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# The interpretation of morphogen gradients

**Hilary L. Ashe[1] and James Briscoe[2]**

Morphogens act as graded positional cues that control cell fate specification in many developing tissues. This concept, in which a signalling gradient regulates differential gene expression in a concentration-dependent manner, provides a basis for understanding many patterning processes. It also raises several mechanistic issues, such as how responding cells perceive and interpret the concentration-dependent information provided by a morphogen to generate precise patterns of gene expression and cell differentiation in developing tissues. Here, we review recent work on the molecular features of morphogen signalling that facilitate the interpretation of graded signals and attempt to identify some emerging common principles.

## Introduction

The transformation of the spatial distribution of naïve cells in a developing tissue into an organised arrangement of cell differentiation is fundamental to the development of multicellular organisms. More than a century ago, evidence began to accumulate that cells receive 'positional information' that instructs them to develop in specific ways, depending on their location within a tissue (Wolpert, 1996). Over the intervening decades, the potential for signalling gradients to provide this positional information has become a much-investigated and -debated subject, and the term 'morphogen' has been coined to describe such signals. Today the morphogen concept continues to form the basis of many models of pattern formation (Lewis et al., 1977; Green and Smith, 1991; Gurdon and Bourillot, 2001; Tabata and Takei, 2004). Typically, in current models it is proposed that a signal produced from a defined localised source forms a concentration gradient as it spreads through surrounding tissue (Fig. 1A). The graded signal then acts directly on cells, in a concentration-dependent manner, to specify gene expression changes and cell fate selection. Thus, the concentration of ligand provides cells with a measure of their position relative to the source of the signal and organises the pattern of cell differentiation. Experimental evidence from tissues in both vertebrates and invertebrates indicates that several molecules appear to function as graded signals. The roles of these signals range from the establishment of the initial polarities of embryos to specification of cell identity in specific tissues, notably limb appendages and the nervous system in both vertebrates and *Drosophila*. The examples we focus on in this review are introduced in Fig. 1. Evidence in support of these signals acting as graded morphogens has been summarised in recent reviews (Gurdon and Bourillot, 2001; Tabata and Takei, 2004).

Although the morphogen concept has provided an enduring and valid framework for understanding pattern formation, it raises many mechanistic issues. Much attention has focused on how the distribution of a morphogen through a tissue establishes and maintains a gradient of activity (Vincent and Dubois, 2002; Tabata and Takei, 2004); however, how the signal is perceived and interpreted in a graded manner by the receiving cells has received less consideration. Nonetheless, this represents an equally important element of the morphogen hypothesis. Crucial to understanding the mechanism of morphogen activity is determining how a graded signal is transformed into alterations in gene expression programmes, such that the positional information supplied by the morphogen produces the appropriate spatial pattern of cellular differentiation. To understand how this is accomplished, several questions have to be addressed. How does the signal transduction pathway transmit graded information intracellularly to control concentration-dependent differential gene expression? How is a continuous gradient transformed into discrete changes in gene expression that ultimately determine the choice of cell fate from the available alternatives? And how does graded signalling accommodate fluctuations in biological conditions to achieve the necessary robustness required for accurate developmental patterning? By focusing on specific examples, we review recent work that addresses these questions and, where possible, we highlight some of the general principles that appear to be shared between different morphogen gradients.

[1]Faculty of Life Sciences, The University of Manchester, Oxford Road, Manchester M13 9PT, UK. [2]Developmental Neurobiology, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.

Authors for correspondence (e-mail: hilary.ashe@manchester.ac.uk; james.briscoe@nimr.mrc.ac.uk)

## Morphogen signal transduction pathways are linear and transmit graded information

### How many thresholds does a morphogen control?

At a minimum, to meet the definition of a morphogen, a graded signal must be able to direct the generation of at least two distinct cell types at different concentrations. Theoretical analysis has raised the possibility that graded signals can achieve up to 30 thresholds (Lewis et al., 1977); however, empirical evidence has typically identified between three and seven distinct thresholds. For example, the Dorsal (Dl) gradient appears to specify at least four, and as many as seven, distinct thresholds of gene expression along the dorsoventral (DV) axis of *Drosophila* embryos (Stathopoulos and Levine, 2002a). A concentration gradient of activin is able to induce five cell states in *Xenopus* blastula cells (Green et al., 1992), and a similar number of neuronal subtypes appears to be produced by graded Sonic Hedgehog (Shh) signalling in the neural tube (Ericson et al., 1997; Pierani et al., 1999). In each of these cases, additional signals are believed to promote or cooperate in the forming of some of the threshold responses, so whether a single morphogen acting alone produces each of the observed threshold responses remains unknown. In other well-studied cases, fewer defined thresholds have been clearly identified, for example Wingless (Wg) signalling in the *Drosophila* wing imaginal disc promotes three thresholds of gene expression (Tabata and Takei, 2004), whereas graded Decapentaplegic (Dpp) signalling is responsible for at least three threshold responses in *Drosophila* embryos and the wing disc (Ashe et al., 2000; Affolter et al., 2001).

### Small morphogen concentration changes are sensed

In the case of the vertebrate morphogens activin, bone morphogenetic protein (Bmp) 4 and Shh, the dose responses of cells have been assayed (Green et al., 1992; Wilson et al., 1997; Ericson et al., 1997). For activin and Shh, the full range of responses is elicited over a 25- to 50-fold concentration range with relatively

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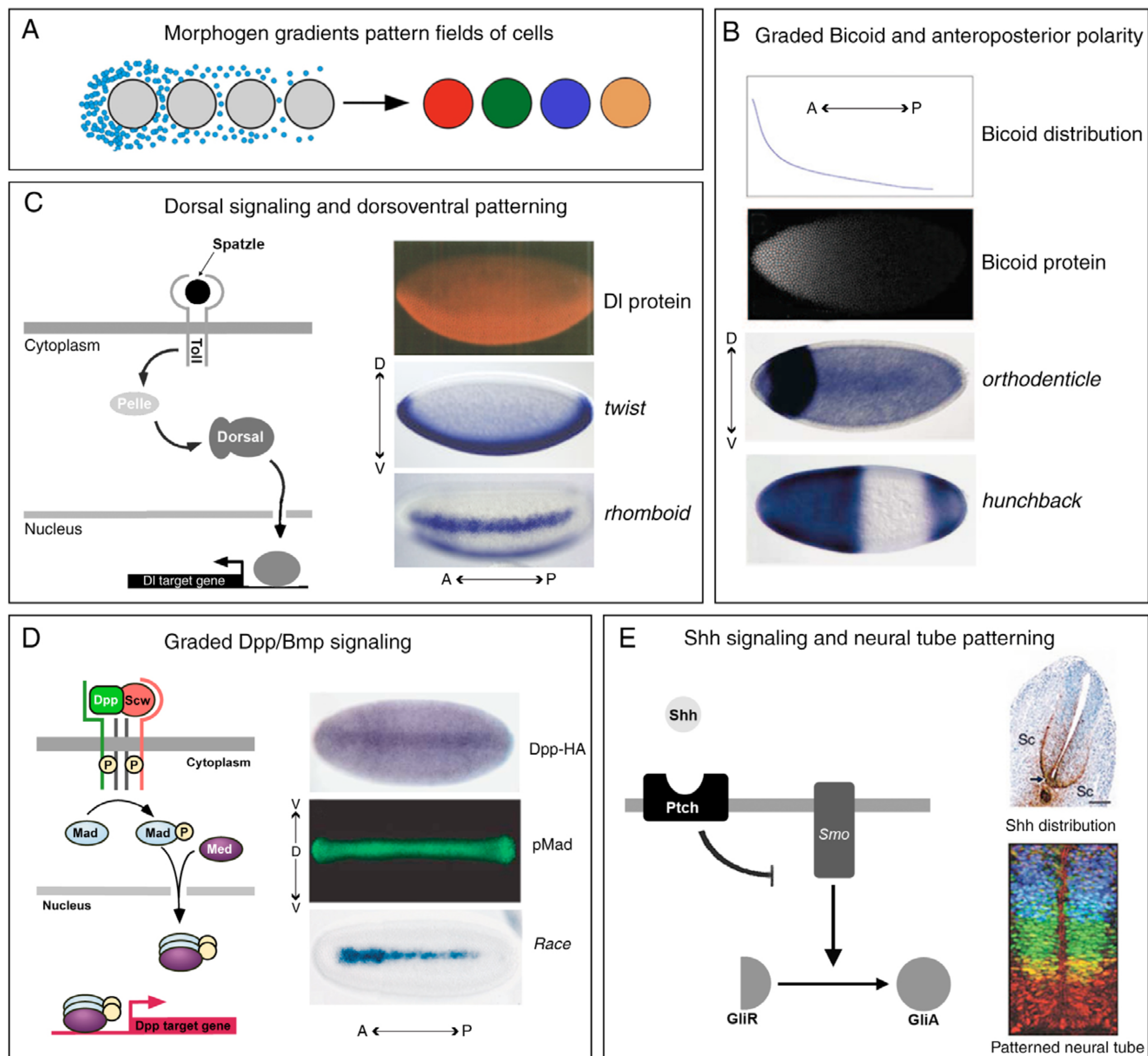


**Fig. 1. Morphogen gradients pattern developing tissues.** (**A**) Theoretical morphogen gradient. A gradient of a signalling molecule (blue) within tissue (grey cells) provides positional information, instructing cells to adopt distinct cell fates (coloured cells), according to the concentration of signal to which they are exposed. (**B**) The graded distribution of the transcription factor Bicoid establishes anteroposterior (AP) polarity in the developing *Drosophila* embryo. Immunostaining reveals the gradient of Bicoid distribution in the embryo. Expression of *orthodenticle* and *hunchback* genes are induced by high and low levels of Bicoid, respectively. (**C**) The dorsoventral (DV) axis of the early *Drosophila* embryo is patterned by graded Dorsal (Dl) activity (left). The ligand Spatzle binding to its transmembrane (TM) receptor Toll initiates signal transduction that, through the action of the kinase Pelle, activates the NF-κB-like transcription factor Dl. (Right) Graded distribution of Dl protein; *twist* and *rhomboid* are induced by high and low levels of Dl, respectively. (**D**) In both *Drosophila* and vertebrates, Dpp/BMP signalling operates in a graded manner to pattern several developing tissues. A Dpp/Screw (Scw) heterodimer activates its heteromeric complex containing receptor TM serine/threonine kinases. The activated receptor phosphorylates Mad/Smad transcription factors that, with Med/Smad4 transcription factor, then translocate to the nucleus where they can activate, in combination with other proteins, target gene expression. In the *Drosophila* embryo, high Dpp levels are distributed along the dorsal midline (top panel), resulting in a peak of phosphorylated Mad (pMad) (middle panel) and the induction of target genes such as *Race* (bottom panel). In the *Drosophila* embryo, a stepped distribution of Dpp is observed, resulting in a stepped activation of Mad (see text for details). (**E**) Graded Sonic hedgehog (Shh) signalling patterns the ventral neural tube. In the absence of Shh ligand, the TM protein Patched (Ptch) inhibits Smoothened (Smo), consequently Gli factors are converted to transcriptional repressors (GliR). Shh binds to Ptch, relieving repression of Smo, which signals to block the production of GliR proteins, promoting the generation of Gli activators (GliA). (Right) A Shh gradient can be visualised in the ventral neural tube (top panel), which regulates homeodomain protein expression (bottom panel). (B) Reproduced, with permission, from Ochoa-Espinosa et al. (Ochoa-Espinosa et al., 2005) (Bicoid protein) and Ephrussi and St Johnston (Ephrussi and St Johnston, 2004) (*orthodenticle* and *hunchback* mRNAs). (C) Reproduced, with permission, from Rushlow et al. (Rushlow et al., 1989) (Dl protein); Berkeley Drosophila Genome Project In Situ Database (http://www.fruitfly.org/cgi-bin/ex/insitu.pl) (*twist*); and Erives and Levine (Erives and Levine, 2004) (*rhomboid* neuroectoderm enhancer directed *lacZ* expression). (D) Reproduced with permission from Shimmi et al. (Shimmi et al., 2005) (Dpp-HA); Wang and Ferguson (Wang and Ferguson, 2005) (pMad); and Wharton et al. (Wharton et al., 2004) (*Race*). (E, Shh gradient) Reproduced, with permission, from Gritli-Linde et al. (Gritli-Linde et al., 2001).

small, two- to threefold, changes in concentration being sufficient to switch cells between alternative fates. Moreover, for other graded signals, the evidence also suggests that comparatively moderate changes in signalling strength are sufficient to alter significantly the response of cells. For example, a gradient of Dpp regulates the dose-dependent expression of target genes in dorsal regions of *Drosophila* embryos. Altering the gene dose of *dpp* by decreasing the gene copy number to one or by increasing it to three or four has significant effects on the position at which target genes are expressed (Ashe et al., 2000). Consistent with this, the injection of *dpp* transcripts into *Drosophila* embryos is sufficient to promote the development of ectodermal cells with incremental two- to fourfold increases in the injected concentration eliciting progressively more-dorsal cell fates (Ferguson and Anderson, 1992).

### Signalling pathways are linear

Most morphogens are protein ligands that bind to transmembrane receptors and initiate intracellular signal transduction cascades to regulate the transcription of specific target genes. Clearly, the concentration information supplied by the ligand needs to be encoded and transmitted through each step of the signalling pathway. Conceptually, a simple mechanism for producing discrete cell fate decisions is one in which differences in ligand concentration are perceived intracellularly as qualitative differences in signal transduction, perhaps by employing distinct types of receptors to initiate different downstream signalling events. However, evidence suggests that, where this has been tested, it is not a generally applicable mechanism. For example, a single type of ligand-binding receptor is sufficient to specify the concentration-dependent responses characteristic of graded activin signalling in *Xenopus* cells. Therefore, the absolute number of activated receptors determines the response of cells: a threefold difference in the number of activated receptors is sufficient to specify distinct responses in these assays (Dyson and Gurdon, 1998). Moreover, the transmembrane protein Smoothened is necessary and sufficient to induce the multiple neuronal subtypes characteristic of Shh signalling in the vertebrate ventral neural tube (Hynes et al., 2000; Wijgerde et al., 2002). In the case of Bmp signalling in the *Drosophila* dorsal ectoderm, the presence of Dpp and a second Bmp-type ligand, Screw, facilitates the activation of different receptor complexes that contain distinct combinations of receptors molecules. Despite this, within the cell the same transcriptional effectors transduce signals emanating from each receptor complex (Shimmi et al., 2005).

Employing a single species of receptor to transmit concentration-dependent information does not preclude the downstream induction of different branches of a signalling pathway at different ligand concentrations. For example, in tissue-culture models, a different set of proteins appears to be phosphorylated and activated at low concentrations of platelet-derived growth factor compared with high concentrations (Rankin and Rozengurt, 1994). However, this type of mechanism does not appear to be favoured in the perception of morphogen gradients. Instead, linear signalling pathways seem to be the rule. An elegant demonstration of this comes from the ability of a Dl nuclear gradient to pattern the DV axis of the early *Drosophila* embryo. In the embryo, graded activation of the Toll transmembrane receptor by Spatzle leads to the induction of Pelle kinase, and ultimately nuclear translocation of Dl (Fig. 1C) (Stathopoulos and Levine, 2002a). Ectopic gradients of either Toll or Pelle can provide positional information and control multiple patterning thresholds, providing evidence of a linear signalling pathway in which differences in the number of activated Toll receptors are transduced to a gradient of Pelle activity that, in turn, establishes a gradient of nuclear Dl (Stathopoulos and Levine, 2002b). Studies of other morphogen signalling pathways indicate that each pathway culminates in the post-translational regulation of the activity of a single transcription factor or family of related transcription factors that have overlapping functions. For example, a constitutively active form of β-catenin/Armadillo (Arm), the transcriptional mediator of Wg signalling, is sufficient to induce the expression of both short range and long targets of Wg signalling in the *Drosophila* wing disc (Zecca et al., 1996). Similarly, Smad1 and Smad2, the mediators of Bmp4 and activin signalling, respectively, are sufficient to transduce the graded responses to these signals (Wilson et al., 1997; Shimizu and Gurdon, 1999). Active versions of the Gli3 protein, the Shh transcriptional mediator (see Fig. 1E), can recapitulate the patterning activity of Shh in the chick neural tube (Stamataki et al., 2005).

### Morphogen concentration determines the level of transcriptional effector activated

The apparent lack of signalling pathway branching, together with the sufficiency of single transcriptional effectors to mediate the full range of responses to a morphogen, indicate that changes in the extracellular morphogen concentration should be reflected directly by differences in the activity of the relevant transcriptional effectors. In general, this appears to be the case. For example, the threefold difference in activin concentration that causes a switch in gene expression in *Xenopus* cells is mimicked by a comparable change in the level of nuclear Smad2 (Shimizu and Gurdon, 1999). Similarly, the graded activity of Bmp4 can be recapitulated with corresponding concentration changes in ectopic Smad1 (Wilson et al., 1997). Moreover, the incremental two- to threefold changes in Shh concentration, which determine alternative neuronal subtypes, are mimicked by equivalent small changes in Gli activity levels, indicating that a gradient of Gli activity reflects graded Shh signalling (Stamataki et al., 2005). Together, these observations suggest that morphogen gradient interpretation requires target genes to be able to interpret two- to threefold changes in transcriptional effector in order to generate distinct transcriptional responses. Consistent with this, the Bicoid (Bcd) target gene *hunchback* (*hb*) appears able to discriminate between approximately twofold changes in Bcd concentration (Struhl et al., 1989).

In other cases, it is less clear if there is a direct correlation between changes in the extracellular ligand concentration and changes in transcriptional strength. For example, although the graded activation of Mothers against Dpp (Mad), the transcriptional effector of the Dpp pathway (see Fig. 1D), depends on its ligand, studies in the *Drosophila* wing disc have revealed that a sudden transition in the level of activated Mad occurs that does not coincide with a similar, abrupt change in the distribution of Dpp (Teleman and Cohen, 2000). It is possible that this is because the Dpp visualised in these experiments does not accurately reveal the distribution of Dpp that is able to engage and activate its receptors. Alternatively, the deviation may be due to the modulation of the activated Mad profile by additional factors, such as Daughters Against Dpp (Dad), which is an inhibitory Smad, the Saxophone receptor, the levels of the SARA adaptor protein or the Smurf ubiquitin ligase (Teleman and Cohen, 2000). For other graded signals, the quantitative relationship between ligand concentration, pathway activity and transcriptional effectors remains to be determined.

The linearity of signalling pathways implies that the signal transduction machinery transmits concentration-dependent information with sufficient fidelity to mediate differential responses.

Consequently, changes in ligand concentration control proportionate quantitative changes in the activity of the transcriptional effectors. This provides a contrast to those signalling pathways that display bistable or ultra-sensitive responses that confer monotonic, switch-like responses (Monod and Jacob, 1961; Ferrell, 2002). A well-studied example of this type of response is the maturation of oocytes, which is induced at a crucial threshold of progesterone signalling through the activation of a mitogen-activated protein kinase (Mapk) cascade (Ferrell and Machleder, 1998). Similar switch-like behaviour may be elicited by other signals, such as some receptor tyrosine kinase receptor pathways that activate the Ras-Mapk pathway. Although this type of binary switching behaviour is relevant in some biological settings, it does not allow the transduction of a graded signal responsible for controlling multiple cell fate decisions at different concentration thresholds. This raises the possibility that the molecular mechanisms of signalling pathways capable of transmitting concentration-dependent responses are distinct from those that provide simple monotonic responses. Addressing this issue will require a detailed analysis and comparison of the mechanisms of signal transduction and of the strategies employed to transfer graded information accurately from receptor to the nucleus.

## Strategies employed in the regulation of differentially responsive genes

The mechanisms of gene regulation by morphogen signalling must provide a means to translate small differences in signal strength into threshold responses in which all-or-none changes in gene expression allow the selection of discrete cell identities in the developing tissue. More than a generation ago, strategies that could explain this phenomenon were proposed (Monod and Jacob, 1961), and some of these ideas are beginning to re-emerge from more recent molecular studies. We attempt to categorise these strategies into general design features that can account for differential gene regulation by graded signalling (Fig. 2). Clearly, there are overlaps between these categories and the list is not exhaustive. It is apparent that most, if not all, of the well-studied morphogen pathways use a combination of these mechanisms to control target gene expression. To illustrate the key features of each of the strategies, we have outlined examples of their use in the interpretation of specific morphogen gradients.

### Binding-site affinity

A major mechanism that has been extensively investigated exploits differences in the affinity of the transcriptional effector for binding to sites with different DNA sequences (Fig. 2A). A paradigm for this is the Dl gradient in the early *Drosophila* embryo, which directs DV patterning and gastrulation through the concentration-dependent activation and repression of target genes (Stathopoulos and Levine, 2004). Extensive studies of specific enhancers that respond to different thresholds of Dl have revealed a detailed picture of the mechanism of gene regulation. Based on their responsiveness to Dl, target genes have been classified into different categories. Type I genes, such as *twist* (*twi*) are activated in the presumptive mesoderm where there are peak levels of nuclear Dl (Fig. 1C). The enhancers of these genes tend to have low-affinity Dl-binding sites that are only occupied at the highest Dl concentration, thus limiting the expression of type I genes to the presumptive mesoderm (Jiang and Levine, 1993). By comparison, enhancers of type II genes, such as *rhomboid* (Fig. 1C), contain high-affinity Dl-binding sites that are bound and activated by the lower levels of Dl that are present in the ventral neuroectoderm (Ip et al., 1992a). Recent computational analysis of a large set of Dl-responsive enhancers from the genomes of *D. melanogaster* and related species has confirmed that Dl affinity is a major determinant of the expression domains of Dl target genes (Papatsenko and Levine, 2005). However, a high-affinity Dl-binding site does not necessarily lead to the activation of transcription when Dl is present. In some cases, Dl bound to a high-affinity site can also repress transcription, indicating that enhancer architecture also plays a significant role in determining the responsiveness of genes to Dl (Stathopoulos and Levine, 2004). Moreover, cooperative interactions between Dl and other factors also significantly influence the responsiveness of some genes.

A second example is interpretation of the Bcd gradient, which is responsible for regulating gene activity along the anteroposterior (AP) axis in the *Drosophila* embryo. Early studies of Bcd interpretation identified the affinity of Bcd-binding sites as a key determinant for setting the limits of expression of the *hb* target gene (Fig. 1B). Decreasing Bcd affinity leads to a more anterior restricted expression pattern where Bcd levels are higher. Thus, a model was proposed for the interpretation of the Bcd gradient, in which genes with anterior restricted expression have low-affinity Bcd-binding sites in their enhancer and consequently require a high Bcd concentration for occupancy and activation. Conversely, the higher-affinity sites in the *hb* enhancer allow expression at more posterior positions where the Bcd concentration is lower (Driever et al., 1989; Struhl et al., 1989). In support of this model, the *orthodenticle* gene, which is regulated by a low Bcd affinity enhancer, has a narrow expression pattern (Gao et al., 1996) (Fig. 1B).

It is not only in the precellular embryo that the response of genes to graded transcription factor activation uses binding-site affinity. This mechanism is also relevant in more conventional settings post cellularisation, such as the interpretation of the extracellular gradient of Dpp in the *Drosophila* embryo. In response to peak levels of Dpp signalling at the dorsal midline of the embryo, the target gene *Race* is expressed in a narrow stripe of cells in the presumptive amnioserosa (Fig. 1D). The enhancer responsible for this activity contains low-affinity binding sites for Mad, the transcriptional effector of Dpp. Altering these sites to increase their affinity for Mad broadens the associated expression pattern to that characteristic of genes that are responsive to a lower threshold of Dpp signalling (Wharton et al., 2004).

### Combinatorial inputs

Binding-site affinity can account for some of the morphogen gradient readouts; however, in general, affinity alone is insufficient to direct the full complement of transcriptional responses. For example, although the affinity of Bcd-binding sites sets the limits of expression of the *hb* target gene (Driever et al., 1989; Struhl et al., 1989), a computational study of a larger sample size of Bcd cis-regulatory modules indicates that for most there is a poor correlation between the strength of Bcd-binding clusters and the expression limits of a gene. Moreover, only a few target genes appear to be activated by Bcd alone, and the expression of these genes is restricted to the most anterior parts of the embryo that contain peak Bcd levels (Ochoa-Espinosa et al., 2005), as observed for a synthetic reporter containing only Bcd-binding sites (Crauk and Dostatni, 2005). For many genes, the major determinant for the interpretation of positional information is not the absolute Bcd affinity. Instead, other elements in target gene promoters and the integration of positive and negative transcriptional inputs from proteins bound to these elements can determine the interpretation of the Bcd gradient. For genes activated in the middle and posterior regions of the embryo, most enhancers of Bcd target genes tend to have additional inputs from the Hb, Caudal (Cad) and/or Krüppel (Kr) transcription factors (Ochoa-Espinosa et al., 2005). Hb and Cad are maternally expressed and zygotically activated and repressed by Bcd at the transcriptional and translational levels, respectively (Driever and

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Nusslein-Volhard, 1989; Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). Both Hb and Cad augment Bcd-dependent transcriptional activation (La Rosee et al., 1997; Simpson-Brose et al., 1994). Therefore, the Bcd gradient may function with Hb and/or Cad to establish a broad domain where enhancer activation can occur, and the balance of positive and/or negative inputs from these and other transcription factors would determine the limits of an expression domain (Ochoa-Espinosa et al., 2005). The transcriptional repressor Kr may be one such negative input that sets a sharp posterior border of some Bcd targets (Kraut and Levine, 1991). As well as binding sites for other transcriptional effectors, the arrangement of Bcd-binding sites also influences gene expression, and the data indicate that Bcd binds cooperatively to DNA. Therefore, Bcd binding to a high-affinity site potentiates binding to an adjacent low-affinity site (Burz et al., 1998). Expression of a Bcd protein with a mutation that disrupts cooperativity in the embryo leads to an anterior shift in the expression patterns of target genes, such as *hb*, and reduced sharpness of their posterior borders (Lebrecht et al., 2005).

The integration of inputs from Dl and from other transcription factors also influences the response of genes along the DV axis of the *Drosophila* embryo. An analysis of Dl target gene enhancers in different Drosophilids (Papatsenko and Levine, 2005) has revealed that, just as type I threshold genes tend to have lower Dl affinity than do type II genes, there is a similar trend in these promoters for the affinity of another transcription factor, Twi. Moreover, type II thresholds tend to have a fixed orientation of, and spacing between, Dl and Twi sites. This is consistent with the occurrence of synergistic interactions between Dl and Twi transcription factors being important for the activation of type II targets in the neurectoderm where Dl and Twi levels are low (Papatsenko and Levine, 2005). Type II enhancers

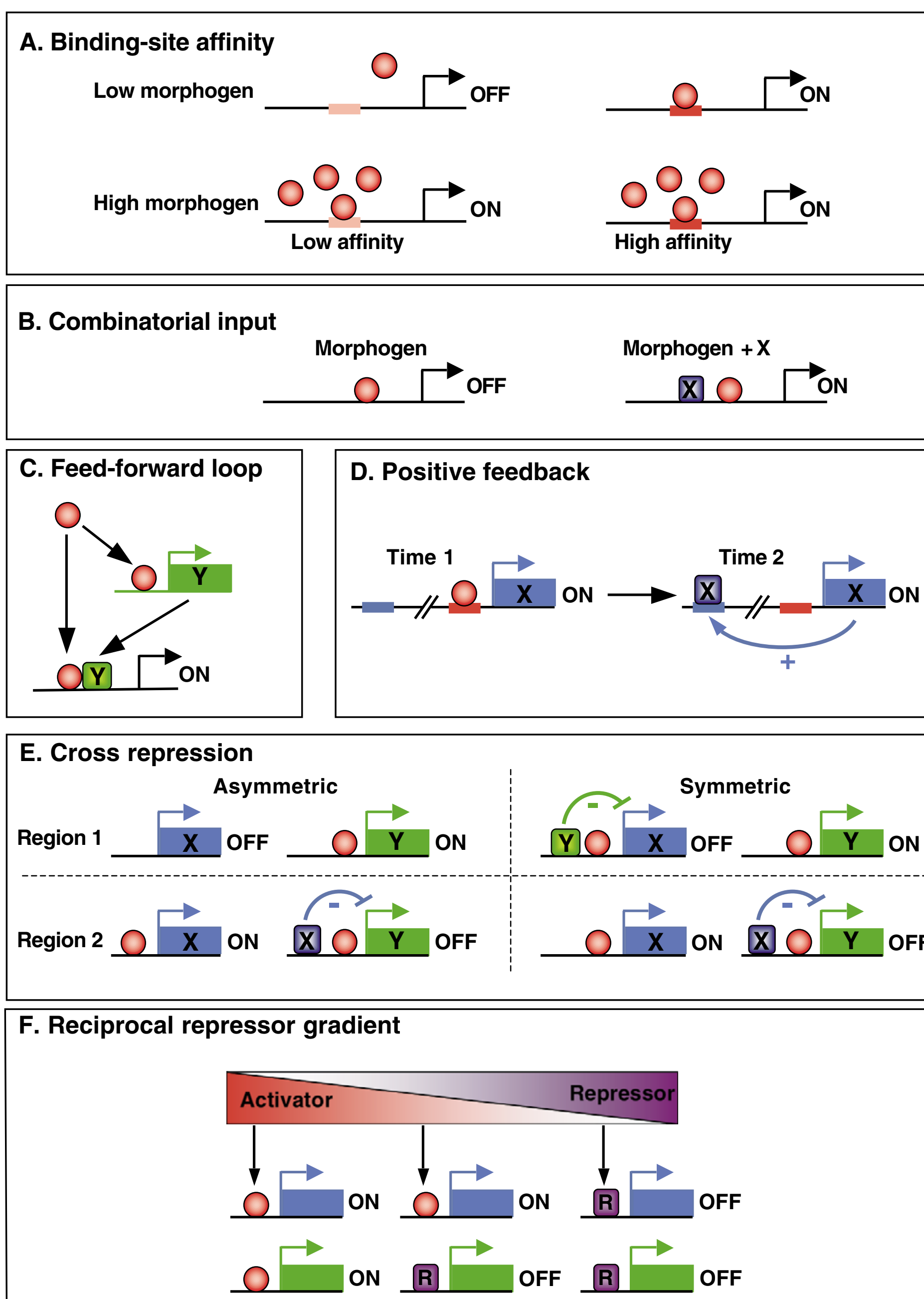


**Fig. 2. Strategies employed to interpret graded signals.** (**A**) Binding-site affinity. The number and affinity of transcription factor binding sites determine threshold responses. Low amounts of transcriptional effector are sufficient to bind to and activate transcription from high-affinity binding sites; lower-affinity binding sites require larger amounts of transcriptional effector. (**B**) Combinatorial inputs. The integration of multiple positive and/or negative inputs with the transcriptional effector of the morphogen establishes a threshold response. Other regulatory elements (X) can also determine the response of a target gene. (**C**) Feed-forward loop. A regulatory circuit in which the transcriptional effector activated by the morphogen controls the expression of a second regulator (Y); the combination of the two regulate the transcription of a target gene. (**D**) Positive feedback. A gene (X) induced by the morphogen autoregulates to enhance its own expression. (**E**) Cross repression. Repressive interactions between morphogen-regulated genes (X and Y) establish discrete changes in gene expression. Repressive interactions can be asymmetric (for example ventral dominance in the *Drosophila* neurectoderm) or symmetric, resulting in reciprocal cross repression (for example in the vertebrate neural tube). (**F**) Reciprocal repressor gradient. The transcriptional effector sets up an inverse transcriptional repressor gradient that is interpreted by target genes. The ratio of repressor (R) to activator defines the threshold response of target genes, depending on the binding sites present in the enhancer.

commonly have an additional positive input from the Suppressor of Hairless [Su(H)] activator (Erives and Levine, 2004) and a negative input from the Snail repressor. *snail* is a Dl target gene that is activated in the presumptive mesoderm (Ip et al., 1992b), thereby excluding expression of type II genes from the mesoderm (Kosman et al., 1991). In contrast to type II enhancers, there is a negative correlation between the quality of Dl and Twi sites at type I enhancers, i.e. a good Dl site is associated with a poor Twi site, and vice versa. This suggests that, at those enhancers where there are peak levels of Dl and Twi, the activators function in a compensatory manner (Papatsenko and Levine, 2005). Importantly, studies with synthetic enhancers also indicate that the presence of Twi and Dl sites leads to a sharper expression pattern, in comparison with the weaker, fuzzy pattern that is observed with Dl alone (Szymanski and Levine, 1995). Thus, it is clear that in addition to the affinity of binding sites for the main transcriptional effector of a morphogen, the integration of positive and negative inputs onto an enhancer is an important determinant of threshold responses (Fig. 2B).

### Feed-forward loops

The inclusion of combinatorial inputs into the control of differential gene expression allows complex regulatory relationships to develop between responding genes. One such relationship is the feed-forward loop (Fig. 2C), an example of which has recently been described for the activation of *Race* by Dpp signalling. In addition to the affinity of Mad-binding sites in the *Race* enhancer (Wharton et al., 2004), the transcription factor Zerknüllt (Zen) plays a crucial role in *Race* activation. Zen and Mad bind to adjacent sites in the *Race* enhancer, and a direct interaction between them is necessary for *Race* activation (Xu et al., 2005). *zen* is itself a Dpp-regulated gene that depends on peak levels of Dpp signalling (Rushlow et al., 2001). Thus, for *Race* to be induced, peak levels of Dpp signalling need to activate high levels of Mad and to induce Zen expression, which function together to activate *Race* (Xu et al., 2005). This type of regulatory genetic network, in which transcription factor X activates transcription factor Y, and together X and Y activate target Z, is termed a feed-forward loop (Lee et al., 2002).

The Mad-Zen feed-forward loop may represent a general strategy that is used to activate other peak Dpp target genes (Xu et al., 2005). It is certainly the case that feed-forward loops operate in other morphogen-responsive gene networks. For example, Twi, which functions with Dl to regulate genes along the DV axis, is itself encoded by a Dl-responsive gene (Jiang and Levine, 1993). The recurrence of feed-forward loops in the interpretation of early *Drosophila* embryo morphogen gradients suggests that his type of regulatory circuit is particularly suitable for gradient interpretation. Data from other systems reveal that feed-forward loops are useful for discriminating between erratic external signals to ensure that activation only occurs in response to persistent signalling, thus providing a means to buffer against small fluctuations in signal (Shen-Orr et al., 2002). Moreover, the co-incidence requirement inherent in feed-forward loops can also provide highly sensitive responses to small changes in signal level (Goldbeter and Koshland, 1984), a feature that would allow threshold responses to be generated in response to small changes in the initial signalling strength.

### Positive feedback

The autoregulation of, or positive-feedback loops (see Fig. 2D) in, responding genes can also play a role in gradient interpretation and provide a mechanism for the generation of all or none responses at threshold levels of signalling. A well-characterised example of this is the regulation of *Hoxb4* in the vertebrate hindbrain (Gould et al., 1998; Gould et al., 1997). A gradient of retinoic acid (RA) confers positional information along the AP axis of the forming vertebrate hindbrain and is responsible for determining the anterior limit of the induction of *Hoxb4*. RA activates nuclear RA receptors (RARs), and these receptors bind to a defined enhancer region in the *Hoxb4* locus to activate its expression. At early stages of hindbrain development, this mechanism establishes a diffuse anterior expression border of Hoxb4. A second enhancer element, the late enhancer element, within the *Hoxb4* locus is responsive to Hoxb4 protein itself. Therefore, at later developmental stages, following RA-mediated induction of *Hoxb4*, this element responds to the induced Hoxb4 and is sufficient to direct expression of this gene up to the normal anterior boundary of gene expression. Thus, graded RA activity initiates *Hoxb4* expression, *Hoxb4*-mediated autoregulation by Hoxb4 refines and maintains its expression as hindbrain development progresses. Hoxb4 regulates RARβ in a similar manner, indicating that a reciprocal positive feedback circuit exists between these proteins that generates and maintains the discrete boundaries of *Hoxb4* expression (Serpente et al., 2005).

### Cross repression

Repressive interactions between morphogen-regulated genes are also important for gradient interpretation (Fig. 2E). A well-studied example is the contribution of cross repression to the partition of the *Drosophila* neuroectoderm into three columns along the DV axis (Cowden and Levine, 2003). This subdivision is mediated by three homeobox transcription factors (Vnd, Ind and Msh) that demarcate the ventral, intermediate and dorsal columns, respectively. Distinct thresholds of Dl signalling induce these genes, but the production of the distinct columns of gene expression, which are delimited by abrupt switches in the expression of each homeodomain protein, depends on asymmetric cross-regulatory interactions between these proteins. In this way, the homeodomain proteins expressed in the more ventral domains repress those expressed more dorsally. Thus, incremental increases in Dl signalling result in the sequential activation of each gene and in the corresponding repression of the genes induced by lower levels of Dl activity – a process that has been termed 'ventral dominance'.

The vertebrate nervous system displays a variation on this regulatory motif that involves the use of mutual cross-repression, or reciprocal negative feedback, between pairs of genes. Cells in the vertebrate neural tube respond to graded Shh signalling by regulating the expression of a series of transcription factors that include the homeodomain orthologues of Vnd, Ind and Msh (Briscoe and Ericson, 2001). On the basis of their mode of regulation by Shh signalling, these transcription factors are divided into two groups, termed class I and II proteins. The expression of each class I protein is extinguished at distinct thresholds of Shh activity; conversely, expression of the class II proteins depends on Shh signalling. In vivo, the expression patterns of these genes divides the ventral neural tube into sharply demarcated domains reminiscent of those seen in *Drosophila,* with the ventral limit of most class I proteins corresponding to the dorsal limit of expression of a class II protein. This is achieved by selective cross-repressive interactions between the complementary pairs of class I and class II proteins expressed in adjacent abutting domains (Briscoe et al., 2001; Briscoe et al., 2000). In both the vertebrate and *Drosophila* nervous system (Cowden and Levine, 2003), the repressive interactions establish gene-response thresholds and generate the sharp boundaries of gene expression that ensure each progenitor domain expresses a distinct set of transcription factors. This mechanism converts a gradient of positional information into discrete all-or-none changes in gene expression.

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The principle of cross-regulatory interactions is also observed in other developing tissues, indicating that it may represent a general strategy deployed to interpret graded positional information. The Bcd gradient specifies the expression domains of the Gap genes, which position the downstream pair-rule and segment polarity genes necessary for segmentation of the embryo (Jäckle et al., 1986; Kraut and Levine, 1991). Both asymmetric and reciprocal repressive interactions between Gap genes appear to form an intricate circuit. Strong reciprocal repression between pairs of genes ensures mutual exclusivity of expression, while asymmetric repression of anterior Gap genes by more posterior genes leads to an anterior shift in their posterior boundaries (Jaeger et al., 2004; Monk, 2004) (Fig. 2E). These findings highlight a dynamic feature of Bcd gradient interpretation, whereby spatial domains of gene expression can be repositioned by subsequent asymmetric repressive interactions between Gap genes.

### Reciprocal repressor gradient

A common feature of many morphogen gradients is the establishment of an inverse gradient of a transcriptional repressor that is reciprocal to the transcriptional effector activated by the signal (Fig. 2A). In the case of Shh and Wnt signalling, the primary transcriptional effectors of these pathways mediate transcriptional repression in the absence of signalling, but are converted to transcriptional activators upon signalling (Giles et al., 2003; Jacob and Briscoe, 2003). The net effect of signalling, then, is formation of a gradient of transcriptional activator with an opposing repressor gradient, a strategy that could augment changes in transcriptional activity mediated by the morphogen. A variation in this strategy is employed in the interpretation of the Dpp gradient in the *Drosophila* wing imaginal disc. Here, the main role of Dpp signalling appears to be the creation of a reciprocal gradient of the Brinker (Brk) repressor protein. Mad and Medea directly repress Brk in a complex with the Schnurri transcription factor (Pyrowolakis et al., 2004), and sensitivity to Brk repression sets the expression limits of the Dpp threshold responses, including *spalt* (*sal*) and *optomotor-blind* (*omb*) (Muller et al., 2003). *omb* expression is repressed in *mad* mutant clones because of the derepression of Brk. However, in *brk mad* double mutant clones there is ectopic activation of *omb*, indicating that, for *omb* expression, the only requirement for Dpp signalling is to repress Brk. By contrast, expression of maximal levels of *sal* does require a positive input from the Smads (Affolter et al., 2001; Barrio and de Celis, 2004). In other developmental contexts, Mad and Brk have been found to compete for the same binding sites (Affolter et al., 2001), although the relevance of this to the establishment of the wing target gene expression domains is unclear.

## How are interpretation strategies influenced by properties of the morphogen gradient?

It is apparent that, in each case, interpretation of morphogen signalling involves a combination of different mechanisms; it is difficult to deduce with certainty why one strategy is employed over another. However, in some cases, clues about this may come from specific features of the gradients themselves.

### Interpretation of a step gradient

Although the standard view of a morphogen gradient is a continuous gradient, the embryonic Dpp gradient is unusual in that it contains a threshold, or step, in its distribution at the dorsal midline (Ashe, 2005). This step is mirrored by a similar plateau of high nuclear Smad concentration, which is flanked by a shoulder of lower concentration (Raftery and Sutherland, 2003). This unusual gradient distribution may help to generate sharp borders of the peak and intermediate Dpp threshold responses, which coincide with the stepped Smad nuclear gradient. In fact, the step gradient may have obviated the need for a repressor to assist in the specification of these Dpp threshold responses. Although sharp borders of threshold responses tend to involve an additional repressor input, such as those described in the patterning of the *Drosophila* and vertebrate nervous systems (Cowden and Levine, 2003; Briscoe and Ericson, 2001), based on current knowledge, the establishment of the peak Dpp threshold *Race* requires only inputs from activators (Zen and Smads) (Wharton et al., 2004; Xu et al., 2005).

### Temporal integration

The interpretation strategy and regulatory circuit may also govern the temporal response of cells to ongoing morphogen signalling. A striking correlation between the strength and duration of signalling and the spatial distribution of induced genes has been observed in a number of experiments. For example, at a low concentration or short duration, activin signalling induces Xbra at short range in *Xenopus* blastula cells, whereas with increasing time or activin concentration, the field of Xbra-expressing cells appears to move away from the source of signal (Gurdon et al., 1995). Likewise, studies of the relationship between time and concentration of Shh signalling and the induction of different digits in the vertebrate limb indicate that increasing the duration of Shh signalling results in the generation of increasingly posterior digits (Harfe et al., 2004; Yang et al., 1997). One possibility that would account for the temporal integration of morphogen signalling is that signal duration is sensed by cells in a similar manner to increasing signal strength – more signal results in the activation of increasing amounts of the transcriptional effector. Alternatively it is possible that the concentration and duration of signalling are not directly equivalent. The identification of feed-forward loops and cross-regulatory networks downstream of graded signals could offer an explanation. Accordingly, the regulatory interactions between morphogen target genes would result in a sequential induction of genes, providing a mechanism to explain changes in the temporal response of cells to morphogens.

It is possible that the regulatory circuits also provide an explanation for the hysteretic, or persistent, feature of the response of cells to a gradient (Lewis et al., 1977). This attribute, which has also been termed the 'ratchet effect' (Gurdon et al., 1995), results in cells retaining gene expression profiles characteristic of the highest concentration of signal to which they have been exposed. The induction of a gene in a positive-feedback loop becomes self sustaining, while cross-repression allows the persistence of the expressed gene and the inhibition of its negative regulator. The prolonged maintenance of gene expression profiles after a gradient has dissipated could relieve a requirement for a long-lasting signalling gradient to be established and could allow the consolidation of the positional identity of a cell.

### Robustness and correction mechanisms in the interpretation of gradients

Quantitative aspects of morphogen activity appear at odds with normal biological processes. Small changes in the concentration of an extracellular signalling molecule can have dramatic consequences on cell fate, yet embryonic development is able to cope with stochiometric fluctuations in gene expression and with changes in environmental and genetic conditions, such as changes in temperature and gene dose. Investigations of the mechanisms that underlie the precision and robustness of different signalling pathways have largely focused on morphogen distribution and the

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regulation of the signal transduction. Such studies have found that a number of mechanisms appear to operate to increase the reliability of graded signalling (Eldar et al., 2004; Freeman, 2000). In addition, gene regulation strategies, such as feed-forward and positive-feedback loops, may also contribute to the reliability of gene expression in response to morphogen signalling. Moreover, it is apparent that several mechanisms also exist to correct and refine initial morphogen patterning (Box 1), which facilitate the elimination (Namba et al., 1997), rearrangement (Wijgerde et al., 2002) or respecification (Standley et al., 2001) of mislocated cells. Understanding the molecular basis of these mechanisms and analysing the contribution they make to the precise and reliable patterns generated by morphogens requires considerable additional work.

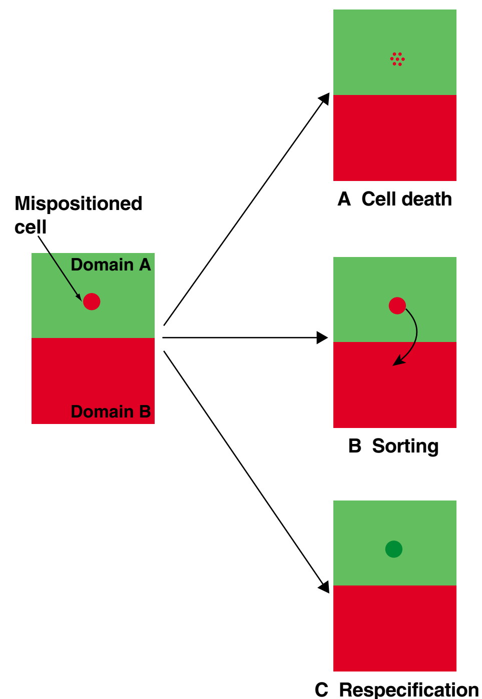
## Future perspectives

Much progress has been made towards identifying and understanding morphogen gradients. Emerging from these studies are a number of principles and shared strategies that we have attempted to outline in this review. Questions relating to the molecular mechanisms of morphogen activity still need to be addressed. One challenge is to understand how quantitative information is faithfully transferred through signalling pathways. The realisation of this goal depends on the development of reagents and techniques that will allow live in vivo assays to be performed at the single-cell level. In most cases, mechanistic inferences about how the quantitative differences in the activation of genes are interpreted have been drawn from a limited number of target genes, so it is unclear how general the conclusions are. However, with the advent of computational approaches to enhancer identification (Vavouri and Elgar, 2005) and of ChIP-chip technology (Taverner et al., 2004), it will be possible to address interpretation on a genome-wide scale. In this way, the relative contributions of different transcription factors throughout the duration of signalling can be crucially assessed and incorporated into network solutions for gradient interpretation (Stathopoulos and Levine, 2005). Moreover, the relationships between the regulatory motifs deployed in the regulation of different genes need to be compared and placed in the context of the entire genetic network controlled by a morphogen. How the specific gene expression programmes then specify different cell fates also needs to be determined, and progress has been made in this area with respect to gastrulation of *Drosophila* embryos in response to the early Dl gradient (Stathopoulos and Levine, 2004) and to the control of neuronal subtype identity in the vertebrate neural tube (Lee and Pfaff, 2001). Again, whole-genome expression profiling can potentially identify all in vivo morphogen targets from which a framework for cell fate specification can be generated (Stathopoulos and Levine, 2004). Details on the precision and refinement of gradient interpretation are still vague in most cases, yet this is an important issue for reliable embryonic development in the real-world conditions that are experienced by most embryos that develop outside of a cosseted laboratory environment. Coupled with this is the issue of how interpretation can remain accurate when gradients are scaled to accommodate the variability in tissue sizes during development. No doubt significant progress in these areas and the resolution of many other fascinating issues will be forthcoming.

## Note added in proof

A recent study (Rogulja and Irvine, 2005) demonstrates that the slope of a morphogen gradient can influence cell proliferation in a developing tissue. This supports models of morphogen action in

**Box 1. Error correction mechanisms refine tissue patterns established by morphogen signals**




A mispositioned cell (red circle) that expresses markers of Domain B (red) is situated in Domain A (green). Three main mechanisms (A-C) have been proposed to correct this type of error.

**(A) Selective elimination of mispositioned cells**
In *Drosophila* embryos with one or four copies of *bicoid* (*bcd*), an altered Bcd gradient affects the expression of downstream genes (Struhl et al., 1989), yet survival to adulthood is largely unaffected. This is due to increased apoptosis in the head of 4×*bcd* embryos and in the abdomen of 1×*bcd* embryos to normalise cell numbers (Namba et al., 1997). The molecular mechanism by which the presence of excess tissue is recognised and eliminated is not understood, although it may reflect a general aspect of development as similar processes appear to operate in other tissues (Adachi-Yamada and O'Connor, 2002; Moreno et al., 2002; Gibson and Perrimon, 2005; Shen and Dahmann, 2005).

**(B) Sorting of mispositioned cells towards the correct domain**
Differential cell adhesion has been implicated in refining and maintaining the patterns of gene expression (Dahmann and Basler, 1999). For example, Hh signalling in both the *Drosophila* wing disc (Rodriguez and Basler, 1997) and the vertebrate neural tube (Wijgerde et al., 2002) appears to control differential adhesive properties allowing the segregation of Hh-responding cells from non-responders. The identity of the molecules and mechanisms which mediate these processes remains to be determined.

**(C) Respecification of mispositioned cells so that they acquire the fate of their location: the 'community effect'**
Heterotopic transplantations in the vertebrate hindbrain, for example, indicate that, in contrast to coherent groups of cells, individual cells are unable to retain their original identity (Trainor and Krumlauf, 2000). Also during *Xenopus* muscle development, groups of 100 or more precursors must be in contact to promote muscle differentiation; in this case, fibroblast growth factor signalling appears to be the key signal (Standley et al., 2001).

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which the slope of the gradient, in addition to the concentration of the signal, influences cellular responses, and suggests a mechanism to coordinate tissue growth with tissue patterning.

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