Precise domain specification in the developing *Drosophila* embryo

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A simple morphogen gradient based on the protein *bicoid* is insufficient to explain the precise (i.e., similar in all embryos) setting of anteroposterior gene expression domains in the early *Drosophila* embryo. We present here an alternative model, based on quantitative data, which accounts for all of our observations. The model also explains the robustness of hunchback boundary setting in unnatural environments such as published recently [Luccheta *et al.*, Nature **434**, 1134 (2005)]. The model is based on the existence of a secondary gradient correlated to bicoid through protein degradation by the same agent.

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I. INTRODUCTION

The specification of gene expression domain in a developing embryo is the central problem of developmental biology. For *Drosophila Melanogaster*, the early events for the anteroposterior differentiation are well known: bicoid (bcd) mRNAs are deposited and localized by the mother at the anterior pole of the embryo. This localized source serves as a "fountain" for bcd proteins, which diffuse from there and are degraded throughout the embryo by some protease. The combined processes of diffusion from a source and degradation generate a stable, stationary (time independent) gradient of the bicoid protein with high concentration at the anterior and low concentration at the posterior pole. Downstream genes *read* the *bcd* concentration and set their boundaries according to different thresholds. The readout process can be direct, as in the case of hunchback (hb) or indirect, as for the gap genes which are activated by reading a combination of bicoid and hunchback (and each others'). This is a cascading gene interaction network where bcd and hb, at the top, play a very special role. The general scheme for this kind of genetic network is called (simple) "morphogen gradient model" [1] [Figs. 1(a)–1(c)].

This picture has emerged over the past 20 years following several fundamental discoveries: (i) bcd is a maternally active gene that encodes a transcription factor for many downstream genes expressed in the early embryo; (ii) bcd mRNAs are localized at the anterior pole of the embryo; (iii) bcd protein concentration profile forms a gradient across the embryo; (iv) modifying bcd dosage shifts downstream gene expression domains, in "agreement" with a threshold reading process [2,3] [Fig. 1(d)].

This simple morphogen gradient model suffers, however, from two important weaknesses: precision and scaling [4]. Precision here is to be understood as the degree of similarity between gene expression domains in different embryos. Scaling signifies the proportionality between gene expression domains and the embryo size.

A. Precision

The bcd concentration profile depends on various parameters such as the diffusion coefficient; bcd half-life, which itself depends on the degrading agent concentration, and the quantity of mRNA deposited by the mother at the pole. Any embryo-to-embryo variation in these parameters will modify the bcd profile and thus affect the spatial extension of domains in these embryos. The problem with the simple morphogen gradient model is the lack of feedbacks and error

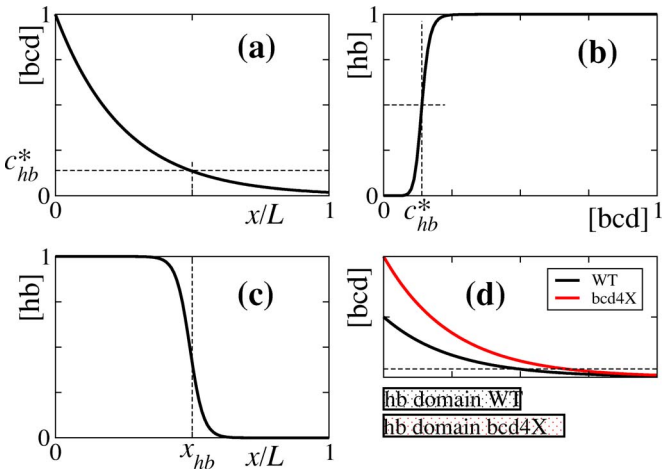

FIG. 1. (Color online) (a)–(c): Outline of the morphogen gradient model. (a) The concentration of a transcription factor protein (such as bcd) varies across the embryo (of size L); in this sketch, we suppose an exponential variation: $[\mathrm{bcd}]=\exp(-x/\lambda)$. (b) The transcription factor activates a downstream gene (such as hb) according to a Hill law $[\mathrm{hb}]=[\mathrm{bcd}]^n/\{(c^*_{hb})^n+[\mathrm{bcd}]^n\}$. The Hill coefficient n is supposed high enough for the activation to be switchlike: for bcd concentration below the threshold concentration c^*_{hb}, there is little hb production; hb production, on the other hand, is at its maximum when $[\mathrm{bcd}]\gtrsim c^*_{hb}$. (c) The hb gene is activated (and hb protein produced) only in the part of the embryo where $[\mathrm{bcd}]>c^*_{hb}$. Here, only cells whose position $x<x_{hb}$ transcribe the hb gene where x_{hb}, the boundary of hb domain, is given by $x_{hb}=-\lambda \ln c^*_{hb}$. (d) Dosage modification experiment: if the amplitude of the morphogen gradient is doubled (compared to the wild type), the position at which the gradient crosses a given threshold is pushed further upward. In this example, hb expression domain boundary (boxes) would be shifted by $\lambda \ln 2$.

correcting mechanisms. No usable machine, however, can be made without feedbacks.

B. Scaling

The shape of the bcd gradient is set by the above cited parameters, and none of them depends on the size of the embryo. This means that even if the mother were able to control precisely these parameters, the spatial extension of the hb domain will not be proportional to the *embryo size*.

As an example, if hb is activated at a bcd concentration threshold normally found at 230 microns, that concentration will occur at the same average distance from the anterior end of the egg regardless of its length. In a 450-μm long embryo, the hb domain will represent 51% of the embryo length (EL), whereas in a 500-μm embryo, it will occupy only 46% EL. This "error" is equivalent to five nuclei. Any mechanism used to create the scaling has to sense the posterior end of the egg, and this is clearly lacking in a simple morphogen gradient. Of course, in the *Drosophila* embryo, there are known posterior gradients, such as *nanos*, which participates in the boundary setting of hb, and *caudal*, but we know that these genes do not have any role in the scaling of hb [4].

In our previous investigation [4], we have shown that indeed, bcd is an extremely noisy signal and varies widely from embryo to embryo. The positional information bcd can transfer to hb, based solely on a threshold reading mechanism, has a standard deviation of 7% EL (approximately seven nuclei). If the human nose were positioned by such a morphogen, we would find it in some individuals on the torso and in some on top of the head. Moreover, there is no correlation between the positional information of bcd and the egg size. Hb, on the other hand, displays astonishing precision and scaling: at cycle 14, its boundary is set at 0.49 ± 0.01 EL, always proportional to the embryo size. In fact, hb plays the role of the "real" morphogen [5], filtering out all the errors of bcd and transmitting a pure signal to downstream genes. Boundaries of downstream genes show strong correlation to hb fluctuations, whether in wild type (WT) embryos or mutants where hb itself looses its precision [6]; on the other hand, no correlation with bcd fluctuation can be observed. We use the term *fluctuation* or *noise* through this paper as an equivalent of "embryo-to-embryo variability."

Clearly, there are correcting mechanisms present in the developing embryo which compensate for bcd errors at the very first stage of boundary setting. There are two possibilities for such a mechanism, either zygotic or maternal. Zygotic models are based on the subsequent interactions between hb and other gap genes such as Kr, kni, and gt. A look at the genetic network of anteroposterior early *Drosophila* differentiation shows that indeed many such feedbacks are thought to exist [7]. Some of them, upon closer inspection, are wrong (Kr) [6], but others do have a noticeable effect on the mean position of the hb boundary. No zygotic gene, however, has an effect on the *precision* and *scaling* properties of hb. Even removing 80% of the *Drosophila* genome had no visible effect on hunchback [4]. Another counter argument for zygotic feedback is the timing of the events: hb is among the very first activated genes, and from the very beginning, it displays high precision.

The other possibility is a maternal control: if the mother provides another signal to the embryo, and if this second signal has the same source of fluctuation as that of bcd (i.e., the two signals are correlated), then in principle the two noises can cancel out each other. We have previously shown that *nanos*, or more generally, genes downstream of oscar, do not play the role of this secondary signal. Some alleles of the maternal gene stauffen, however, disrupt the precision of the hb boundary, inducing fluctuation of the same magnitude as that of bcd. Sta itself is not a transcription factor, but plays a role during oogenesis in the localization of anterior *and* posterior mRNAs [8].

In the remainder of this paper, we will first investigate certain aspects of the bcd gradient. We will then explore the error correcting capabilities of a (hypothetical) secondary signal. We will show that such a model is in extremely good agreement with ours and others' observations.

II. THE BCD GRADIENT

Let us revisit the establishment of the bicoid gradient. As mentioned above, bcd proteins are produced at the anterior pole at a rate J, diffuse through the embryo with a diffusion coefficient D, and are degraded by some agent at a rate ω. The concentration $B(x,t)$ of bcd is given by the diffusion (Fick's) equation $\partial_t B = D\partial_x^2 B - \omega B$ with the boundary conditions $\partial_x B|_{x=0} = -J$ and $\partial_x B|_{x=L} = 0$. L is the embryo length. The second boundary condition expresses the fact that bcd molecules cannot cross the posterior extremity of the embryo. After a transitory time a stable, stationary ($\partial B/\partial t = 0$) state is reached which obeys

$$\frac{d^2B}{dx^2} - \frac{1}{\lambda^2}B = 0, \tag{1}$$

where $\lambda = \sqrt{D/\omega}$ is the diffusion length, i.e., the average distance a molecule diffuses before degradation. The stationary solution reads

$$B(x) = C_1 \exp(-x/\lambda) + C_2 \exp(+x/\lambda).$$

The amplitudes are $C_1 = J\lambda/[1-\exp(-2L/\lambda)]$ and $C_2 = \exp(-2L/\lambda)C_1$. For bcd gradient in *Drosophila*, the average diffusion length is $\lambda = 0.26L$. We can thus drop the positive exponential and approximate the gradient by

$$B(x) \approx (J\lambda)\exp(-x/\lambda).$$

The error in the approximation is 0.03% at the anterior and 2% at the posterior pole.

The measurement of the bcd gradient is most conveniently achieved by immunofluorescence staining techniques: in each embryo the local intensity of fluorescence $I(x)$ staining is extracted by image analysis techniques (see Appendix). Figure 2 displays such a measurement in one embryo.

In order to assess embryo to embryo variability, the most robust parameter to measure is the diffusion length λ, which is independent of the fluorescent staining noise and can be

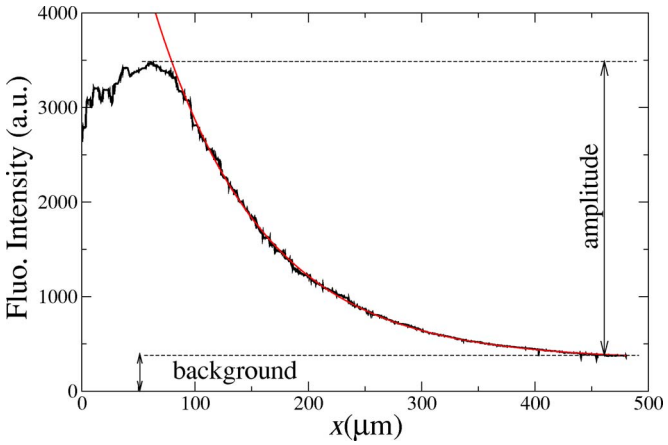

FIG. 2. (Color online) The profile of the bcd gradient in a bcd6X embryo measured by immunofluorescence. The fluorescence intensity data (black) is fitted to an exponential (red) $a\exp(-x/\lambda)+b$.

obtained by directly fitting the function $a\exp(-x/\lambda)+b$ to $I(x)$. As reported before, the embryo to embryo variability of the diffusion length is $\delta\lambda=0.05$ EL. It follows that if hb was activated directly by bcd, the embryo to embryo variability of its boundary would be $\delta x_{hb}=0.07$ (approximately seven nuclei).

There exists a possibility for this signal to be less noisy than it appears, at least for hb activation at midembryo: If in an embryo, the bcd-degrading agent has a higher concentration, then λ is smaller and a smaller *proportion* of molecules produced at the anterior pole reach the midembryo; if, however, in the same embryo, more bcd mRNA is deposited at the pole, the synthesis rate at the origin would be higher. The combination of these two effects can induce the same *number* of bcd molecules to reach the middle of the embryo. Individual absolute amplitudes (Fig. 2) cannot be measured directly from the images, because of the additional uncertainty induced by fluorescent staining, but if the above hypothesis were true, we should observe a negative *correlation* between the amplitude a of the fitted signal and its slope λ. No significant correlation has been found, however: on $N=91$ samples analyzed, the spearman correlation coefficient obtained was $r_{sp}=-0.09$ ($P=0.4$).

As we mentioned above, individual signal amplitudes cannot be measured for embryos. It is possible however to compare the *average* signal amplitudes in different backgrounds if embryos are stained at the same time in the same conditions. Then, the experimental error induced by staining is similar for all embryos and by averaging the amplitudes over enough embryos in the same background, a good estimation of the signal strength in one background *compared* to the other can be obtained (see Appendix).

Table I shows the bcd average amplitude in wild type embryos and in embryos derived from mothers with two or four more copies of bcd (see Appendix). There is *a priori* no reason for a transgene to have the same efficiency than the endogenous locus as observed here: each transgene addition increases the total transcription rate by approximately half of the endogenous expression.

Table II displays the bcd amplitude in embryos developing at different temperatures. Bcd amplitude decreases and its diffusion length increases as the temperature drops below 25 °C, implying a slowing down of both protein synthesis and degradation.

TABLE I. Average Relative (to WT) amplitude of the bcd gradient in embryos derived from mothers with variable numbers of bcd transgenes. Uncertainties are standard errors (standard deviation/$\sqrt{N}$). The relative amplitude is approximately $RA=2\times0.5+n\times0.25$, where 2 is the number of WT copies of bcd, n the number of transgenes, 0.5 the efficiency of a WT gene, and 0.25 the efficiency of a transgene.

Background	Relative Amplitude	N
bcd2X(WT)	1.0±0.1	16
bcd4X	1.6±0.1	17
bcd6X	1.9±0.1	21

III. SECOND MORPHOGEN HYPOTHESIS

In the simplest model of anteroposterior specification, hb is activated when the bcd signal is above a given threshold c_0. In this model, hb boundary would be as (embryo-to-embryo) variable as bcd. As we mentioned above, the bcd error can be corrected maternally if a second morphogen were present in the embryo and its fluctuations correlated to that of bcd. Then, the errors of these two signals can cancel each other. Let us again insist that even though nanos participates in the hb boundary setting (indirectly, by degrading maternal hb mRNA), it is *not* the second morphogen considered here: its removal (with or without the maternal hb) does not affect the precision of hb.

The bcd variability we measure is in fact the variability of its exponential decay length λ (which has a standard deviation of 0.05 EL). The decay length in turn depends on the diffusion coefficient D of bcd molecules and the degradation rate ω. The diffusion coefficient, a passive parameter which is related to viscosity should not vary from embryo to embryo. On the other hand, the degradation rate depends on the quantity of degrading agents the mother deposits in the embryo which can be highly variable and the main source of fluctuations in the bcd gradient. In order for the second morphogen to be correlated to bcd, it will be enough for it to be degraded by the same agent which degrades bcd. Then, in a given embryo, if ω is higher (lower) than average, both morphogens will have a smaller (bigger) diffusion length.

TABLE II. Average bcd gradient amplitude at various temperature, and its exponential decay length (%EL). Amplitudes are relative to the 25 °C condition.

Temperature	Relative Amplitude	λ	N
29 °C	0.80±0.03	0.28	32
25 °C	1.0±0.04	0.26	19
18 °C	0.84±0.03	0.37	24
9 °C	0.68±0.06	0.80	15

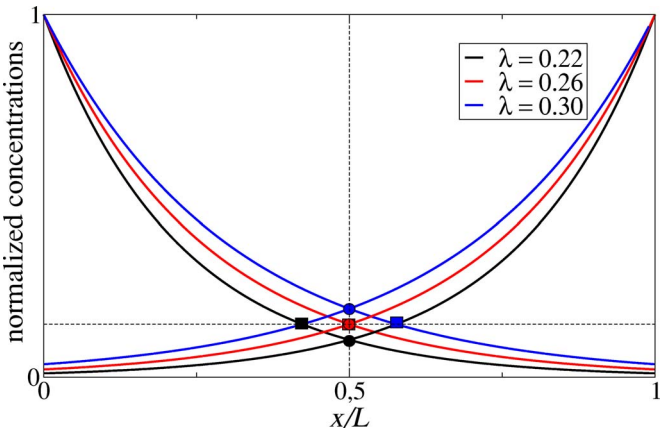

FIG. 3. (Color online) Setting of the hb boundary in a two gradient model (circles) and a simple gradient model (rectangles). Three couples of anterior $[B(x)]$ and posterior $[P(x)]$ gradient (normalized to [0, 1]) with diffusion length $\lambda=0.22$, 0.26, 0.30 are shown. In a two gradient model, the position of the hb boundary, given by the condition $B(x_{hb})=P(x_{hb})$, marked by circles, does not vary and remains at $L/2$. Compared to a simple gradient model where the hb boundary is specified by the anterior gradient crossing a given threshold, marked by rectangles; the hb position would follow variations of λ.

The other fact we mentioned is the scaling property of the hb boundary. Whatever the activation mechanism, it has to be influenced by the distance to both poles [9]. Bcd has a vanishing value at the posterior pole and cannot play such a role.

The simplest model of a second morphogen (which we will designate by the letter P), taking into account the above ideas, is the following: (i) P is produced from a localized source of mRNA at the *posterior* pole, diffuses, and is degraded with the same rates as bcd, so it makes an exponential gradient going from high values at the posterior to small values at the anterior; (ii) it is an *inhibitor* of hb. More precisely, hb is activated in the region where $B(x)>P(x)$, so the boundary position of hb is given by the following condition (Fig. 3):

$$B(x_{hb}) = P(x_{hb}). \tag{2}$$

This condition is easily realized, for example, if bcd and P compete for the same sites on the regulatory region of the hb gene. In principle, we should speak about the activities of these two proteins and not their actual concentration. P can have twice the activity of bcd and be present at half the concentration. Without loss of generality, however, and to keep the model as simple as possible, we will use concentrations instead of activities.

Repeating the arguments of the introduction, $P(x)$ obeys the diffusion equation $d^2P/dx^2-\lambda^{-2}P=0$ with the boundary conditions $dP/dx|_{x=0}=0$ and $dP/dx|_{x=L}=J$ (production at the posterior pole). Thus, $P(x)$ reads (Fig. 3)

$$P(x) \approx (J\lambda)\exp[(x-L)/\lambda], \tag{3}$$

where in *each embryo* the diffusion length for both morphogens are the same. This hypothesis is a consequence of P being degraded by the same agent as bcd. Let us now consider the plausibility of this model and its many prediction.

A. Correcting for errors and scaling

If hb was activated only by bcd through a thresholding mechanism $B(x_{hb})=c_0$, its embryo-to-embryo fluctuation would be $\delta x_{hb}=-\delta\lambda \ln c_0=0.07$ (in EL units). If, however, hb was activated by two gradients as explained above, the condition (2) reads

$$\exp(-x_{hb}/\lambda) = \exp[(x_{hb}-L)/\lambda],$$

and thus, the hb boundary is given by (see Fig. 3)

$$x_{hb} = L/2.$$

We have here two remarkable facts. First, the position of hb is set independently of λ, the source of fluctuations: The errors in the two gradients have canceled out each other. Second, the hb position is automatically proportional to the embryo size L. This simple model explains two of the elusive behaviors of the hb activation.

Let us also note that by this mechanism, when both posterior and anterior gradients have the same slope, error correction is optimal at midembryo, precisely where the hb boundary is actually set. In principle, the two gradient mechanism can set the boundary at any position if the condition for the gene activation were $uB(x_{hb})=P(x_{hb})$. Then, the position of the hb boundary would be

$$x_{hb} = \lambda(\ln u)/2 + L/2.$$

But this would not be proportional to the embryo size L. Even worse, fluctuations in λ would not be corrected any more and

$$\delta x_{hb} = \delta\lambda(\ln u)/2. \tag{4}$$

In the general case, anterior and posterior gradients can have different slope, and the position of a given gene's boundary will be given by $u\exp(-x/\lambda_1)=\exp[(x-L)/\lambda_2]$, i.e.,

$$x = \frac{\lambda_1}{\lambda_1+\lambda_2}(L+\lambda_2 \ln u).$$

Again, scaling can be achieved only if $u=1$. Moreover, if we suppose the embryo-to-embryo fluctuations of slopes $\delta\lambda_i$ to be due to the degrading agent concentration fluctuations δC and, thus, $\delta\lambda_i/\lambda_i=(1/2)\delta C/C$, then

$$\delta x = [(\lambda_1 \ln u)/(\lambda_1+\lambda_2)]\delta\lambda_2$$

and the error is corrected again only if $u=1$.

A last issue is error correction for amplitude variations. There exists *a priori* an embryo-to-embryo variation in the quantity of mRNA deposited by the mother which we have neglected in the above discussion. This source of fluctuation, which cannot be measured by fluorescent staining and is independent of variation in diffusion length, would add to errors in the hb boundary. This error also can be corrected by the two gradient mechanisms, if there is a correlation between mRNAs of localized posterior and anterior morpho-

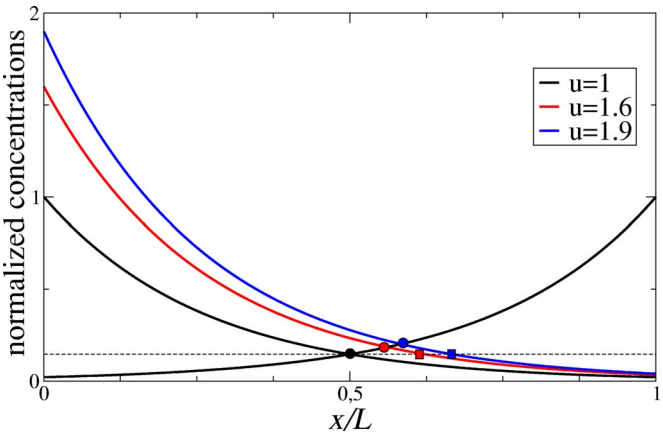

FIG. 4. (Color online) The hb boundary shifts when the strength u of the anterior gradient (but not of the posterior gradient) is varied. The shift expected from a two gradient model (circles) is half of a simple gradient model (rectangles). Concentrations (y axis) are relative to WT.

gens, i.e., the same quantity is deposited at both poles. It is probably no coincidence that the only gene which disrupts the precision of hb is one that is responsible for mRNA localization at *both* poles.

B. Effect of bcd gene copies

The most convincing argument for bicoid being indeed a morphogen was given by Driever and Nusslein-Wolhard in 1988 when they showed that providing more or less copies of the bcd gene to the embryo by the mother shifts the position of downstream genes toward anterior or posterior in the embryo [3]. This argument, however, does not hold quantitatively if bcd were the sole morphogen. By modifying the number of genes in the mother, the amplitude of bcd in the derived embryos becomes u times higher than in WT ($u=1$ for WT). Then the condition $B(x_{hb})=u\exp(-x/\lambda)=c_0$ of the simple morphogen gradient model implies that the expected shift (compared to WT) in the average hb position will be $\Delta x_{hb}=\lambda \ln u$. In a two gradient model, however, the expected shift in the hb boundary will be only half of this value: $\Delta x_{hb}=(\lambda/2)\ln u$ (Fig. 4). Table III shows the comparison between measured shifts in embryos with various background and values expected from simple and two gradient models. As it can be observed, only the two gradients model is in agreement with the measurement. Note that if we had assumed a normal activity for transgenes ($u=2$ for bcd4X and $u=3$ for bcd6X), the discrepancy of the simple morphogen model would be much higher, but the two gradient model predictions will still be acceptable.

C. Temperature compensation

As showed in Table II, the bcd amplitude and diffusion length are functions of temperature. There is no reason *a priori* for a simple morphogen gradient to be temperature compensated, i.e., specify the same boundary position for hb whatever the temperature. Without more knowledge of the detailed activation rates, however, this possibility cannot be ruled out, at least for the activation of a single gene: the hb activation threshold can vary in such a manner as to compensate the variation in the other two parameters. This, however, remains a fragile process with no feedback. A two gradient mechanism, on the other hand, corrects naturally for temperature variations: if, for example, bcd and P compete for the same regulatory region, they are affected in a similar way by temperature variation and the condition $B(x_{hb})=P(x_{hb})$ remains valid at all temperatures.

D. Precision in a nonuniform temperature gradient

Using a microfluidics device, Luccheta *et al.* have been able to keep one half of an embryo at one temperature (18 °C) and the other half at another temperature (25 °C) [10]. Even though development time is highly different in the two halves, the hb boundary is still set at midembryo with high precision. As we will show below, this is what a two gradient model predicts. The nature of this regulation is summarized in Fig. 5, where the posterior half is maintained at 18 °C and the anterior half at 25 °C. The source (mRNAs) for the posterior gradient being at 18 °C, synthesis of P is reduced at the posterior pole. The lower temperature in the posterior half of the embryo, however, induces also an increase in the diffusion length in this part. As a consequence, the *number* of P molecules reaching the midembryo are the same as the number of bcd molecules coming from the anterior pole.

More precisely, a variation in temperature affects the synthesis rate J and the diffusion length λ for which we possess quantitative data. The diffusion equation for bcd reads in this nonuniform temperature gradient as follows:

$$\frac{d^2B}{dx^2}-\frac{1}{\lambda^2(x)}B(x)=0 \tag{5}$$

with $\lambda(x)=\lambda_1$ if $x<L/2$ and $\lambda(x)=\lambda_2$ if $x>L/2$, where subscripts 1 and 2 refer to diffusion length in the anterior and

TABLE III. Measured shift in the hb boundary (relative to its position in WT) compared to predictions of simple and two gradient models. The quantity u denotes the strength of the bcd amplitude in different genetic background. Bcd amplitudes in mothers with transgenes have been measured (Table I).

Background	u	Measured	Simple gradient	Two gradient
bcd1X	0.5	−0.08±0.01	−0.18	−0.09
bcd4X	1.6	0.07±0.015	0.12	0.06
bcd6X	1.9	0.10±0.02	0.17	0.08

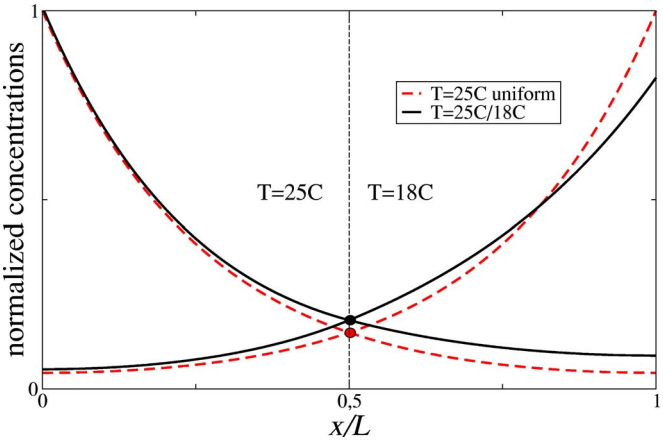

FIG. 5. (Color online) Anterior and posterior gradients for the setting of the hb boundary in a nonuniform temperature gradient. The anterior half of the embryo is maintained at 25 °C and its posterior half at 18 °C. Values for synthesis rates and diffusion lengths are set according to Table II. The position $B(x_{hb})=P(x_{hb})$ is marked by a circle. For comparison, the two gradients are also shown in a uniform temperature field. Concentrations (y axis) are relative to uniform temperature 25 °C.

posterior halves of the embryo, set by temperature in these parts. Two of the boundary conditions read as before $d^2B/dx^2|_{x=0}=-J_1$ and $d^2B/dx^2|_{x=L}=0$. J_1 is the synthesis rate of bcd at the anterior pole set by local temperature there. There are two additional conditions of continuity of concentration and flux at the boundary between high and low temperature: $B(L/2^-)=B(L/2^+)$ and $dB/dx|_{x=L/2^-}=dB/dx|_{x=L/2^+}$. The solution for the gradient is now two connected exponentials

$$B(x) = C_1 \exp(-x/\lambda_1) + D_1 \exp[(x-L)/\lambda_1], \quad x \leqslant L/2, \qquad (6)$$

$$= C_2 \exp(-x/\lambda_2) + D_2 \exp[(x-L)/\lambda_2], \quad x > L/2. \qquad (7)$$

The four amplitudes C_i, D_i are determined by the linear system of four equations given by boundary conditions. The same equation (5) holds for the gradient $P(x)$ except that two of the boundary conditions are reversed: $d^2P/dx^2|_{x=0}=0$ and $d^2P/dx^2|_{x=L}=J_2$. Note that J_2 is the synthesis rate at the posterior pole, set by local temperature there.

Figure 5 shows the solutions of the above equations when the anterior half of the embryo is held at 25 °C and its posterior half at 18 °C, similar to the experiment performed by Lucchetta *et al.* ([10], Fig. 4). All values for diffusion lengths and synthesis rates are measured experimentally (Table II). As it can be observed, the position of the hb boundary x_{hb} given by the condition $B(x_{hb})=P(x_{hb})$ is equal to its value for an embryo in a uniform temperature field. Moreover, precision is still conserved and variations of 5% in the diffusion lengths ($\delta\lambda=0.05$) induce only ten times smaller variations in the hb boundary ($\delta x_{hb}=0.005$).

IV. CONCLUSION

The simple morphogen model, where bcd, in a concentration dependent manner, specifies that gene expression domains lack feedback mechanisms and cannot quantitatively account for many phenomena. These phenomena include high precision and scaling properties of downstream genes; smaller than the expected shift when the amplitude of bcd is changed; temperature compensation, specially when embryos are maintained in the nonuniform temperature field.

We have shown in this paper that all these phenomena can be accounted for if we suppose the existence of a second posterior morphogen correlated to bcd. The correction mechanism in this "two gradient model" is based on a simple principle: if a signal is noisy, duplicate it by taking its mirror image and subtract the second from the first. Then, at one position inside the embryo (the midembryo being the optimum choice), the noises of the two gradients cancel each other completely. This is where the hb boundary is set and this precise signal can then be transmitted to downstream genes. It is remarkable how such a simple model can explain so many different observations, either obtained by us or recently by Luccheta *et al.*

The second gradient remains, however, a hypothesis and it would seem surprising that more than 20 years after the genomewide screen, there are still genes not uncovered. Until (and if) the second morphogen is found, the two gradient model is only a plausible framework, similar to the "simple gradient" model until 1987 and the discovery of bcd. It is, however, significant that the only mutation we have found which disrupts the precision of boundary setting is the *maternal* gene Stauffen, which is responsible for localizing mRNAs at both anterior and posterior poles. More work is needed at this level to understand the nature of molecular events caused by Stauffen mutation. We believe however that the exact mechanism cannot be very different from the general scheme we have presented here.

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APPENDIX: MATERIAL AND METHODS

1. Staining and image analysis

Embryos were collected at cycle 14 and immunostained following published protocols [11], except for the final rinsing time, which is an important step in reducing the nonspecific antibodies attachment. The best results were obtained by three days' rinsing (signal-to-noise ratio of $\approx$20 can be obtained). Most results were obtained by one day's rinsing ($S/N \approx 10$). When studying temperature effects, embryos were collected for 1 h at 25 °C and then allowed to reach cycle 14 at the set temperature (20 h at 9 °C). Antibodies were a gift of J. Reinitz and David Kossman [12]. High-resolution (1317×1015 pixels, 12 bits/pixel) images of stained embryos in a given condition were taken. Images were focused at midembryo to avoid geometric distortions. Intensity profiles were extracted by sliding a rectangle, the size of a nucleus, along the inner edge of the embryo, itself detected by intensity thresholding. The average was com-

puted on the brightest half pixels of the rectangle in order to compensate for the space between nuclei. The coordinates of the rectangle were projected on the main axes of the embryo, and the intensity $I(x)$ recorded separately for dorsal and ventral side.

2. Amplitude quantification

The bcd concentration $B_i(x)$ in the embryos i reaches a high value at x_i^*, which we call its *amplitude* A_i $[A_i=B_i(x^*)]$ (Fig. 2) and drops to vanishing level at the posterior pole $B_i(L)\approx 0$. In the fluorescent signal, we measure in this embryo, a part β_i is due to nonspecific antibody binding and another part α_i to specific ones. Therefore, the fluorescent intensity in this embryo reads

$$I_i(x) = \alpha_i B_i(x) + \beta_i,$$

where α, β, and $B(x)$ are random variables (varying from embryo to embryo) with given averages and standard deviations. If all embryos from various backgrounds are stained at the same time in the same conditions, then α and β have the same distribution for all embryos and depend only on staining conditions. $B(x)$, on the other hand, depends only on the genetic background of the embryos. Using the exponential variation of bcd in each embryo, the term β_i can be evaluated as $I_i(L)$. Therefore, the *measured* quantity we call "signal" $S_i=I_i(x^*)-I_i(L)=\alpha_i A_i$ depends only on two random variables. Averaging over all embryos in a given background, and assuming independence of α and A

$$\langle S\rangle_{\text{bckgrnd1}} = \langle\alpha\rangle\langle A\rangle_{\text{bckgrnd1}}. \tag{8}$$

To compare the relative amplitude of bcd in two different backgrounds, one has only to evaluate the ratio of the average signals in these two backgrounds

$$\frac{\langle A\rangle_{\text{bckgrnd1}}}{\langle A\rangle_{\text{bckgrnd2}}} = \frac{\langle S\rangle_{\text{bckgrnd1}}}{\langle S\rangle_{\text{bckgrnd2}}}.$$

In order to decide if the differences in amplitudes are significant, note that $\delta A/A=\delta S/S-\delta\alpha/\alpha<\delta S/S$. Therefore, if the differences in measured average signals $\langle S\rangle$ are significantly different (from a statistical point of view), then so are the estimated amplitudes $\langle A\rangle$. The best indicator for the significance of the random variable $\langle S\rangle_{\text{measured}}$ is its *standard error*, i.e., the standard deviation of the random variable S divided by the square root of the number of samples. The significance of differences can be further evaluated by a Student's t-test.

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