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REVIEW

Dorsal Gradient Networks in the *Drosophila* Embryo

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Here, we describe one of the major maternal regulatory gradients, Dorsal, and threshold outputs of gene expression that result from the graded distribution of this transcription factor. The analysis of a large number of authentic and synthetic target genes suggests that the Dorsal gradient directly specifies at least four, and possibly as many as seven, different thresholds of gene activity and tissue differentiation. These thresholds initiate the differentiation of the three primary embryonic tissues: the mesoderm, neurogenic ectoderm, and dorsal ectoderm. Moreover, primary readouts of the Dorsal gradient create asymmetries that subdivide each tissue into multiple cell types during gastrulation. Dorsal patterning thresholds represent the culmination of one of the most complete gene regulation network known in development, which begins with the asymmetric positioning of the oocyte nucleus within the egg chamber and leads to the localized activation of the Toll-Dorsal signaling pathway in ventral regions of the early embryo.

Key Words: **Dorsal; gradient; *Drosophila* embryo; gene network.**

Introduction

The Dorsal gradient specifies as many as seven threshold responses in the early *Drosophila* embryo. In this review, we will focus on what is known about the *cis*-regulatory elements of Dorsal targets and how their organization qualitatively determines gene expression outputs. Most reviews on dorsoventral (DV) patterning have emphasized the complex genetic pathway that ultimately triggers the localized activation of the Toll receptor and the relocalization of Dorsal from the cytoplasm to the nucleus (e.g., Belvin and Anderson, 1996). Only a very brief summary of the Toll pathway is provided in order to place Dorsal transcription thresholds in the broader context of DV patterning in the *Drosophila* egg and early embryo.

Brief Review of the Toll Signaling Pathway

The oocyte nucleus is localized in an anterior–dorsal position within the stage 10 egg chamber. *gurken* mRNA, which encodes an EGF-related signaling molecule, is localized around the periphery of the nucleus. This localized source of Gurken specifies a simple dorsal/ventral switch in the patterning of the follicular epithelium (Nilson and Schupbach, 1999). High levels of Gurken activate the EGF receptor, Torpedo or DER, in dorsal follicle cells. These cells follow a dorsal pathway of differentiation, which includes the elaboration of specialized chorion structures such as the dorsal appendages. In contrast, the EGF receptor is not activated in follicle cells located far from the source of Gurken in ventral regions. The absence of EGF signaling permits the differentiation of ventral follicle cells. One manifestation of this ventral fate is the localized transcription of *pipe*, which encodes a putative heparan sulfate 2-O-sulfotransferase (Sen *et al.*, 1998). Pipe enzymatic activity is thought to modify extracellular components located in the perivitelline fluid (PVF) that separates the oocyte and follicle cells.

Localized Pipe activity somehow leads to the activation of an extracellular protease, Nudel. Nudel, in turn, triggers a serine protease cascade on the ventral surface of newly fertilized eggs. This cascade includes three proteases: *gastrulation defective*, *snake*, and *easter* (reviewed by LeMosy *et al.*, 1999). The localized activation of the Easter protease in ventral regions of the PVF leads to the localized process-

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ing of an inactive form of the Spätzle (Spz) ligand, which is distributed throughout the PVF. The activated, proteolytically processed ligand selectively activates the ubiquitous Toll receptor in ventral regions of the precellular embryo, approximately 90 min after fertilization (e.g., Roth, 1994).

The exact mechanism responsible for the formation of the broad Dorsal nuclear gradient is unclear. It has been suggested that diffusion of processed Spz creates an extracellular ligand gradient, which in turn leads to a gradient in Toll activation and Dorsal nuclear transport (e.g., Roth, 1993). There is also evidence that Spz-Toll complexes formed in ventral regions might be able to diffuse into lateral regions within the plasma membrane of the precellular embryo (Huang *et al.*, 1997). Regardless of mechanism, Dorsal nuclear transport appears to depend on the absolute number of fully activated Toll receptors (Anderson *et al.*, 1985; Huang *et al.*, 1997). A large number of activated receptors lead to the complete transport of Dorsal from the cytoplasm to nuclei in ventral regions of precellular embryos. In more lateral regions, there are fewer fully activated Toll receptors, and consequently, lower levels of Dorsal enter nuclei. Studies on activin signaling in the *Xenopus* embryo suggest that the number of fully activated receptors also determines cell fate in the animal cap (Shimizu and Gurdon, 1999).

The Dorsal gradient is rather shallow in the presumptive mesoderm in ventral regions, but is very steep in lateral regions where the neurogenic ectoderm will form (e.g., Kosman *et al.*, 1991). The single biggest drop is seen near the future boundary between the mesoderm and neurogenic ectoderm. Perhaps an approximate twofold difference in the levels of Dorsal determines whether a naïve embryonic cell forms mesoderm or neurogenic ectoderm. Moreover, the steep slope of the Dorsal gradient generates as many as five different thresholds of gene activity, which pattern the future ventral midline and nerve cord.

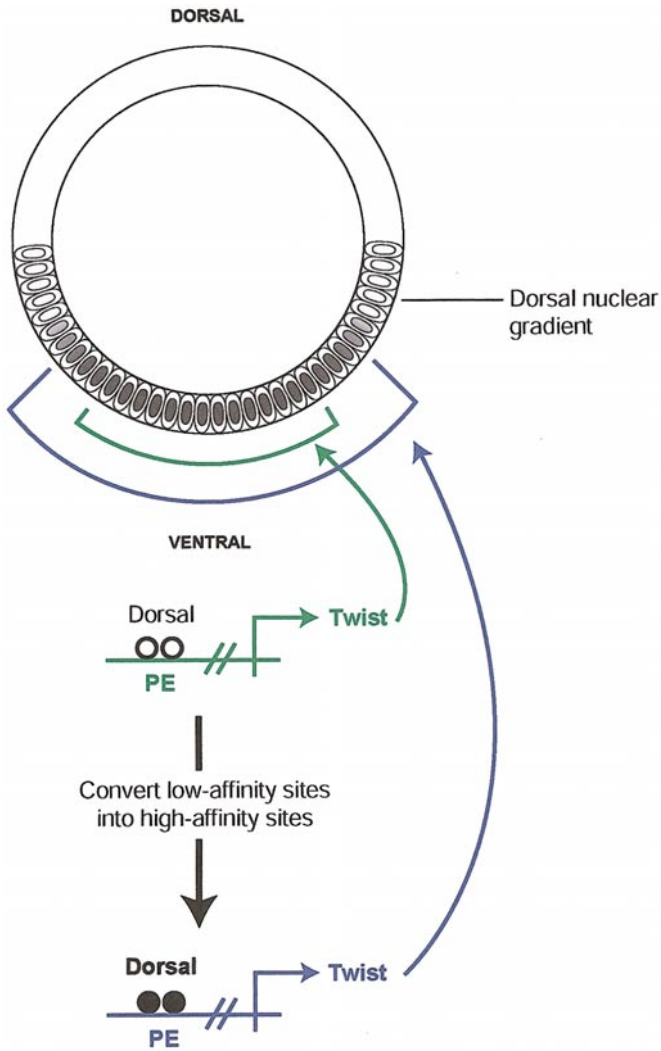

FIG. 1. The importance of Dorsal binding affinities. The circle represents a cross-section through a cellularizing embryo. There are 72 nuclei located at the periphery of the embryo that enclose the internal yolk. The Dorsal nuclear gradient is represented by the crescent in ventral and lateral regions. Peak levels are located in the ventralmost regions. The 250-bp PE sequence is located just upstream of the *twist* transcription start site. It is sufficient to direct the expression of a lacZ reporter gene in ventral regions of transgenic embryos (in response to high levels of the Dorsal gradient). The PE contains two low affinity Dorsal binding sites; these are indicated by the open circles. Single nucleotide substitutions convert each site into an optimal Dorsal recognition sequence. This causes an expansion in the lacZ reporter gene, indicating that both high and intermediate levels of the Dorsal gradient can activate the modified *twist* PE.

Specification of the Mesoderm

twist is one of the earliest target genes activated by the Dorsal gradient. It encodes a bHLH regulatory protein that is essential for the specification of the mesoderm (Thisse *et al.*, 1988). *twist* transcripts are first detected during nuclear cleavage cycle 12/13, within 20–30 min after the formation of the Dorsal nuclear gradient. The analysis of the *twist* 5′ regulatory region identified an ~250-bp enhancer, the PE, which is located about 180 bp upstream of the transcription start site (Thisse *et al.*, 1991; Pan *et al.*, 1991; Jiang *et al.*, 1991). This enhancer contains two low affinity Dorsal binding sites and directs the expression of a lacZ reporter gene within the ventralmost 12–14 nuclei of transgenic embryos, where there are peak levels of Dorsal (Fig. 1). Experimental manipulation of the *twist* PE demonstrated the importance of operator occupancy in establishing different dorsoventral limits of target gene expression (Jiang and Levine, 1993).

Dorsal binds DNA as a homodimer and each of the two recognition sequences in the PE contains imperfect dyad symmetry (GGG——CTC and GGG——GCC). Single nucleotide changes were made within each site to create exact symmetry: GGG——CCC and GGG——CCC. DNaseI foot-

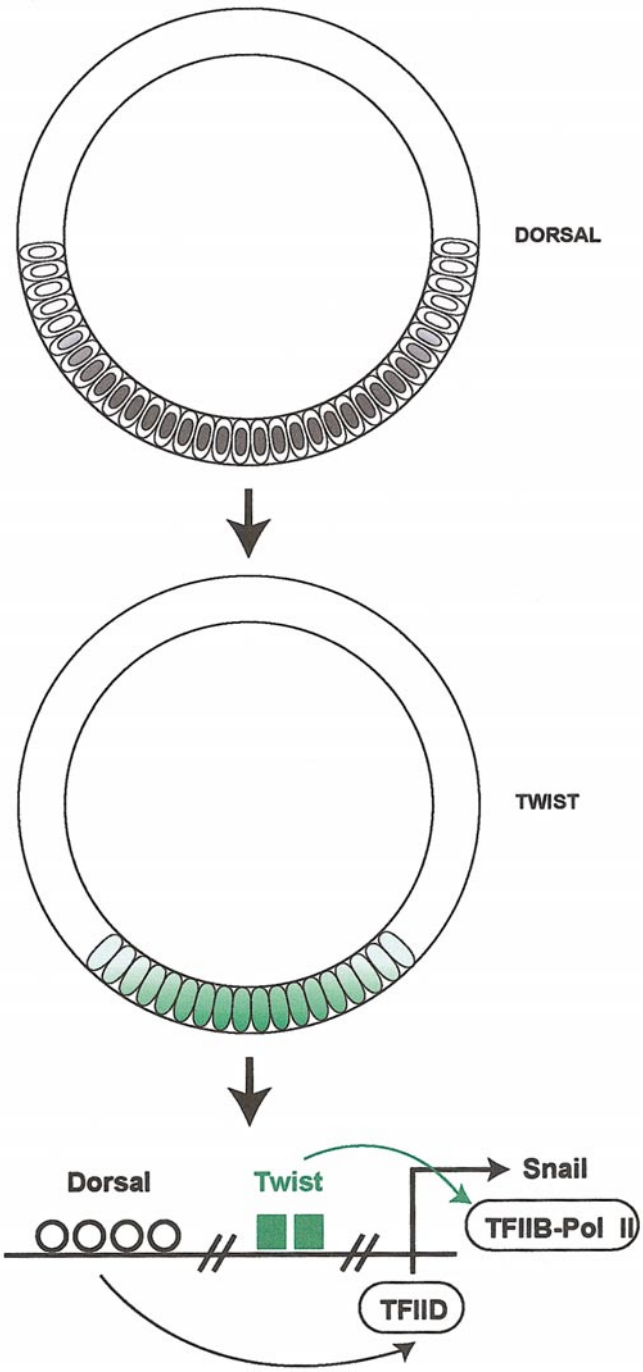

FIG. 2. Dorsal–Twist synergy. The circles represent cross-sections through cellularizing embryos. The broad Dorsal nuclear gradient triggers a slightly steeper pattern of *twist* expression. The Dorsal and Twist proteins function in a synergistic fashion to activate *snail* expression. The *snail* 5′ *cis*-regulatory region contains a distal cluster of low affinity Dorsal binding sites and at least two Twist sites located near the transcription start site. It is possible that Dorsal and Twist interact with distinct rate-limiting components of the Pol II transcription complex. For example, Dorsal might recruit TFIID to the core promoter, while Twist augments the rate of transcription by interacting with TFIIB or a component of the Pol II complex that functions "downstream" of TFIID. Alternatively, one of the activators might interact with a component of the Pol II complex, while the other recruits histone acetyltransferases or Swi/Snf remodeling complexes that decondense chromatin.

print assays indicated that the modified sites possess at least a 5-fold higher affinity for Dorsal than the native sites. The modified PE-lacZ transgene, containing just 2 nucleotide changes, exhibits a substantially broader pattern of expression than the native PE (Jiang and Levine, 1993). Staining is now detected in 18–20 cells, similar to the normal pattern of *twist* expression. These experiments suggest that the binding affinities of Dorsal operator sites specify different limits of gene expression in response to the Dorsal nuclear gradient. However, as discussed below, this is just part of the story.

It would appear that *twist* responds in a fairly straightforward fashion to the Dorsal nuclear gradient, although the lateral limits of the *twist* pattern are somewhat sharper than the Dorsal gradient (Kosman *et al.*, 1991; Leptin, 1991). This could be explained, in part, by cooperative interactions between Dorsal dimers on the adjacent, low affinity operator sites within the *twist* PE, although such cooperativity has not been demonstrated.

snail is another Dorsal target gene that is activated in the presumptive mesoderm. It exhibits a similar threshold response as *twist*, as they are both expressed in the ventralmost 18–20 cells of the embryo, but Twist protein extends into lateral regions beyond the limits of the *snail* pattern (Kosman *et al.*, 1991). The key difference between the two target genes is that the *snail* expression pattern exhibits sharp lateral limits, which demarcate the boundary between the presumptive mesoderm and neurogenic ectoderm.

The sharp *snail* expression pattern depends, in part, on the multiplication of the Dorsal and Twist gradients. The idea is that the broad Dorsal gradient triggers a slightly steeper *twist* pattern, and then the Dorsal and Twist proteins function synergistically within the limits of the *snail* 5′ regulatory region to activate expression (Ip *et al.*, 1992b; Fig. 2). Consistent with this model is the demonstration that the *snail* promoter region contains both Dorsal and Twist binding sites. There is a cluster of low affinity Dorsal sites, similar to those observed in the *twist* PE, located ~1 kb upstream of the transcription start site. There are also 2 Twist binding sites in a proximal region of the *snail* promoter. Several lines of evidence suggest that Dorsal and Twist do indeed function synergistically to activate *snail*. First, mutations in either the Dorsal or the Twist sites cause a catastrophic reduction in the expression of *snail*-lacZ fusion genes in transgenic embryos (Ip *et al.*, 1992b). Second, the insertion of synthetic Twist binding sites within the *twist* PE results in a substantial broadening in

the PE/lacZ staining pattern, which is expanded from 12 to 14 nuclei in the ventralmost regions to 18–20 nuclei (Jiang and Levine, 1993), similar to the normal *snail* expression pattern. Finally, a completely synthetic lacZ reporter gene that contains multimers of a high affinity Dorsal binding site exhibits weak and fuzzy expression in the presumptive ventral mesoderm (18–20 cells). The addition of multimerized Twist sites does not cause a significant expansion in the lacZ staining pattern, but instead results in both enhanced levels of expression and sharper lateral limits, similar to the native *snail* pattern (Szymanski and Levine, 1995).

These results suggest that Dorsal and Twist function synergistically to activate *snail*. But what is the mechanism? It does not appear to involve direct Dorsal–Twist protein–protein interactions, but instead, is more likely to entail "postbinding" synergy, whereby Dorsal and Twist make separate contacts with the pol II transcription complex. Dorsal binding sites are sufficient to activate a lacZ reporter gene in transgenic embryos, whereas Twist strongly enhances Dorsal activity, but is unable to activate lacZ on its own (Szymanski and Levine, 1995). There might be two steps in the activation of Dorsal target genes (Fig. 2). Step one may involve the Dorsal-mediated recruitment of the TFIID initiator complex to the core promoter (Zhou *et al.,* 1998). Step two might involve optimizing interactions between TFIID and pol II, possibly through protein–protein interactions between Twist and TFIIB (e.g., Burley and Roeder, 1998). Evidence for this type of two-step mechanism of transcriptional activation is suggested by the removal of Twist sites in the context of otherwise normal *snail*-lacZ transgenes. There is no significant change in the limits of expression, but staining is sporadic yielding a "salt and pepper" pattern of expressing and nonexpressing cells (Ip *et al.*, 1992b).

It is hard to imagine that the Dorsal and Twist gradients, even when multiplied, are sufficient to account for the sharp, on/off lateral limits of the *snail* expression pattern. Sharp stripes of segmentation gene expression depend on the interplay of transcriptional activators and repressors (e.g., Small *et al.*, 1991). For example, the posterior border of *eve stripe 2* depends both on limiting amounts of the Bicoid gradient and on the Krüppel repressor (Small *et al.,* 1992). Recent studies on the Dpp signaling pathway have shown that sharp limits of target gene expression, such as *pannier* in the embryo and *omb* in wing disks, depend on a transcriptional repressor, Brinker (Campbell and Tomlinson, 1999; Jazwinska *et al.,* 1999a,b). It is conceivable, probably even likely, that the *snail* borders depend on one or more repressors expressed in lateral regions of the embryo. Evidence for a neurogenic repressor that helps restrict the *snail* pattern was obtained by creating an ectopic anteroposterior Dorsal gradient in transgenic embryos. Snail is activated in ventral regions by the normal Dorsal gradient, and in anterior regions by high levels of the ectopic gradient. There is a gap in the endogenous *snail* pattern immediately posterior to the ectopic expression of *snail* in head regions (Huang *et al.*, 1997). It was suggested that low levels of the ectopic gradient activate a neurogenic repressor, which normally helps sharpen the *snail* borders. Unfortunately, the identity of the repressor is not known.

In summary, the Dorsal nuclear gradient initiates the differentiation of the mesoderm by directly activating at least two target genes, *twist* and *snail*. The *twist* promoter region includes a proximal enhancer, the PE, which directs expression within a subdomain of the presumptive mesoderm. The expression pattern directed by the *twist* PE is similar to that observed for *folded gastrulation* (*fog*), a gene required for the invagination of the ventralmost regions of the mesoderm (Costa *et al.,* 1994). Thus, Dorsal might specify two distinct patterning thresholds within the mesoderm. These thresholds depend on the binding affinity of Dorsal operator sites, and on the synergistic action of Dorsal and Twist.

The Neurogenic Ectoderm

The slope of the Dorsal gradient is steepest in lateral regions that form the neurogenic ectoderm. At least three, and possibly as many as five, different thresholds of gene activity are established in direct response to this steep portion of the gradient. The analysis of a number of authentic and synthetic target genes suggests that most of these thresholds depend on similar high affinity Dorsal operator sites. Distinct patterning thresholds depend on the organization of Dorsal binding sites and on the other transcription factors bound within the target *cis*-regulatory DNAs.

The first patterning threshold within the neurogenic ectoderm is responsible for specifying a specialized cell type, the mesectoderm, just beyond the sharp snail borders that define the limits of the presumptive mesoderm. Several genes are expressed in a single row of cells on either side of the presumptive mesoderm, including single-minded (*sim*) and genes contained within *the Enhancer of Split* [*E(spl)*] complex (e.g., Martin-Bermudo *et al.*, 1995). *sim* encodes a member of the bHLH-PAS family of transcription factors, and is essential for the differentiation of the mesectoderm (Crews *et al.*, 1988). After gastrulation, the *sim*-expressing cells converge at the ventral midline and form specialized neurons and glial cells associated with the ventral nerve cord. The *sim* promoter region contains a series of linked Dorsal and Twist binding sites (Kasai *et al.,* 1998). It also includes binding sites for the Snail repressor, which keeps *sim* off in the ventral mesoderm and restricted to lateral lines in the presumptive mesectoderm (Kasai *et al.,* 1992).

Dorsal–Twist synergy generates slightly different thresholds of *snail* and *sim* expression. This might result from the different arrangements of Dorsal and Twist binding sites in the two promoter regions. In the case of *snail*, low affinity Dorsal sites are located ~1 kb away from Twist sites (Ip *et al.,* 1992b), whereas they are tightly linked within the *sim* promoter region (Kasai *et al.,* 1998). This linkage might permit cooperative DNA binding interactions between Dor-

sal and Twist so that lower levels of Dorsal can activate *sim* (Shirokawa and Courey, 1997; Fig. 3A). *sim* expression is also regulated by Notch signaling (Morel and Schweisguth, 2000). Therefore, it may be that combinatorial control of *sim* expression by Notch, Dorsal, Twist, and Snail generates the unique threshold response exhibited by *sim* in its restriction to a lateral stripe of one cell's width (see Fig. 4). The putative repressor that helps sharpen the *snail* border may also regulate the distal *sim* border to further limit *sim* expression (Fig. 3B).

A second threshold of gene activity is represented by *rhomboid*, which is expressed in lateral stripes that encompass the ventralmost 8–10 nuclei of the presumptive neurogenic ectoderm (Bier *et al.*, 1990). These stripes are regulated by a 300-bp enhancer, the NEE, located ~1.7 kb upstream of the *rhomboid* transcription start site (Ip *et al.*, 1992a). The NEE contains both high and low affinity Dorsal binding sites, as well as closely linked Twist sites. It also contains E box sequences that bind additional bHLH proteins (Fig. 3A). The latter proteins, Daughterless and Scute, are maternally expressed and ubiquitously distributed throughout the early embryo (e.g., Gonzalez-Crespo and Levine, 1993). Daughterless/Scute heterodimers interact with Dorsal to ensure efficient occupancy of linked Dorsal operator sites (Jiang and Levine, 1993). These cooperative DNA binding interactions permit low levels of Dorsal to activate rhomboid in lateral regions. When the Daughterless/Scute E boxes are converted into Twist E boxes, then the modified rhomboid NEE directs a narrower pattern of expression (Gray and Levine, 1996). Thus, interactions between Dorsal and two different types of bHLH proteins, Daughterless/Scute and Twist, determine two different thresholds of gene activity within the neurogenic ectoderm. As in the case of *sim*, the NEE contains *snail* repressor sites that exclude rhomboid expression from the ventral mesoderm (Figs. 3A and 3B).

Dorsal Ectoderm

The Dorsal gradient also establishes a transcription threshold at the boundary between the presumptive neurogenic ectoderm and dorsal ectoderm. This is exemplified by the expression pattern of *short gastrulation* (*sog*), which exhibits broad lateral stripes that appear to encompass the entire presumptive neurogenic ectoderm (Francois *et al.*, 1994). In contrast, the narrower *rhomboid* stripes are confined to the ventral half of the neurogenic ectoderm (Fig. 3A). The *sog* stripes are regulated by a 393-bp intronic enhancer that contains four optimal Dorsal binding sites that are evenly spaced across the limits of the enhancer (Markstein *et al.*, 2002). This enhancer was identified by the computational search of the entire *Drosophila* genome using the Dorsal binding sites located in the 5′ regulatory region of *zen*, which is expressed in the presumptive dorsal ectoderm in response to the Dorsal gradient (Doyle *et al.*, 1989). In principle, *zen* can be activated throughout the embryo by one or more ubiquitous transcription factors. However, expression is kept off in ventral and lateral regions by the Dorsal gradient. The same low levels of Dorsal that activate *sog* repress *zen*.

The *zen* promoter region contains a 600-bp silencer sequence, or ventral repression element (VRE), that is located ~1 kb upstream of the transcription start site (Cai *et al.*, 1996; Fig. 3A). The VRE is able to mediate long-range silencing of heterologous enhancers and promoters in ventral regions of the early embryo. For example, it can repress the ventral expression directed by an *eve stripe 2*/lacZ fusion gene when located 3′ of lacZ, nearly 5 kb away from the transcription start site and stripe 2 activators (Cai *et al.*, 1996). The *zen* VRE contains three optimal, high affinity Dorsal binding sites. It also contains three AT-core recognition sequences that bind at least two different regulatory proteins, including Cut and Dead Ringer (Cut and Dri). Dorsal-Cut/Dri complexes recruit Groucho, a corepressor containing WD40-repeats, and Capicua, an HMG-box transcription factor, which in turn mediate long-range transcriptional repression (Jimenez *et al.*, 2000; Valentine *et al.*, 1998; Dubnicoff *et al.*, 1997).

Cut and Dri binding sites are separated from neighboring Dorsal operator sites by 10 bp, one turn of the helix. This type of helical phasing can facilitate protein–protein interactions between neighboring transcription factors. Indeed, when a 5-bp spacer sequence is inserted between the second Dorsal operator site and the second AT-core motif, then the *zen* VRE no longer mediates transcriptional repression. Instead, the mutagenized VRE mediates activation in response to high levels of Dorsal in the ventral mesoderm (Cai *et al.*, 1996). Repressor function returns when the 5-bp spacer inserted between the Dorsal-2 and AT-2 sites is replaced with a 10-bp spacer sequence that restores the helical phasing of the two proteins.

These results suggest that direct protein–protein interactions between Dorsal and neighboring Cut/Dri complexes are required for converting the Dorsal activator into a repressor. In addition, these interactions may be essential for the efficient occupancy of Dorsal operator sites by the lowest levels of the Dorsal gradient. Disrupting these interactions causes a substantial change in the threshold response. The normal VRE mediates repression in ~40 nuclei spanning ventral and lateral regions. The mutagenized VRE lacking the AT "corepressor" motifs mediates activation in the ventralmost 18–20 nuclei. Further evidence for strong Dorsal–corepressor interactions is the observation that the low affinity Dorsal binding sites from the *twist* PE can mediate repression in both ventral as well as lateral regions when inserted into the *zen* VRE in place of the native, high affinity Dorsal operator sites (Jiang *et al.*, 1992). Thus, it would appear that Dorsal–corepressor interactions permit the efficient occupancy of low affinity Dorsal operator sites in response to the lowest levels of the Dorsal gradient. Conversely, replacing the low affinity Dorsal sites in the *twist* PE with the optimal sites from the *zen* VRE causes a relatively modest expansion in the expression limits, from 12–14 nuclei to 18–20 nuclei (Jiang and Levine, 1993).

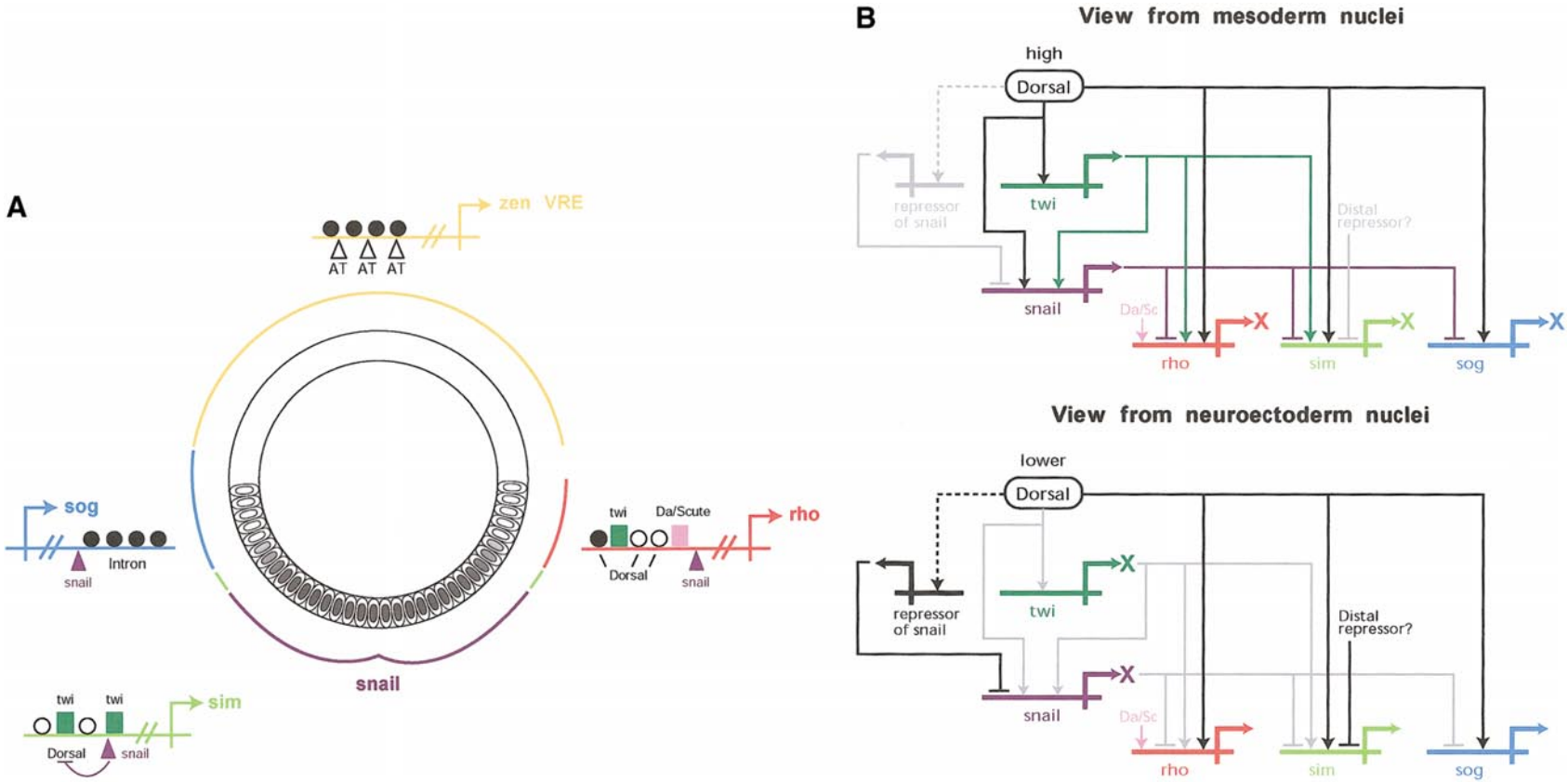

FIG. 3. Multiple Dorsal gradient thresholds. The Dorsal gradient generates between four and seven different thresholds of gene expression along the DV axis of the early embryo. Four of these thresholds are represented by the *snail*, *sim*, *rhomboid*, and *sog/zen* expression patterns. (A) *sim* expression appears to depend on linked low affinity Dorsal binding sites (open circles) and Twist sites. *sim* expression is excluded from the ventral mesoderm by the Snail repressor. The *rhomboid* expression pattern depends on linked high affinity (filled circles) and low affinity Dorsal bindings, along with linked Twist binding sites and general "E box" sequences that may be recognized by ubiquitous bHLH activators such as Daughterless (Da) and Scute. The broader *sog* lateral stripes depend on four evenly spaced, optimal Dorsal binding sites. The same low levels of Dorsal that activate *sog* also repress *zen*, which contains optimal Dorsal binding sites and linked "corepressor" binding sites (AT). The *sog* intronic enhancer and *zen* VRE silencer appear to contain a more rigid organization of factor binding sites than the *rho*, *sim*, and *snail cis*-regulatory DNAs. (B) Models of the Dorsal *cis*-regulatory networks that pattern mesoderm and neuroectoderm (Bolouri and Davidson, 2002). Higher levels of nuclear Dorsal present in ventral regions of the embryo activate *twist* and *snail* expression to help define mesoderm. While the levels of nuclear Dorsal present in ventral nuclei are sufficient to activate *rhomboid*, *sim*, and *sog*, these genes are not expressed (X) due to dominant repression by Snail. In lateral regions of the embryo which form the neuroectoderm, lower levels of Dorsal are sufficient to activate *rhomboid*, *sim*, and *sog* but not *snail* or *twist*. Daughterless (Da)/Scute (Sc), which is ubiquitously expressed, aids in the regulation of *rhomboid*. Twist may diffuse from the ventral region into lateral regions to affect expression of *rhomboid* and *sim*. Also depicted is the putative *snail* repressor, which helps create such a sharp *snail* border, possibly itself a Dorsal target that is expressed in response to lower levels of nuclear Dorsal. This same repressor may also be responsible for defining the distal border of *sim*. Alternatively, the restriction of *sim* expression to one cell's width may result from localized Notch signaling, and therefore the repressor that regulates the distal *sim* border may not affect *sim* directly but regulate Notch activation.

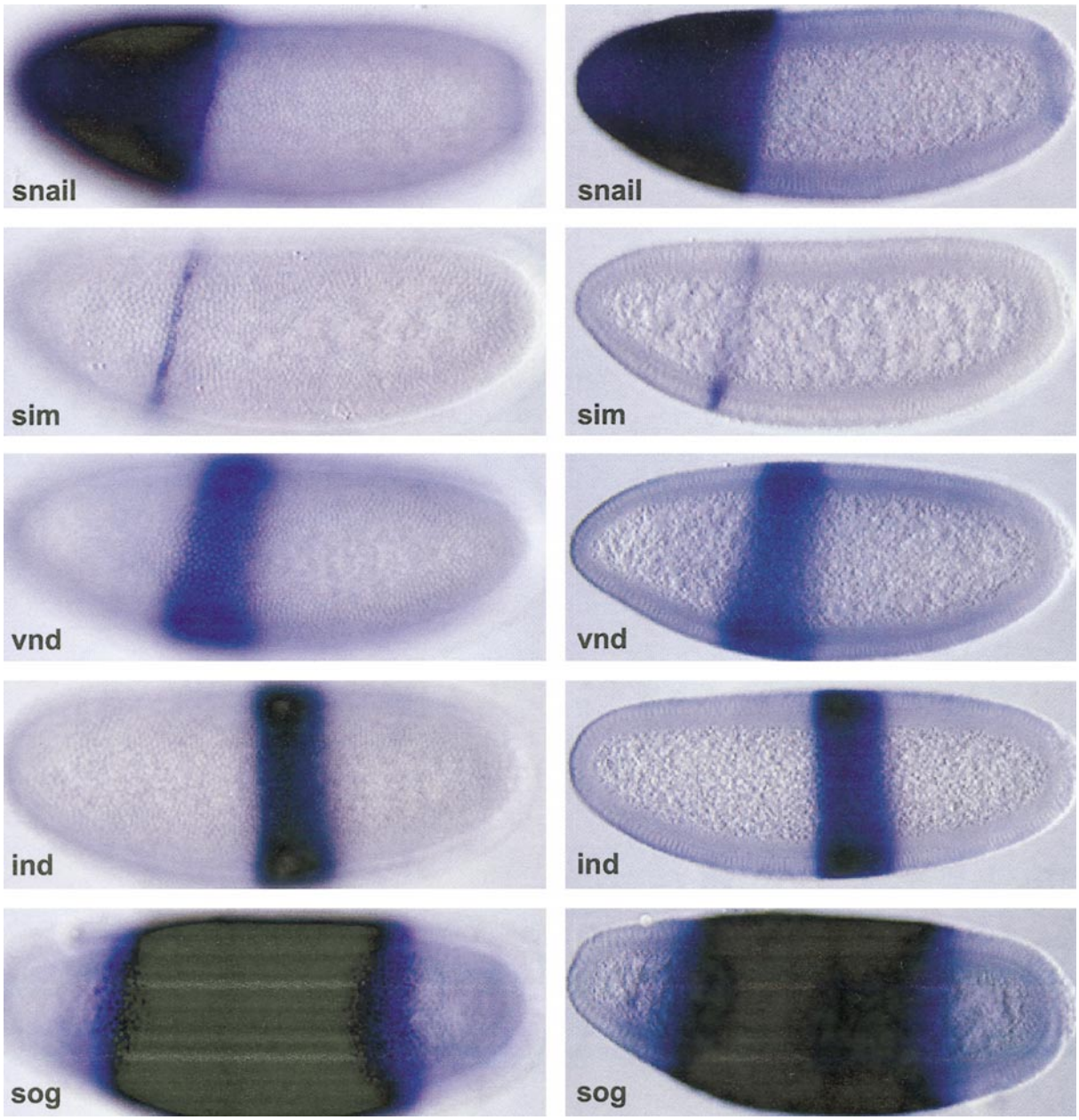

FIG. 4. Autonomy of an anterior–posterior Dorsal nuclear gradient. A broad AP Dorsal gradient generates the full spectrum of dorsal–ventral gene expression along the length of the embryo. Cellularizing embryos were stained to show the expression of different Dorsal target genes (i.e., *snail*, *sim*, *vnd*, *ind*, and *sog*). Embryos are oriented with anterior to the left and dorsal up. For each gene expression pattern, surface views are shown on the left and parasagittal views on the right.

These results underscore the importance of protein–protein interactions in determining different threshold responses to the Dorsal gradient. Similarly, MCM1-α2 interactions appear to compensate for changes in the binding affinities of α2 operator sites in the promoter regions of a-specific genes in yeast (Smith and Johnson, 1992).

It is conceivable that the lateral stripes of *sog* expression also depend on cooperative DNA binding interactions between Dorsal and other transcription factors bound to the *sog* intronic enhancer. Perhaps the exact spacing of these binding sites is essential for the activation of *sog* by the lowest levels of the Dorsal gradient. Indeed, the organization of the binding sites contained in the *zen* VRE and *sog* intronic enhancer may be more rigid than the distribution

of sites in the *rhomboid* lateral stripe enhancer, which is activated by higher levels of the Dorsal gradient. The Dorsal binding sites contained in the *zen* VRE and *sog* intronic enhancer are virtually identical (Markstein *et al.*, 2002). Yet, Dorsal activates *sog* and represses *zen*. Consequently, the nature of the Dorsal recognition sequences does not determine whether a target gene is activated or repressed. Rather, promoter context determines how the Dorsal gradient regulates target genes. The *zen* silencer contains binding sites for "corepressor" proteins that convert Dorsal into a potent transcriptional repressor (Cai *et al.*, 1996; Valentine *et al.*, 1998; Dubnicoff *et al.*, 1997). In contrast, the *sog* enhancer lacks corepressor sites, and is therefore activated, not repressed, by Dorsal.

Additional Thresholds?

How many transcriptional thresholds are generated by the Dorsal gradient established by the Spz ligand? This question is important because only two or three thresholds have been documented by other morphogens, including EGF (e.g., Golembo *et al.*, 1999), Dpp (e.g., Ashe *et al.*, 2000), Hedgehog (Struhl *et al.*, 1997), and Wnts (Zecca *et al.*, 1996). We have reviewed evidence that the Dorsal gradient directs at least four thresholds: mesoderm (*twist* and *snail* expression patterns), mesectoderm (*sim* expression), ventral half of the neurogenic ectoderm (*rhomboid*), and the entire neurogenic ectoderm (*sog*, a positive target of the gradient and *zen*, a negative target). A fifth potential threshold is observed for the *twist* PE, which mimics the folded gastrulation expression pattern in the ventralmost regions of the embryo where there are peak levels of the Dorsal gradient.

Recent studies on the dorsoventral patterning of the *Drosophila* ventral nerve cord raise the possibility that the Dorsal gradient directs at least one additional threshold of gene activity. Three interacting homeobox genes, *vnd*, *ind*, and *msh*, have been implicated in the patterning of the nerve cord, which is formed from three rows of neuroblasts on either side of the ventral midline (e.g., Weiss *et al.*, 1998). The ventralmost row is patterned, in part, by *vnd*, the intermediate row by *ind*, and the dorsalmost, or outer, row is specified by *msh*. These genes, particularly *vnd* and *ind*, exhibit early lateral stripes of expression in cellularizing embryos, similar to the time when Dorsal directly activates the *rhomboid* NEE. However, the *vnd* and *ind* patterns are distinct from both the lateral lines of *sim* expression in the presumptive mesectoderm and the lateral stripes of *rhomboid* expression in the ventral half of the neurogenic ectoderm. The *vnd* stripes are about half the width of the *rhomboid* stripes and encompass the ventralmost four to five cells. The dorsal limits of these stripes are therefore positioned between the *sim* and *rhomboid* patterns. We are currently testing a putative early embryonic *vnd* enhancer element which should drive expression similar to the endogenous *vnd* pattern, as this genomic sequence is proximal to *vnd* and includes multiple high affinity Dorsal binding sites and optimal Twist binding sites. It is conceivable that the *ind* expression pattern represents a seventh Dorsal transcription threshold since the dorsal limits of the *ind* lateral stripes appear to extend somewhere between the *rho* and *sog* patterns (Fig. 4). However, the Dorsal-responsive enhancer that drives *ind* expression, if it exists, remains to be found as it has not been identified to date using the available computational approaches.

Just two of the five to seven transcription thresholds depend on high vs low affinity Dorsal operator sites. The remaining three to five thresholds depend on protein–protein interactions with other classes of transcription factors, including Twist, Daughterless/Scute, and Cut/Dri. It is conceivable that the lowest threshold readout depends on a fixed organization of binding sites, similar to the enhanceosome that regulates the β-interferon gene in mammals (Merika and Thanos, 2001). In contrast, higher threshold readouts may be obtained with enhancers containing a less rigid structure and lacking helical phasing between interacting proteins.

Dorsal–Twist synergy appears to establish three of the transcription thresholds. When the Twist sites map far from low affinity Dorsal sites, expression is restricted to the presumptive mesoderm, as seen for *snail*. Tight linkage of Twist and low affinity Dorsal sites might cause a slight expansion of the pattern into the mesectoderm, as seen for *sim*. Finally, a synthetic lacZ reporter gene containing tightly linked Twist and high affinity Dorsal sites exhibits a broader pattern of expression that extends through the ventralmost four to five cells of the neurogenic ectoderm. This is similar to the native *vnd* pattern. The broader *rhomboid* pattern might result from the presence of both Twist and Da/Scute E boxes in the NEE. As discussed earlier, the latter proteins are ubiquitously expressed throughout the early embryo, whereas Twist is restricted to ventral regions.

The distinct *cis*-regulatory regions associated with the various Dorsal target genes are sufficient to generate sequential patterns of gene expression in response to the Dorsal gradient (Huang *et al.*, 1997). This has been demonstrated by creating an ectopic anteroposterior dorsal gradient. A constitutively activated form of the Toll receptor ($Toll^{10b}$) was expressed in anterior regions of transgenic embryos by using the mRNA localization signal from the *bicoid* 3′ UTR. The localized, activated Toll receptor leads to the formation of a broad anterior–posterior (AP) Dorsal nuclear gradient. This ectopic gradient triggers the full spectrum of dorsal–ventral patterning responses along the AP axis, including sequential patterns of *snail*, *sim*, *vnd*, *ind*, and *sog* expression (Fig. 4).

Summary of Dorsoventral Patterning Thresholds

Dorsal gradient thresholds initiate the differentiation of the mesoderm, mesectoderm, neurogenic ectoderm, and dorsal ectoderm, and help prepattern the mesoderm and neurogenic ectoderm. It is conceivable that the Dorsal

gradient establishes two cell types within the presumptive mesoderm through the differential regulation of *fog* and *twist/snail*. Moreover, Dorsal appears to initiate DV polarity within the presumptive nerve cord through the differential regulation of *vnd*, *ind*, and *msh*. In contrast, Dorsal gradient thresholds only indirectly pattern the dorsal ectoderm through the differential regulation of two target genes, *sog* and *dpp* (Fig. 4). *sog* encodes a secreted protein that inhibits Dpp signaling activity (Srinivasan *et al.*, 2002). The diffusion of Sog from a localized source within the neurogenic ectoderm is thought to create a peak of Dpp signaling at the dorsal midline, which is responsible for triggering the differentiation of the amnioserosa (e.g., Ashe and Levine, 1999). Lower levels of Dpp signaling in dorsolateral regions specify dorsal epidermis.

It is possible to trace the asymmetric position of the oocyte nucleus to the formation of a broad Dorsal nuclear gradient and the specification of multiple tissues and cell types along the dorsoventral axis of gastrulating embryos. The fact that the single, broad Dorsal nuclear gradient can generate at least five or six different thresholds of gene expression and cell fate specification raises the possibility that other signaling systems also trigger three or more thresholds of differential gene activity. For example, the Dpp gradient emanating from the A/P compartment boundary in the developing wing imaginal disk specifies at least two transcription thresholds, manifested by differential patterns of *spalt* and *omb* expression (Nellen *et al.*, 1996; Strigini and Cohen, 1999). It may generate additional thresholds. Alternatively, it is conceivable that the Toll–Dorsal signaling pathway is particularly well suited for generating multiple thresholds. Only one or two cytoplasmic kinases appear to transduce Toll activity (Belvin and Anderson, 1996). Consequently, there may be a fairly linear correlation between the number of activated Toll receptors and the amount of Dorsal that enters nuclei. In contrast, other signaling systems, particularly receptor tyrosine kinase pathways, include multiple enzymatic steps between the activation of the receptor and the modification of downstream transcription factors (e.g., Duffy and Perrimon, 1996). Such pathways might produce inherent on/off responses to extracellular ligands, and thereby preclude the formation of broad nuclear activity gradients, as seen for Dorsal. According to this view, some signaling mechanisms are better suited than others for generating multiple transcription thresholds.

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