INTRODUCTION

Dorsal is distributed in a broad concentration gradient in the precellular Drosophila embryo (reviewed by Belvin and Anderson, 1996; Rusch and Levine, 1996). It establishes dorsoventral patterning by regulating a number of target genes in a concentration-dependent fashion. At least five thresholds of gene activity have been described (Huang et al., 1997). Peak levels of Dorsal are required for the activation of the snail gene in the presumptive mesoderm in ventral regions of the early embryo (Ip et al., 1992a), whereas progressively lower levels of Dorsal activate single-minded (sim), ventral nervous system defective (vnd), rhomboid (rho) and short gastrulation (sog) in the neurogenic ectoderm (Kasai et al., 1998; Ip et al., 1992b; von Ohlen and Doe, 2000; Francois et al., 1994). These differential patterns of gene activity subdivide the embryo into primary tissues: mesoderm, neurogenic ectoderm and dorsal ectoderm.

The restricted activation of the Toll receptor results from the localized processing of the Spätzle (Spz) ligand in ventral regions of the precellular embryo (Mizuguchi et al., 1998; Sen et al., 1998). Toll signaling is transduced by the Pelle kinase, which causes the direct or indirect phosphorylation and degradation of Cactus, so that Dorsal is released from the cytoplasm and enters nuclei (Grosshans et al., 1994; Galindo et al., 1995; Towb et al., 1998). A constitutively activated form of Pelle was created by fusing the Pelle kinase catalytic domain to the signal peptide, the extracellular domain and the transmembrane peptide of a constitutively activated form of the Torso (Tor) receptor, Tor4021 (Y327C) (Sprenger and Nüsslein-Volhard, 1992). The resulting ‘Pelle-Tor4021’ chimera caused a dominant ventralized phenotype when its RNA was injected throughout the embryo in a manner independent of both Toll receptor and Tube adapter protein (Grosshans et al., 1994; Galindo et al., 1995). This is consistent with the idea that activation of Pelle leads to efficient degradation of Cactus, so that Dorsal can enter nuclei in both dorsal and ventral regions of the mutant embryo. However, mutant phenotypes were assessed predominantly on the basis of cuticular defects. Consequently, it is not known whether physiological levels of activated Pelle kinase are sufficient for the activation of type I target genes, such as twist and snail, which depend on peak concentrations of the Dorsal gradient (Huang et al., 1997). It is conceivable that the Toll-Dorsal signaling pathway is branched. For example, activation of the Toll receptor might induce Pelle and an additional kinase, which together activate type I target genes (e.g. Karin, 1999).

twist is one of the first genes activated by the Dorsal gradient (Thissè et al., 1991; Jiang et al., 1991). It encodes a bHLH regulatory protein implicated in mesoderm differentiation in a
broad spectrum of animals (Reuter and Leptin, 1994; Baylies and Bate, 1996; Harfe et al., 1998). This conservation of Twist contrasts with the apparently specific use of Dorsal as a dorsoventral determinant in insect embryos (Chen et al., 2000). Thus far, Rel-containing transcription factors have not been implicated in the dorsoventral patterning of vertebrate embryos, even though fly and frog embryos employ many common signaling components such as transforming growth factor β (TGFβ) and Chordin/Sog inhibitors (Ferguson, 1996).

Linearity in the Toll-Dorsal signaling pathway was analyzed by creating ectopic anterior-posterior gradients of Twist and activated Pelle kinases (Pelle-Tor4021 and Pelle-Tor). Twist- and Pelle-coding sequences were attached to the bicoloc (bcd) 3′UTR and expressed in the maternal germline using the hsp83 promoter (Huang et al., 1997). The Pelle-Tor4021 fusion gene is sufficient to establish sequential patterns of snail, sim, vnd and sog expression across the anteroposterior axis of transgenic embryos. These results suggest that the levels of activated Pelle kinase determine dorsoventral patterning thresholds, and argue against branching in the Toll signaling pathway. The twist transgene was able to activate snail, sim and vnd expression in mutant embryos containing low, uniform levels of the Dorsal protein. However, gene expression supported by Twist in the absence of a Dorsal gradient is erratic, in that expression patterns are out of order or are incorrect. These observations contrast with the recent demonstration that most Bicoid gradient thresholds are generated also by Hunchback, an immediate target of the Bicoid activator. As Twist largely fails to compensate for the loss of the Dorsal gradient, we conclude that Dorsal and Twist function in a highly interdependent manner to specify the mesoderm and ventral regions of the neurogenic ectoderm.

MATERIALS AND METHODS

Plasmid constructions, P-element transformation and in situ hybridization

The construction of Pelle-Tor4021 and Pelle-Tor fusion proteins in pSELECT vector (Promega) have been described previously (Galindo et al., 1995). The chimeric proteins contain the extracellular and transmembrane domains of Tor and Tor4021 residues 1-455 fused to residues 163-501 of Pelle. To generate anteroposterior Pelle-Tor and Pelle-Tor4021 gradients, we placed these fusions under the control of the hsp83 promoter and regulation by the bcd 3′UTR as follows. Blunted-NcoI/SacI fragments containing the Pelle-Tor and Pelle-Tor4021-coding sequences were cloned into the blunt-end-HindIII/SacI digested hsp83/pUC19 (Huang et al., 1997). A three-way ligation of a KpnI/blunted-SacI digested fragment containing hsp83-Pelle-Tor or hsp83-Pelle-Tor4021, blunted-SacII/XbaI fragment containing the bcd 3′UTR, and KpnI/XbaI digested Casper AUG β-gal P-element transformation vector (Huang et al., 1997) generated the Pelle-Tor4021 and Pelle-Tor P-element transformation vectors used in this study. A bluntend HindIII/HincII fragment containing the entire twist open reading frame (ORF) and a 5′ β-globin leader was isolated from the Twist/pNB40 plasmid (kindly provided by Maria Leptin) and cloned into the EcoRV site of pBSK+/KpnII, creating Twist-KpnII/BSK+. pBSK+KpnII is pBSK+ (Stratagene), which was modified to contain an additional KpnI site in place of SacI. A KpnI fragment containing the twist ORF from twist-KpnII/BSK+ was inserted into the KpnI site of the pCASPER-hsp83-KpnI-bcd3′UTR vector (a modified form of pCASPER-hsp83-KpnI-bcd3′UTR vector (manuscript submitted this month)). twist was thereby placed under the control of the hsp83 maternal promoter and bcd3′UTR localization sequence to generate P-element transformation vector P(Twist-bcd). The construction of P(bcdToll10b/bcd) and P(hspToll10b/bcd) P-element transformation vectors have been previously described (Huang et al., 1997) which places the Toll10b sequence under control of the bcd 3′UTR localization sequence and the bcd and hsp83 promoters, respectively. The bcd promoter mediates maternal expression at levels lower than the hsp83 maternal promoter. P-element mediated transformation and in situ hybridization using digoxigenin-labeled RNA probes were performed as described previously (Jiang et al., 1991).

Genetic crosses

All mutant fly stocks were obtained from the Bloomington Stock Center (Indiana) and wild-type embryos correspond to the yw fly stock, unless otherwise noted. The ectopic Toll10b, Pelle-Tor4021, Pelle-Tor and Twist-bcd anteroposterior Dorsal gradients were examined in the absence of the endogenous dorsoventral Dorsal gradient by introducing the Toll10b, Pelle-Tor4021, Pelle-Tor and Twist-bcd transgenes into embryos homozygous for a null mutation in gastrulation defective (gdf) (Konrad et al., 1988). Male flies from several independent lines carrying these P-element transposons were individually crossed to gd7/FM3 females. gd7/P-element+/+ males were crossed to gd7/FM3 females and embryos from gd7/gd7; (P-element)/+ females were collected for analysis by in situ hybridization.

The ectopic Toll10b anteroposterior Dorsal gradient was examined in the absence of Twist by crossing the P(hspToll10b/bcd) transgene into a twist-/twist- mutant background. Sp/CyO; P(hspToll10b/bcd)/+ males were crossed to cn twi1 bw/CyO females obtained from Bloomington stock center. Virgin females of the genotype cn twi1 bw/CyO; P(hspToll10b/bcd) were mated to cn twi1 bw/CyO males. twist-twist- mutant embryos represented one fourth of the embryos examined. Similar crosses were done to introduce the P(Twist-bcd) transgene into a snail mutant background (yw; snailIIG05/CyO fze-lacZ, kindly provided by Tony Ip).

The ectopic Twist anteroposterior gradient was examined in a background of low uniform Dorsal levels by misexpressing the twist-bcd transgene in embryos homozygous for a partially activating mutation of Toll (Schneider et al., 1991). Male flies from several independent lines in which the P(Twist-bcd) insertion was mapped to the second chromosome and balanced by CyO were individually crossed to Sp/CyO; Toll10b/TM3 females, which were isolated by crossing Toll10b/TM3 males to double balancer Sp/CyO; P(Prd)/TM3 females. twist-bcd/CyO; Toll10b/TM3 males were crossed to Toll10b/TM3 females, and embryos from twist-bcd+/+; Toll10b/TM3 females were collected from at least three individual lines for analysis by in situ hybridization.

Whole-embryo extracts and western blotting

Flies were allowed to deposit eggs on fresh apple juice agar plates with yeast paste for 2 hours, and then removed. Embryos were collected after aging an additional 2 hours at room temperature and homogenized in RIPA Buffer [50 mM TrisHCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS and protease inhibitors]. Extracts were centrifuged at 16,000 g for 15 minutes. The clear protein supernatant was carefully separated from floating lipid and precipitate, and quantified by Bradford assay. Equivalent amounts of embryo extracts were subjected to SDS-PAGE and western blotting using an affinity-purified, rabbit polyclonal antibody to Pelle protein at a 1:2000 dilution (kindly provided by James Manley) (Shen and Manley, 1998).

RESULTS

The snail, sim, vnd and sog expression patterns represent four different Toll-Dorsal signaling thresholds. snail is activated
only by peak levels of the Dorsal gradient (Ip et al., 1992a), sim and vnd are activated by intermediate levels (Kasai et al., 1998; von Ohlen and Doe, 2000), and sog is activated by the lowest levels of the gradient (Francois et al., 1994). These expression patterns were visualized in mutant and transgenic embryos via in situ hybridization using digoxigenin-labeled antisense RNA probes (Huang et al., 1997).

**Activated Pelle generates multiple Toll-Dorsal patterning thresholds**

Dorsal target genes are essentially silent in mutant embryos that lack an endogenous dorsalventral Dorsal nuclear gradient (Fig. 1A-C). Mutant embryos were collected from females that are homozygous for a null mutation in the gastrulation defective (gd) gene. Mutant embryos lack a dorsalventral Dorsal nuclear gradient. The embryos were hybridized with different digoxigenin-labeled antisense RNA probes, and visualized by histochemical staining. Each image is representative of the predominant pattern exhibited in the majority of embryos stained. In addition, at least three independent transgenic lines were analyzed for this and subsequent figures. Embryos are oriented with anterior to the left and dorsal up. (A-C) snail, vnd, and sog patterns, respectively, in mutant embryos. There is no staining above background levels. (D-F) snail, vnd, and sog patterns, respectively, in mutant embryos that contain the Toll10b transgene [Pbcd(bcd)]). snail expression is detected at the anterior pole, a band of vnd staining is detected in anterior regions (E), and a broad band of sog staining is detected in the anterior third of the embryo (F). The absence of vnd and sog expression at the anterior pole is probably due to repression by Snail (D).

**Synergistic activities of Dorsal and Twist**

There are several similarities between Dorsal and the major determinant of anteroposterior patterning, Bicoid, even though the two morphogens are unrelated. Both gradients activate regulatory genes that are essential for patterning the early embryo. Bicoid activates the zinc finger gene Hunchback, while Dorsal activates the bHLH gene Twist. It has recently been shown that the loss of the normal Bicoid gradient can be largely compensated by an anteroposterior Hunchback gradient (Wimmer et al., 2000). Hunchback restores posterior head segments and the thoracic segments lost in bicoid–/bicoid– mutant embryos. We have investigated the possibility that Twist plays a similar role in dorsoventral patterning.

**Twist gradients in “apolar” embryos**

Twist plays a similar role in dorsoventral patterning. Twist gradients in ‘apolar’ embryos that lack dorsoventral polarity. snail (Fig. 1D), vnd, (Fig. 1E) and sog (Fig. 1F) are sequentially expressed along the anteroposterior axis of mutant embryos that contain a constitutively activated form of the Toll receptor (Toll10b) misexpressed at the anterior pole using the bicoid (bcd) promoter and 3′ UTR (Fig. 1). These expression patterns depend on an ectopic anteroposterior Dorsal nuclear gradient (Huang et al., 1997). The repression of the vnd and sog patterns at the anterior pole is probably mediated by Snail, which normally excludes expression of these genes in the ventral mesoderm of wild-type embryos (Mellerick and Nirenberg, 1995; Rusch and Levine, 1996).

The activated Pelle-Tor4021 kinase also directs sequential anteroposterior patterns of snail, vnd, and sog expression in gd1/gd1– mutant embryos (Fig. 2C-E). As in the case of Toll10b, the activated Pelle kinase was misexpressed at the pole using the bcd 3′ UTR. The snail, vnd and sog expression patterns are similar to those obtained with the Toll10b transgene (see Fig. 1D-F). The vnd and sog expression patterns are probably repressed at the anterior pole by Snail (Fig. 2D,E). These results suggest that the levels of Pelle kinase activity are sufficient to determine different Dorsal transcription thresholds.

**Dorsal and Twist patterns are periodic**

Similar experiments were carried out with a Pelle-Tor fusion gene that contains the Tor signal peptide, extracellular domain and transmembrane peptide, but lacks the amino acid substitution (Y327C) in the Tor4021 protein that induces dimerization (Fig. 2F-H). The Pelle-Tor fusion protein fails to induce snail expression (Fig. 2F), but succeeds in activating vnd (Fig. 2G) and sog (Fig. 2H). The activities of the Pelle-Tor4021 and Pelle-Tor proteins are summarized in Fig. 2B.

Western assays were carried out to determine whether the different activities of Pelle-Tor4021 and Pelle-Tor might result from the differential stability of the two fusion proteins (Fig. 2A). The normal Pelle kinase has a molecular weight of ~75 kDa (lane 1, Fig. 2A), whereas the Pelle-Tor4021 and Pelle-Tor fusion proteins are considerably larger, ~140 kDa (lanes 2 and 3, respectively). The Pelle-Tor fusion protein (lane 3) is expressed at somewhat higher levels than the Pelle-Tor4021 protein, but nonetheless fails to activate snail expression. One interpretation of these results is that recruitment to the plasma membrane is not sufficient for the induction of peak Pelle kinase activity. Rather, full induction might require both recruitment and protein dimerization, which is achieved with the Pelle-Tor4021 fusion protein, but not with Pelle-Tor (Grosshans et al., 1994; Galindo et al., 1995). The Pelle-Tor4021 protein is constitutively activated in the absence of the Tor ligand, whereas full activation of Pelle-Tor might require ligand (see Discussion).

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**Fig. 1.** snail, vnd and sog expression in transgenic embryos with an ectopic, anterior-posterior Dorsal nuclear gradient. Cellularizing mutant embryos were collected from females homozygous for a null mutation in the gastrulation defective (gd) gene. Mutant embryos lack a dorsalventral Dorsal nuclear gradient. The embryos were hybridized with different digoxigenin-labeled antisense RNA probes, and visualized by histochemical staining. Each image is representative of the predominant pattern exhibited in the majority of embryos stained. In addition, at least three independent transgenic lines were analyzed for this and subsequent figures. Embryos are oriented with anterior to the left and dorsal up. (A-C) snail, vnd, and sog patterns, respectively, in mutant embryos. There is no staining above background levels. (D-F) snail, vnd, and sog patterns, respectively, in mutant embryos that contain the Toll10b transgene [Pbcd(bcd)]. snail expression is detected at the anterior pole, a band of vnd staining is detected in anterior regions (E), and a broad band of sog staining is detected in the anterior third of the embryo (F). The absence of vnd and sog expression at the anterior pole is probably due to repression by Snail (D).
which induces a broad anteroposterior Dorsal nuclear gradient (Huang et al., 1997). This transgene is similar to the one used in Fig. 1D-F, except that Toll10b was expressed using the stronger hsp83 promoter rather than the bicoid promoter. In an otherwise normal genetic background, the Toll10b transgene leads to the ectopic activation of snail expression in the presumptive head and anterior thorax (Fig. 3E). However, in twist/twist embryos there is reduced expression of both the endogenous snail expression pattern, and the ectopic pattern in anterior regions (Fig. 3F). Thus, even abnormally high levels of Toll signaling cannot compensate for the loss of Twist. The gap in snail expression seen at the boundary between the ectopic and endogenous patterns (arrow, Fig. 3E) may be due to an unknown repressor that is regulated by low levels of the Dorsal gradient. This gap is more pronounced in twist/twist mutants (arrow, Fig. 3F), suggesting that Twist is not required for the activation of the putative snail repressor.

The preceding results suggest that Twist is necessary for specifying Dorsal gradient thresholds. To determine whether Twist might be sufficient, a twist-bcd transgene was introduced into a number of genetic backgrounds. In wild-type embryos, the twist-bcd transgene induces ectopic activation of the sim gene in anterior regions (Fig. 4D; compare with 4A). This activation is more easily visualized in snail/snail mutant embryos because Snail represses sim expression in the ventral mesoderm (Kasai et al., 1992). There is a delay in the onset of sim expression in snail/snail embryos (Fig. 4B,C), and staining is sporadic in ventral regions where the gene is normally repressed (Fig. 4C; compare with 4A). However, the twist-bcd transgene, when introduced into snail/snail mutant embryos...
Fig. 4. An anterior-posterior Twist gradient induces ectopic expression of sim. Embryos were hybridized with a digoxigenin-labeled sim (A-F) or Sex-lethal (G-I) antisense RNA probe and are oriented with anterior to the left. (A, D) Wild-type embryos that lack (A) or express (D) a twist-bcd transgene. sim is normally expressed in two lines that straddle the presumptive mesoderm (A). Staining does not extend to the anterior pole. In contrast, the twist-bcd transgene induces ectopic expression of sim at the anterior pole (D). This staining is first detected in precellular embryos (not shown), and persists during cellularization (D) and gastrulation (not shown). (B, C) snail–/snail– mutant embryos that lack the twist-bcd transgene. There is a delay in the onset of expression, and staining is not detected in advanced cellularizing embryos (B). Sporadic and weak expression is first detected at the onset of gastrulation in ventral regions (C). B and C display lateral and ventral views, respectively. (E, F) snail+/snail– mutant embryos that express the twist-bcd transgene. There is strong activation of sim in anterior regions of cellularizing embryos (E and F display lateral and ventral views, respectively). Strong ectopic expression persists during germ band elongation (not shown). (G-I) Sex-lethal expression in wild-type embryos and those expressing the twist-bcd transgene. Sex-lethal is ubiquitously expressed in female embryos (H) and is not expressed in male embryos (G). In embryos from females containing the twist-bcd transgene, Sex-lethal is repressed at the anterior end of the embryo (I) in the domain that coincides with ectopic Twist expression. All Sex-lethal expressing embryos exhibit this repression, which accounts for the daughterless phenotype exhibited by females containing the twist-bcd transgene.

A Twist gradient can generate multiple dorsoventral patterning thresholds

In order to determine whether Twist patterning activities require a Dorsal gradient, the twist-bcd transgene was introduced into mutant embryos derived from Tollrm9/Tollrm10 transheterozygous females. This mutant Toll receptor is defective and partially active in a Spätzle-independent fashion (Schneider et al., 1991). Consequently, mutant embryos contain uniform, low levels of the Dorsal protein in both dorsal and ventral nuclei. The twist-bcd transgene causes a substantial reorganization in the patterning of mutant embryos (Fig. 5).

The low, uniform levels of Dorsal present in Tollrm9/Tollrm10 mutant embryos are insufficient to activate snail in precellular or newly cellularized embryos (Fig. 5A). The twist-bcd transgene activates snail throughout anterior regions of precellular embryos (Fig. 5B); however, this staining pattern is refined into a broad anterior stripe after cellularization (Fig. 5C). This ectopic snail expression pattern exhibits a sharp
posterior border, suggesting that the Twist gradient is sufficient for the on/off regulation of snail in the absence of a Dorsal gradient (see Discussion).

sog is normally activated throughout the neurogenic ectoderm by the lowest levels of the Dorsal gradient (Francois et al., 1994; Markstein et al., 2002). The low levels of Dorsal present in Toll\textsuperscript{m09}/Toll\textsuperscript{m10} mutant embryos are sufficient to activate sog everywhere except the extreme termini (Fig. 5D). The twist-bcd transgene leads to the loss of sog expression in anterior regions (Fig. 5E), probably because of repression by Snail. As mentioned earlier, Snail also appears to repress vnd and sog expression in anterior regions of transgenic embryos that contain the Toll\textsuperscript{10B} or Pelle-Tor\textsuperscript{4021} transgenes (see Figs 1 and 2).

The low levels of Dorsal present in Toll\textsuperscript{m09}/Toll\textsuperscript{m10} mutant embryos are insufficient to activate sim, although there is occasional staining in the posterior pole (data not shown). The twist-bcd transgene leads to the efficient activation of sim in anterior regions (Fig. 5F). Staining appears to be restricted to those regions where snail expression is lost (Fig. 5C). These results suggest that a Twist gradient is sufficient to generate multiple dorsoventral patterning thresholds (sim and snail) in the presence of low, uniform levels of Dorsal.

The twist-bcd transgene was introduced into mutant embryos that completely lack Dorsal (Fig. 6). Without the transgene these mutants do not express twist, snail, sim, vnd, or sog (Fig. 6A; data not shown). Introduction of the twist-bcd transgene causes intense expression of twist in the anterior 40% of the embryo (Fig. 6B; compare with 6A). This broad Twist gradient fails to activate snail (data not shown), but succeeds in inducing weak expression of sim (Fig. 6C) and somewhat stronger staining of vnd (Fig. 6D) at the anterior pole. The activation of vnd in mutant embryos is comparable with the expression seen in wild-type and Toll\textsuperscript{m09}/Toll\textsuperscript{m10} embryos (Fig. 6E; data not shown). However, in both wild-type and mutant embryos the vnd pattern is transient, and lost after the completion of cellularization (Fig. 6F; data not shown). These results indicate that Twist can activate dorsoventral patterning genes in the absence of Dorsal.

**DISCUSSION**

**Linear signaling**

The demonstration that the Pelle-Tor\textsuperscript{4021} fusion protein can generate most or all dorsoventral patterning thresholds suggests that the levels of Pelle kinase activity determine Spätzle-Toll signaling thresholds. There is no need to invoke branching in the Toll pathway upstream of Pelle, although it is possible that branching exists downstream of Pelle. For example, there is emerging evidence that IkB kinases might trigger the degradation of Cactus in the immune response (Rutshmann et al., 2000; Silverman et al., 2000; Lu et al., 2001). It is currently unclear whether these kinases are also required for dorsoventral patterning and, if so, whether they are induced by Pelle or separately activated in response to Toll signaling.

Multiple patterning thresholds are also specified by different levels of kinase activity in the Tor, epidermal growth factor receptor (EGFR) and Sevenless (Sev) signaling pathways (Greenwood and Struhl, 1997; Halfar et al., 2001). In the embryo, low levels of Ras1 are sufficient to activate the Tor target gene tailless, whereas higher levels are required for the induction of hückebein (Greenwood and Struhl, 1997). Similarly, in the eye, different levels of EGFR and Sev signaling lead to different levels of MAPK activity: low levels permit the differentiation of R8 photoreceptor cells, whereas higher levels (or more persistent expression) of MAPK activity leads to the specification of an R1-R7 fate (Halfar et al., 2001). In the present study it has been possible to link the levels of an activated cytoplasmic kinase (Pelle) with the expression of well-characterized target genes that are regulated by different concentrations of the Dorsal gradient. It is conceivable that the MAP and Pelle kinases exist in multiple states, which help establish different thresholds of gene activity. For example, even high concentrations of membrane-localized Pelle-Tor fusion protein fail to activate snail. While the Tor\textsuperscript{4021} receptor domain of Pelle-Tor\textsuperscript{4021} induces ligand-independent dimerization, the wild-type Tor receptor domain contained within the Pelle-Tor fusion might mediate only low levels of dimerization because of competition with the endogenous Tor receptor for binding to the Trunk ligand (Casali and Casanova, 2001). Full activation of the Pelle kinase might depend on recruitment to the plasma membrane and dimerization (Grosshans et al., 1994; Galindo et al., 1995), while recruitment to the membrane alone may produce only partial activation of kinase activity. The induction of full Pelle kinase activity, and ultimately the activation of snail expression, might require trans-phosphorylation induced by dimerization of the Toll receptor (Shen and Manley, 1998). In the context of the normal Toll-Dorsal signaling pathway, the transition between a partially activated Pelle kinase and a fully activated kinase...
might help generate a sharp on/off pattern of snail expression. Perhaps the Pelle kinase is converted into the fully activated form (required for snail expression) when Toll signaling exceeds a certain minimal threshold.

**Twist gradient thresholds**

An anteroposterior Twist gradient generates at least two thresholds of gene activity in mutant embryos that contain decreased levels of Dorsal (summarized in Fig. 7B). High levels of Twist activate sim at the anterior pole, whereas lower levels are sufficient to induce the expression of snail in more posterior regions of embryos containing low, uniform levels of the Dorsal protein (Fig. 5C,F). These results demonstrate that twist gene activity is not dedicated to mesoderm formation. Instead, Twist supports expression of two regulatory genes, sim and vnd, which pattern ventral regions of the neurogenic ectoderm (Crews et al., 1988; McDonald et al., 1998). The twist-bcd transgene was shown to induce weak expression of both genes even in mutant embryos that completely lack Dorsal (Fig. 6C,D).

The Twist gradient exhibits some unexpected activities in Tollmut/Tollmut mutant embryos. In particular, both high and intermediate levels of the gradient initially activate snail in a broad domain of precellular embryos (Fig. 5B). However, during cellularization snail is repressed at the anterior pole where there are high levels of Twist (Fig. 5C). Thus, it would appear that high levels of Dorsal + high levels of Twist activate snail expression (e.g. ventral region of wild-type embryos), while low levels of Dorsal + high levels of Twist repress expression (e.g. anterior region of Tollmut/Tollmut embryos containing the twist-bcd transgene). The ratio of Dorsal to Twist might keep Twist patterning activity under control, such that high levels of Twist specify mesodermal targets (i.e. snail), lower levels specify neurogenic targets (i.e. sim and vnd) and the expression of genes such as Sxl remain unaffected.

Alternatively, Twist may differentially interact with a number of bHLH proteins that are present in the early embryo (Moore et al., 2000) to affect its patterning activity. For example, Twist is thought to form a heteromeric activation complex with other bHLH proteins including the ubiquitous, maternal bHLH protein Daughterless (Da) (Gonzalez-Crespo and Levine, 1993; Castanon et al., 2001). The loss of Sex-lethal expression at the anterior pole of twist-bcd embryos (Fig. 4I) may result from a failure of Twist-Daughterless heterodimers to activate this gene. It has been demonstrated that Twist-Daughterless heterodimers possess a different patterning activity from Twist-Twist homodimers (Castanon et al., 2001). It is possible that Twist-Daughterless heterodimers formed in twist-bcd embryos actively repress Sex-lethal expression. Alternatively, Twist might titrate Daughterless levels by forming a sterile heterodimeric complex that is not able to activate Sex-lethal. However, Sex-lethal is normally expressed in ventral regions of wild-type embryos that contain both Twist and the ubiquitous Daughterless, therefore regulation of bHLH patterning activities
must be more complex. In relation to dorsal-ventral patterning, Twist-Twist complexes might be favored in anterior regions of embryos that express the twist-bcd transgene, while Twist-bHLH complexes are formed away from the anterior pole where expression of the transgene is decreased. These complexes might fail to activate snail, or even actively repress transcription as it has been demonstrated that several bHLH proteins can function as repressors of transcription (Brentrup et al., 2000). Regardless of mechanism, the Twist gradient inverts the order of the sequentially expressed snail and sim genes, when compared with the patterns obtained with the normal Dorsal (and Twist) gradient. In the presence of low, uniform levels of Dorsal, high concentrations of Twist specify meseckoderm (sim expression), while lower levels specify mesoderm (snail expression). These observations raise the possibility of evolutionary plasticity in the use of Twist in tissue specification.

**Dorsal-Twist synergy**

*snail* is activated by Dorsal and Twist in cellularizing embryos (Ip et al., 1992a). The sharp lateral limits of the *snail* expression pattern establish the boundary between the presumptive mesoderm and neurogenic ectoderm (Kosman et al., 1991; Leptin, 1991). It has been suggested that the crude Dorsal gradient triggers a somewhat steeper Twist gradient, and the two activators function synergistically within the *snail* 5' cis-regulatory DNA to establish the sharp, on/off expression pattern (Ip et al., 1992a). Dorsal-Twist transcription synergy may provide a means for ‘multiplying’ the Dorsal and Twist gradients to produce the sharp *snail* pattern (see Summary, Fig. 7A). This model suggests that both proteins must be present in a gradient to generate the sharp *snail* border. However, while both Dorsal and Twist are required for the activation of *snail*, we have shown that a Twist gradient is sufficient for the activation of *snail*. It is conceivable that Dorsal binding sites present in the *snail* 5' cis-regulatory region are rendered responsive by the Dorsal activator (whether present at uniform levels or in a gradient). Therefore, the ratio of Dorsal to Twist may be important to produce the sharp lateral limits of *snail* expression.

This study raises some questions about the role of operator binding affinities in the specification of different transcription thresholds. The Dorsal binding sites present in the *snail* 5' regulatory region bind with lower affinity than the sites present in the *rho* lateral stripe enhancer (NEE) (Ip et al., 1992a; Ip et al., 1992b). The analysis of a number of synthetic enhancers prompted the proposal that the activation of Dorsal target genes in the ventral mesoderm versus lateral neurogenic ectoderm depends on the affinity of Dorsal operator sites (Jiang and Levine, 1993; Huang et al., 1997). However, the demonstration that the twist-bcd transgene can activate *snail* expression in *Toll*<sup>tm9/Toll*tm10</sup> embryos suggests that occupancy of the distal Dorsal-binding sites may not be crucial for determining whether the gene is on or off. It is conceivable that Dorsal occupies one or more sites in mutant embryos, but is unable to trigger expression in the absence of Twist. In general, ‘promoter context’ (combinations of regulatory factors) might be more critical for defining Dorsal transcription thresholds than the affinities of Dorsal operator sites.

The relationship between Dorsal and Twist appears distinct from the interplay between Bicoid and Hunchback (Wimmer et al., 2000). It has been suggested that the Bicoid gradient is a relatively recent evolutionary innovation for patterning the anterior-posterior axis of long germ band insects (Dearden and Akam, 1999). By contrast, Hunchback is ancient and is used in the patterning of short germ band insects such as grasshoppers. Most of the patterning activity controlled by the Bicoid gradient appears to be mediated by Hunchback, which is a direct target of the Bicoid activator. Only the patterning of the anterior most head structures requires Bicoid and cannot be compensated by high levels of Hunchback (Wimmer et al., 2000). Thus, the patterning activity of the Bicoid gradient can be explained by the regulation of several target genes, which is consistent with its recent evolution. By contrast, either Dorsal or Twist protein alone produces only a small subset of the five or six dorsal-ventral patterning thresholds generated by the concerted action of both proteins. We conclude that Dorsal and Twist work in a highly interdependent and synergistic fashion to regulate a large number of target genes involved in patterning the dorsoventral axis.

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