cell types through the stochastic, all-or-none activation of their receptors.

Recent studies suggest that members of the transforming growth factor β (TGF- β) superfamily, such as Dpp and activin, can function as long-range morphogen gradients to specify multiple cell types in the *Drosophila* wing imaginal disk (Lecuit et al. 1996; Nellen et al. 1996; Singer et al. 1997) and *Xenopus* mesoderm (Gurdon et al. 1994; Jones et al. 1996). High concentrations of Dpp activate the expression of *spalt*, whereas low levels trigger the expression of *optomotor-blind* (*omb*) in the wing disk. Similarly, high concentrations of activin induce the expression of goosecoid and low levels activate X-Bra in *Xenopus* animal caps (Gurdon et al. 1994; Jones et al. 1996). These differential responses might solely depend on the number of fully activated activin receptors (thick veins, saxophone, and punt in *Drosophila*). Cells located near the source of secreted Dpp at the AP compartment boundary of the wing disk are exposed to high concentrations of ligand and contain a sufficient number of fully activated receptors to induce *spalt* expression. In contrast, cells located farther from the source might contain just a few fully activated receptors, and therefore express *omb* (Nellen et al. 1996).

The findings presented in this study suggest that a fully activated Toll receptor can specify multiple cell fates. However, we cannot exclude other models for the activation of Toll or other receptors in response to ligand gradients in the context of normal development.

Neurogenic repressors

The borders of the *snail* expression pattern are quite

sharp (e.g., Fig. 4A) and help define the boundary between the presumptive mesoderm and neuroectoderm (Alberga et al. 1991; Leptin 1991; Kosman et al. 1991). It has been proposed that this sharp, on/off expression pattern is established through the “multiplication” of the Dl and Twist gradients (Ip et al. 1992a). The crude Dl gradient directs the expression of a steeper Twist pattern (Jiang et al. 1991; Pan et al. 1991; Thisse et al. 1991) and then the two transcriptional activators function synergistically to trigger a sharp on/off *snail* pattern. Previous studies have failed to identify a role for putative repressors in this process. However, sharp stripes of segmentation gene expression depend on both activator synergy and spatially localized repressors (e.g., Arnosti et al. 1996). In the case of *eve*, the Bcd gradient activates *hunchback*, and the two proteins work synergistically to activate stripe 2 in the anterior half of the embryo. Bcd also coordinates the expression of the giant and Krüppel repressors, which define the anterior and posterior borders of the stripe, respectively.

The present study provides the first evidence that one or more repressors might be required for the establishment of the *snail* border (see Fig. 5D), although the identities of these repressors are uncertain. It is conceivable that members of the E(spl) complex encode putative *snail* repressors because previous studies have shown that the *m7* and *m8* genes are expressed in the lateral neuroectoderm of early embryos (Martin-Bermudo et al. 1995; data not shown). E(spl) proteins are related to Hairy and have been shown to function as repressors in lateral inhibition and neurogenesis (Paroush et al. 1994; Dawson et al. 1995; Fisher et al. 1996).

Gradients and thresholds

The slope of the ectopic AP Dl gradient appears to be distinct from the endogenous DV Dl gradient. For example, the ectopic *snail* expression pattern, a measure of high levels of Dl, can encompass more than twice the number of cells as the endogenous pattern (e.g., Fig. 4D). Nonetheless, all five DV patterning thresholds are obtained with the ectopic AP Dl gradient. Therefore, each cell in the embryo autonomously interprets the intranuclear concentration of the Dl protein. Neighboring cells may not be specified by interpreting relative changes in the concentrations of Dl protein.

Despite pronounced changes in the slope of the ectopic AP Dl gradient, the ratio of presumptive mesoderm (*snail* expression) and neurogenic ectoderm (*sog* expression) remains relatively constant (Fig. 6). Previous embryo injection assays suggest that there are no long-range, nonautonomous DV patterning responses directed by the Dl gradient (Roth 1993). For example, a localized source of the Dl gradient does not appear to inhibit a second nearby source of Dl from specifying mesoderm. However, we have shown that the ectopic AP Dl gradient can alter the endogenous pattern (e.g., Fig. 5D). Perhaps transcriptional repressors encoded by different Dl target genes ensure a relatively constant ratio of presumptive mesoderm and neurogenic ectoderm. As dis-

cussed earlier, neurogenic repressors might help specify the mesoderm/neuroectoderm boundary. We suggest that one or more type V target genes, which are repressed by the Dl gradient and exhibit a *zen*-like expression pattern, help establish the boundary between neurogenic ectoderm and dorsal ectoderm.

Materials and methods

Plasmid constructions

The *Toll*^{10b}-coding sequence was inserted into two different expression vectors, one containing the Hsp83 promoter region (Rusch and Levine 1997), and the other containing the *bcd* promoter (Sauer et al. 1996). A 3.5-kb *NsiI*-*XbaI* *Toll*^{10b} cDNA from plasmid pGEM2-*Toll*^{10b} (Norris and Manely 1992) was inserted into each of these expression vectors. In addition, a 900-bp *SacII*-*XbaI* fragment containing the *bcd* 3' UTR was also inserted into these vectors, to localize *Toll*^{10b} RNAs to the anterior pole of transgenic embryos (Macdonald and Struhl 1988). The Hsp83 promoter directs strong maternal expression during oogenesis (Xiao and Lis 1989), whereas the *bcd* promoter mediates weaker expression.

The *Toll*^{10b} expression plasmids were inserted into a derivative of the pCaSpeR P-element transformation vector, and transgenic strains were obtained by injection of *yw*^{67c23} embryos along with delta2,3 transposase, exactly as described previously (Rusch and Levine 1997).

Antibody stainings

The ectopic AP Dl nuclear gradient (Fig. 2C) was visualized through immunofluorescence. Embryos were collected from transgenic females, and incubated with a 1:2000 dilution of a rat serum obtained after immunization with a full-length bacterial Dl protein (see Rushlow et al. 1989). The embryos were subsequently incubated with a 1:200 dilution of a goat anti-rat IgG solution purchased from Jackson laboratories. Incubations and washes were done exactly as described previously (Frasch et al. 1987).

Toll proteins were detected with a rabbit anti-Toll antibody that was kindly provided by Dr. Steve Wasserman (University of Texas, Southwestern, Dallas). The antiserum was prepared after immunization with a Toll-GST fusion protein that includes amino acid residues 1539–2953 of Toll. The protein was visualized by staining transgenic embryos with biotin-conjugated anti-rabbit antibodies and horseradish peroxidase, exactly as described in the Vectastain directions (Vector Labs).

In situ hybridizations were done with digoxigenin-labeled antisense RNA probes and histochemical staining using alkaline phosphatase (Tautz and Pfeifle 1989; Jiang et al. 1991). The *Zen*, *Rho*, and *Snail* hybridization probes have been described previously (Kosman et al. 1991; Rusch and Levine 1994). Synthetic *twist-lacZ* fusion genes (Fig. 5A–C) were visualized using a digoxigenin-labeled *lacZ* antisense RNA probe (Jiang et al. 1991). The *tin* and *sog* probes have been described by Azpiazu and Frasch (1993) and Francois et al. (1994), respectively.

Genetic crosses

The ectopic AP Dl gradient was examined in the absence of the endogenous DV gradient by misexpressing the *Toll*^{10b} transgene in embryos homozygous for a null mutation in *gastrulation defective* (Konrad et al. 1988). Male flies carrying the *Toll*^{10b} P-element transposon [P(*Toll*^{10b})] were crossed to *gd*⁷/FM3 fe-

males (obtained from the Bloomington Stock Center, Indiana). *gd*⁷;P[*Toll*^{10b}] male progeny were crossed to *gd*⁷/FM3 females and embryos from *gd*⁷/*gd*⁷;P[*Toll*^{10b}] females were collected for analysis by in situ hybridization.

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An anteroposterior Dorsal gradient in the *Drosophila* embryo

Audrey M. Huang, Jannette Rusch and Michael Levine

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