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Shape and function of the Bicoid morphogen gradient in dipteran species with different sized embryos

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Abstract

The Bicoid morphogen evolved approximately 150 MYA from a *Hox3* duplication and is only found in higher dipterans. A major difference between dipteran species, however, is the size of the embryo, which varies up to 5-fold. Although the expression of developmental factors scale with egg length, it remains unknown how this scaling is achieved. To test whether scaling is accounted for by the properties of Bicoid, we expressed eGFP fused to the coding region of *bicoid* from three dipteran species in transgenic *Drosophila* embryos using the *Drosophila bicoid* cis-regulatory and mRNA localization sequences. In such embryos, we find that *Lucilia sericata* and *Calliphora vicina* Bicoid produce gradients very similar to the endogenous *Drosophila* gradient and much shorter than what they would have produced in their own respective species. The common shape of the *Drosophila*, *Lucilia* and *Calliphora* Bicoid gradients appears to be a conserved feature of the Bicoid protein. Surprisingly, despite their similar distributions, we find that Bicoid from *Lucilia* and *Calliphora* do not rescue *Drosophila bicoid* mutants, suggesting that that Bicoid proteins have evolved species-specific functional amino acid differences. We also found that maternal expression and anteriorly localization of proteins other than Bcd does not necessarily give rise to a gradient; eGFP produced a uniform protein distribution. However, a shallow gradient was observed using eGFP-NLS, suggesting nuclear localization may be necessary but not sufficient for gradient formation.

Keywords: Scaling; Morphogen; Gradient; *Bicoid*; Evolution; Development; *Drosophila*; *Calliphora*; *Lucilia*

Introduction

The determination of body size is a fundamental problem in developmental biology (Calder, 1984; McMahon and Bonner, 1983; Peters, 1983). Size affects the developmental dynamics of all aspects of biological structure and function, and one approach to further our understanding of these dynamics is through the problem of scaling (Schmidt-Nielsen, 1983; West et al., 1997). In particular, how scaling of the spatial patterns in segmented, multicellular organisms of different sizes (but with similar genetic background) is achieved during embryonic development. In well studied systems such as *Drosophila melanogaster*, and in other insects with long germband embryos, the segments develop almost simultaneously from periodic patterns of gene expression, which are activated by earlier gradients of proteins such as Bicoid (Bcd) that span the developing embryo (Peel et al., 2005). Although, Bcd has emerged as a paradigm for morphogen gradients, how it scales during embryogenesis is not fully understood (Gregor et al., 2005; Lott et al., 2007; Gregor et al., 2007a).

Maternally transcribed *bcd* mRNA is localized to the anterior pole of the *Drosophila* embryo and upon translation the protein forms an anterior to posterior gradient in the

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syncytial blastoderm (Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988a; Frohnhöfer and Nüsslein-Volhard, 1986). Bcd encodes a transcription factor and it is thought to act as a classic morphogen through the concentration dependent activation and repression of target genes such as *hunchback* along the anterior–posterior (A–P) axis (Driever and Nüsslein-Volhard, 1988b; Driever and Nüsslein-Volhard, 1989; Struhl et al., 1989).

bcd is only found in higher dipterans and functional studies indicate that its role is conserved between distantly related members of this taxon, such as *Megaselia abdita*, *Drosophila* and the calyptratae species, *Musca domestica*, *Calliphora vicina* and *Lucilia sericata*, reflecting the overall similarity of their embryogenesis (Heming, 2003; McGregor, 2005; Shaw et al., 2001; Stauber et al., 1999; Stauber et al., 2002; Stauber et al., 2000). However, the regulatory interactions between Bcd and the enhancers of its target genes appear to have co-evolved in various dipterans (Hancock et al., 1999; McGregor et al., 2001; Shaw et al., 2002; Wratten et al., 2006) and so Bcd proteins from different flies may not be functionally equivalent (Schröder and Sander, 1993; Shaw et al., 2002).

A major difference between dipterans relevant to understanding the scaling of the Bcd gradient is the size of their embryos. The length of dipteran embryos has evolved rapidly within and between species and can vary by up to 5-fold, from 300 μM in *Drosophila busckii* to 1500 μM in *C. vicina*. Despite this difference in size, the Bcd gradient and the expression of segmentation genes scale with embryo length (EL) in dipteran embryos (Gregor et al., 2005; Lott et al., 2007; Sommer and Tautz, 1991). However, the mechanism through which this scaling is achieved is not known. We have recently shown that a simple model based on the gradient being formed by a balance of localized synthesis, diffusion, and spatially uniform degradation cannot explain the scaling properties of the gradients observed in different species, unless the protein lifetimes of each species correlate with embryo size (Gregor et al., 2005).

Here we investigated if the scaling of the Bcd gradient in species with different ELs is dependent on particular properties of the Bcd protein from these species. To do this we compared the gradients produced by Bcd from *Drosophila*, *Calliphora* and *Lucilia* as eGFP fusion proteins in transgenic *Drosophila* embryos. We found that *Calliphora* and *Lucilia* Bcd formed *Drosophila* Bcd shaped gradients in *Drosophila* embryos, which suggests the Bcd proteins from these species all have very similar properties with respect to gradient formation. However, Bcd from *Lucilia* and *Calliphora* was unable to rescue *Drosophila bcd* mutants supporting the hypothesis that interactions involving Bcd have co-evolved in these flies. We also tested whether eGFP or eGFP-NLS proteins could form a Bcd-like gradient when expressed maternally and localized to the anterior pole using the Bcd regulatory sequences. While eGFP protein was found almost uniformly throughout the embryo, eGFP-NLS was able to produce a shallow gradient different from that produced by Bcd protein itself. The eGFP-NLS profile suggests that nuclear localization may be necessary but is not sufficient for normal gradient formation.

Materials and methods

Constructs

To clone the complete coding regions of the *Calliphora* and *Lucilia bcd* genes, 5′ and 3′ RACE was performed using primers based on the previously known sequences and cDNA isolated from adult females (Schröder and Sander, 1993; Shaw et al., 2001). The coding regions of *Calliphora* and *Lucilia bcd*, in each case immediately followed by a stop codon, were amplified with primers containing *Sph*I sites and subsequently cloned in frame with eGFP in the modified version of pNBGA1 (Barolo et al., 2000; Gregor et al., 2007a; Hazelrigg et al., 1998), which had a section of *Drosophila bcd* removed by *Bmg*B1 and *Bgl*II digestion and re-ligation. The fragment containing the remaining *Drosophila bcd* genomic region (including all regulatory and localization sequences) with eGFP and either *Calliphora* or *Lucilia bcd* inserted was then subcloned into pCaSpeR4 (Thummel and Pirrota, 1992) using *Bam*HI and either *Kpn*I or *Xho*I respectively (Figure S1). Similarly, eGFP in pNBGA1 was replaced with eGFP followed by a stop codon or eGFP-NLS (amplified from pStinger; Barolo et al., 2000) followed by a stop codon. The *bcd* genomic region including these insertions was then subcloned into pCaSpeR4 using *Bam*HI and *Eco*RI (Figure S1).

A fragment of 2.5 kb from directly upstream of the *Calliphora hb* transcription start site, which contains all 9 characterized Bcd-binding sites and a 240 bp sub-fragment containing 8 of these sites were amplified by PCR from *Calliphora* genomic DNA (McGregor et al., 2001). Primer sequences are available on request. After sequencing of these fragments, enhancer fragment-*lacZ* fusion constructs were made by subcloning each fragment into pCaSpeR-*lacZ*.

Fly transformation

Standard P-element transformation was used to introduce the constructs into *yw* or w^{118} strains of *D. melanogaster* (Spradling and Rubin, 1982). At least three balanced independent lines were established for each construct.

Immunostaining and in situ hybridization

All embryos were collected at 25°C, heat fixed, and protein expression visualized using rat anti-Bcd and rabbit anti-Kr antibodies (gifts from J. Reinitz) (Kosman et al., 1998), and mouse anti-Paired/Pax (gift from N. Patel). Secondary antibodies used were conjugated with Alexa-488, Alexa-546 and Toto3 (Molecular Probes), respectively.

Expression of eGFP-Bcd fusion, eGFP-STOP, eGFP-NLS and *lacZ* reporter constructs was detected with in situ hybridization using anti-sense eGFP and *lacZ* DIG labeled probes respectively (Tautz and Pfeifle, 1989).

Fly stocks and genetics

For experiments with flies expressing *Drosophila bcd* fused to eGFP we used a stock with an insertion on the X chromosome. For substitution of endogenous *bcd* we conducted the mutant crosses of e*GFP-bcd* with bcd^{E1},p^{p}/TM3,Sb to generate e*gfp-bcd*;bcd^{E1},p^{p}.

To determine whether the eGFP-Bcd transgenes derived from *Calliphora* and *Lucilia* rescued the female sterility of the *Drosophila* null mutant bcd^{E1}, each transgene was introduced as a single copy into a homozygous bcd^{E1} background. Embryos derived from such females were scored for hatching and segmentation patterns of cuticles from unhatched embryos were analyzed after preparation in Hoyers-lactic acid. As a control for this experiment we tested three independently isolated lines containing *Drosophila* eGFP-Bcd, as well as five new lines derived by mobilizing the original X chromosome insertion. All but one of the *Drosophila* lines rescued the *bcd* sterility. To determine whether the foreign transgenes could drive expression of the *Calliphora hb-lacZ* reporter

constructs, females homozygous for the eGFP *Calliphora* or the eGFP *Lucilia* Bcd transgene, or heterozygous for the eGFP *Lucilia* Bcd transgene and the CyO, *hb-lacZ* balancer, were mated to males carrying the *Calliphora hb-lacZ* reporter construct and *lacZ* expression examined by in situ hybridization.

Microscopy

Stained embryos were imaged with standard epi-fluorescence microscopy, using Nikon 10× air (*Lucilia* and *Calliphora*) and 20× air (*Drosophila*) objectives. Embryos were mounted in AquaPolymount (Poly-sciences, Inc.). Embryos were placed under a cover slip and the image focal plane of the flattened embryo was chosen at the top surface and at the mid-sagittal plane (Figs. 1, 2, and S2 show a projection of both images).

Live imaging was performed on embryos from transgenic lines expressing eGFP using a custom-built two-photon microscope as previously described (Gregor et al., 2007a). Images were taken with a Zeiss 25× (NA 0.8) oil/water-immersion objective and an excitation wavelength of 900–920 nm. Average laser power at the specimen was 15–35 mW. Embryos were imaged at late cell cycle 14 (when nuclear eGFP-NLS concentration was highest); three high-resolution images (512×512 nm pixels, with 16 bits and at 6.4 μs per pixel) were taken along the A–P axis (focused at the mid-sagittal plane) at magnified zoom and then stitched together in software (MATLAB, MathWorks, Natick, MA); each image is an average of 6 sequentially acquired frames (Gregor et al., 2007b).

Image analysis

Nuclear eGFP profiles were extracted as we previously described (Gregor et al., 2007b). Nuclear centers were hand selected and the average nuclear fluorescence intensity was computed over a circular window of fixed size.

To analyze the profile of the eGFP-NLS construct, we solved numerically the discretized 1D diffusion equation on a grid with reflecting boundary conditions. Grid points were separated by a linear distance of 1/100 of the embryo length. The initial condition was uniform zero concentration throughout the embryo. A nonlinear fitting routine (Levinberg-Marquard within Matlab, MathWorks, Natick, MA) was used to adjust the diffusion coefficient to best match the nuclear fluorescence profile. For the fit, we chose a single free parameter, the diffusion constant, and we allowed a normalization parameter. Background fluorescence was chosen to be zero at the posterior pole for an adjacently imaged Bcd-GFP expressing embryo. The fit was forced to reproduce the posterior fluorescence level of the eGFP-NLS profile after a simulation time of 200 min (roughly the age of the embryos when imaged).

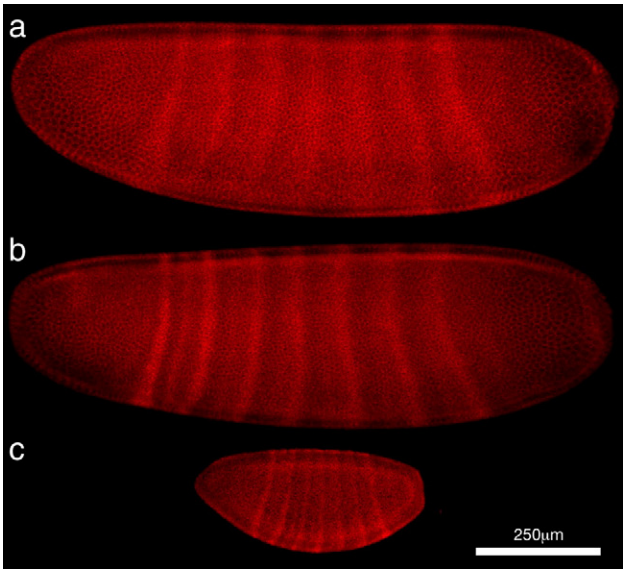

Fig. 1. Immunofluorescence stainings for products of the *paired* gene in *Calliphora* (a), *Lucilia* (b) and *Drosophila* (c).

Results

Calliphora, Lucilia and Drosophila embryos show similar developmental progressions and anterior–posterior patterns

Although, the embryos of *Calliphora* and *Lucilia* are up to three times longer and three times wider than those of *Drosophila*, in all three species the cellularizing blastoderm consists of approximately 6000 nuclei and early development is very similar. As previously shown for *Lucilia* and *Drosophila* (Gregor et al., 2005), the embryos of all three species exhibit the same overall proportionally scaled pattern, such that the size of expression domains and the distances between them are approximately three times higher in the larger embryos. However, the *Calliphora* and *Lucilia* embryos do show a slight anterior-ward shift from the position expected for perfect scaling. To quantify this shift we measured the location of Paired stripe 1, which occurs at 31±1% EL (mean±S.D.; $N=17$ embryos) in *Drosophila* and marks the future position of the cephalic furrow/head fold (Fig. 1). In *Lucilia*, this stripe is found at 25±1% EL (mean±S.D.; $N=9$) from the anterior pole and in *Calliphora* at 27±2% EL (mean±S.D.; $N=17$). Consistent with a small head region in the larger dipteran embryos, the anterior margin of the Krüppel (Kr) expression domain (which corresponds roughly to the beginning of the thorax) is shifted to ~42% EL in *Lucilia* from ~50% observed in *Drosophila* (Figure S2). Expressed in absolute distance, however, the shifts we have found represent relatively subtle deviations from the remarkable scaling of pattern between species whose ELs differ by 3-fold.

To follow the timing of nuclear replications and cytoplasmic movements in larger dipteran embryos we examined movies in which embryos from *Lucilia* and *Calliphora* had been injected anteriorly with fluorescently labeled dextran. These embryos had been previously used to extract diffusion constants in the different species (Gregor et al., 2005). Here the injected dextran serves to highlight cytoplasmic movements and nuclear divisions (Figure S3).

Despite the difference in size, both large and small embryos show remarkably similar periodic 9 to 13 min falls in dextran intensity, as a result of cytoplasmic turbulence during mitosis. This indicates that the duration of interphase and mitosis (the underlying cell cycle) is the same in large and small embryos. Mitosis pauses in all examined species after 13 rounds of division at cycle 14A, and in embryos fixed during cycle 14 and stained with DAPI there are about 100 rows of nuclei along the A–P axis of all species. Because the embryos are 3 times longer, the average internuclear distance in *Lucilia* and *Calliphora* (~24 μm) is three times longer than in *Drosophila*. The nuclear diameter in the larger species is also about three times larger, corresponding to a 27-fold increase in nuclear volume (Gregor, 2005). Note that genome sizes of *Calliphora* and *Lucilia* are approximately 5 times larger than the *Drosophila* genome (Ullerich and Schottke, 2006).

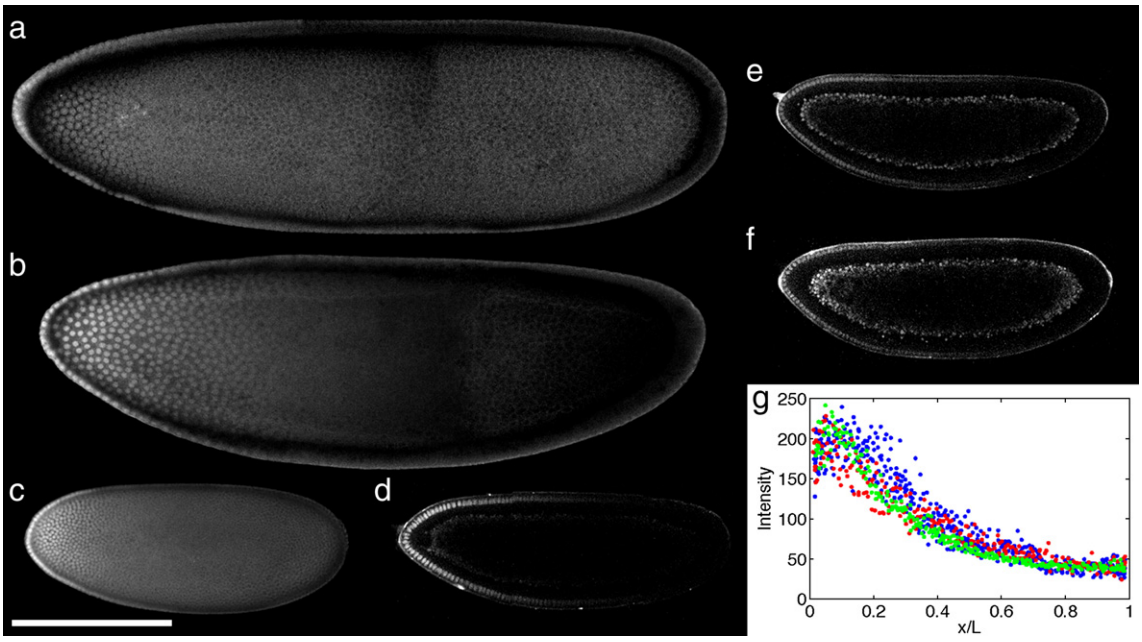

Fig. 2. Immunofluorescence stainings for products of the *bicoid* gene in *Calliphora* (a), *Lucilia* (b) and *Drosophila* (c). *Drosophila* embryos expressing a *Drosophila* eGFP-Bcd fusion (d), Cv-eGFP-Bcd (e) and Ls-eGFP-Bcd (f) fusion proteins respectively. All eGFP expressing embryos were imaged "in vivo" (scale bar: 250 μm). Graph (g) compares nuclear eGFP-Bcd fluorescence intensity profiles in *Drosophila* embryos expressing Cv-Bcd-GFP (blue, $n=7$), Ls-Bcd-GFP (red, $n=5$) and *Drosophila*-Bcd-GFP (green, $n=4$), respectively. Abscissa is relative to egg-length.

The distribution of Bcd is similar in Calliphora, Lucilia and Drosophila embryos

In all three species, syncytial nuclei are uniformly spaced along the A–P axis when the nuclei first migrate into the cortex. By early cycle 14, densities in the future head region of the embryo are lower — a broadened spacing known in *Drosophila* to correlate with anteriorly localized Bcd activity (Blankenship and Wieschaus, 2001). We previously showed that Bcd has a similar distribution in *Drosophila* and *Lucilia* embryos (Gregor et al., 2005). To characterize the distribution of Bcd protein in *Calliphora*, we stained early embryos from this species with various antibodies raised against *Drosophila* Bcd. The only reliably reproducible staining was obtained with a rat polyclonal serum made with the C-terminal region of the *Drosophila* Bcd protein (Kosman et al., 1998) and even with this antiserum the staining was significantly weaker in the larger flies than that observed in *Drosophila* (Fig. 2). The high background in *Calliphora* embryos made it difficult to determine accurately how far the gradient extended into the embryo. The minimal estimate for the posterior extent was 30% EL (the point at which the signal to noise ratio was such that staining intensity was clearly distinct from background intensity level, as estimated at the posterior end of the stained embryo), which when translated into absolute distance indicates that *Calliphora* Bcd, like *Lucilia* Bcd (Gregor et al., 2005), moves significantly farther in *Calliphora* eggs than the corresponding protein does in *Drosophila* (Fig. 2). Our results show that when distributions are expressed as percent EL, a comparable gradient of Bcd is seen in dipteran embryos that vary in size by 3-fold. This means that on average Bcd molecules have to travel 3 times further in embryos of some species compared to others.

Lucilia and Calliphora Bcd make Drosophila Bcd gradients in Drosophila embryos

One explanation for how Bcd travels further in larger embryos is that the Bcd proteins from such species have properties that make them more stable than Bcd proteins from species with smaller embryos. In order to test this hypothesis we first cloned the full-length *bcd* coding regions from *Lucilia* and *Calliphora*.

Previous studies showed that the *bcd* homeodomains of calyptrate species (*Musca*, *Lucilia* and *Calliphora*) have 4 amino acid differences from *Drosophila* and that *Musca* and *Calliphora* each have single additional differences (Schröder and Sander, 1993; Shaw et al., 2001). In addition, the PEST domain, a proline, glutamate, serine and threonine rich domain, thought to be involved in protein turnover (Rechsteiner and Rogers, 1996), has 69% sequence identity between the calyptrate species and *Drosophila*. The N-terminal self-inhibition is conserved between these species, but in the eIF4E/d4EHP binding domain, which is involved in blocking *caudal* translation, a key amino acid in *Drosophila* (L73) is not conserved (Cho et al., 2005; Niessing et al., 2002; Zhao et al., 2003) (Figure S4). The C-terminal regions are more diverged although there are blocks of conservation in the Acidic domain, a transcriptional activation domain (Figure S4) (Janody et al., 2001).

We then used the *Calliphora* and *Lucilia bcd* coding region sequences to make constructs that expressed Bcd from each

species as eGFP fusion proteins (Cv-eGFP-Bcd and Ls-eGFP-Bcd respectively) (Figure S1). To ensure that they would be properly expressed during oogenesis and localized to the anterior region of the *Drosophila* embryo we used the *Drosophila bcd* regulatory regions (Hazelrigg et al., 1998; Gregor et al., 2007a). We previously showed that a similar *Drosophila* construct (hereafter called *Drosophila* eGFP-Bcd) can fully rescue *bcd* mutants (Gregor et al., 2007a), and therefore this was used as a control.

If the broadened expression of Bcd in *Lucilia* and *Calliphora* reflects an autonomous feature of the Bcd protein in these species, we would expect the proteins to form a *Lucilia* or *Calliphora* scale of gradient in *Drosophila* eggs. This did not appear to be the case (Fig. 2). Instead, each made similar gradients to that of the endogenous nuclear *Drosophila* Bcd gradient, which suggests gradient formation in different sizes of embryos is not dependent on species-specific properties of Bcd proteins.

Bcd has evolved functional changes in the course of dipteran evolution

Previous cytoplasm transfer experiments suggested that Bcd from *Lucilia* and *Calliphora* was unable to rescue *Drosophila bcd* mutants (Schröder and Sander, 1993). This seems somewhat surprising given the conserved functional domains of the *Lucilia* and *Calliphora* Bcd proteins and the *Drosophila*-like profile of the gradients they form in *Drosophila* embryos. Therefore, we then tested the activity of the Ls-eGFP-Bcd and Cv-eGFP-Bcd in *Drosophila bcd* mutants, again using *Drosophila* eGFP-Bcd as a control.

Although the activity levels of the transgenic *Drosophila* eGFP-Bcd lines varied with insertion site, 7 of the 8 tested lines rescued the sterility of homozygous null (bcd^{E1}) females, yielding normal patterned larvae that developed to adult stages (not shown). In contrast, none of the lines of Ls-eGFP-Bcd (7) or Cv-eGFP-Bcd (4) tested rescued embryos to hatching larvae. Cuticle preparation showed some thoracic and head structures in all the *Lucilia* lines and in the strongest cases rescued embryos had up to two thoracic segments and tiny residual mouth hooks, structures never observed in embryos from *bcd*E1 mothers without the transgene (Figs. 3a,c). The four *Calliphora* lines showed little sign of rescue even at the cuticle level (Fig. 3b).

To determine if these differences in the ability of *Drosophila* eGFP-Bcd, Ls-eGFP-Bcd and Cv-eGFP-Bcd to rescue *Drosophila bcd* mutant embryos was due to differences in the expression levels of these constructs, we performed in situ hybridizations using an eGFP probe. We found that embryos from *Drosophila* eGFP-Bcd, Ls-eGFP-Bcd and Cv-eGFP-Bcd lines all gave strong anteriorly localized expression, although Ls-eGFP-Bcd gave slightly weaker expression than the other two constructs in the lines tested (Fig. 4). We conclude that the differences in rescue are due to functional differences in the Bcd proteins from each species rather than variation in expression levels. These transgenic results corroborate the results of the earlier cytoplasm transfer experiments between these species (Schröder and Sander, 1993).

The lack of activity of *Calliphora* Bcd in *Drosophila* embryos suggests that it contains amino acid changes that prevent it from regulating the expression of the normal *Drosophila* Bcd target genes. Therefore we asked whether the Cv-eGFP-Bcd could interact with the enhancers of a presumed

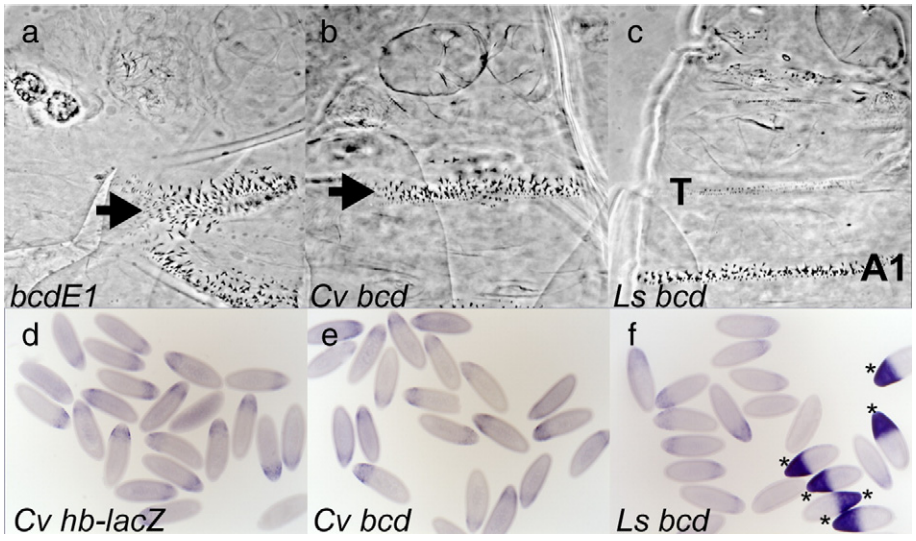

Fig. 3. *Calliphora* and *Lucilia* Bicoid proteins have significantly reduced activity in *Drosophila* eggs. (a–c) Anterior larval cuticle of embryos from mothers homozygous for the null allele, bcd^{E1}, with no transgenic Bcd (a), with a single copy of the Cv-eGFP-Bcd (b), with a single copy of the Ls-eGFP-Bicoid (c). Note the presence of a thoracic denticle belt (T) and abdominal segment 1 (A1) in c that is absent in panels a and b. Abdominal segments of unknown identity are indicated by arrows in panels a and c. *Drosophila* eGFP-Bicoid fully rescues bcd^{E1} (not shown). Ventral views of the larvae are shown with anterior at the top. (d–f) Blastoderm embryos obtained by crossing males carrying a *lacZ* reporter containing the 2.5 kb putative Bicoid responsive region of the *Calliphora hb* gene (*Cv hb-lacZ*) to wild type females (d), females carrying the Cv-eGFP-Bcd transgene (e), or the Ls-eGFP-Bcd transgene (f). For comparison, panel f also includes six embryos marked with asterisks that contain a *lacZ* reporter construct with the Bcd responsive regions from the *Drosophila hb* gene. In *Drosophila* embryos, the *Calliphora* reporter is expressed at low levels compared to the *Drosophila* reporter and is not activated further by addition of Bcd from either *Calliphora* or *Lucilia*.

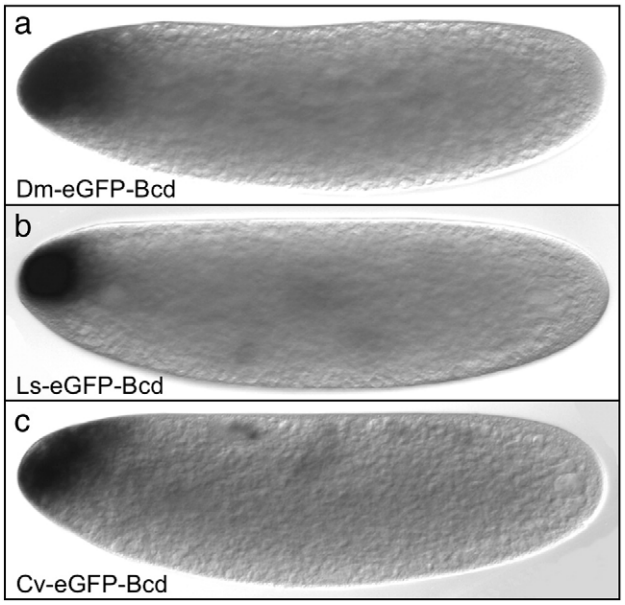

Fig. 4. Expression of eGFP-Bcd construct transcripts in transgenic *Drosophila* embryos. *Drosophila* eGFP-Bcd (a). *Lucilia*, Ls-eGFP-Bcd (b). *Calliphora*, Cv-eGFP-Bcd (c). All embryos are at the syncytial blastoderm stage and are shown dorsal up and anterior to the left.

Calliphora target gene in *Drosophila* embryos, using two reporter constructs of the *Calliphora hunchback* (*hb*) enhancer fused to *lacZ*. One construct, from the *Calliphora hb* transcription start site to 2.5 kb upstream containing all 9 characterized Bcd-binding sites, and a second 240 bp construct containing only the cluster of 8 Bcd-binding sites (McGregor et al., 2001). When introduced into *Drosophila* embryos that contain only *Drosophila* Bcd, both transgenes were only weakly expressed, indicating that the *Calliphora hb* enhancer is not completely functional in *Drosophila* embryos or fully activated by the endogenous *Drosophila* Bcd protein (Fig. 3d). We then tested whether the expression could be activated in *Drosophila* embryos containing Ls-eGFP-Bcd or Cv-eGFP-Bcd. In neither case did either construct appear to alter levels of *lacZ* expression or affect the position of the expression domains (Figs. 3e,f).

Not all anteriorly localized mRNAs form gradients similar to Bcd

It is possible that any localized RNA would produce a stable protein gradient identical to the ones observed above when translated and allowed to diffuse from the anterior end of the embryo. To test this possibility, we introduced a stop codon between the last codon of the N-terminal eGFP and the ATG of *Drosophila bcd* in the construct we had previously used to make a fusion protein identical to endogenous Bcd (Gregor et al., 2007a). Hence the anteriorly localized mRNA from this new construct would only give rise to eGFP (Figure S1). Three transgenic lines expressing eGFP-STOP were obtained and all lines showed fluorescence at very low, apparently uniform, levels along the entire A–P axis of the embryo (Figure S5). This was not the result of mis-localization as the transcripts were anteriorly localized in all three lines (Figure S6). It is, however, possible that the shallowness results from the weakness of the protein expression level, which is barely above the background fluorescence of these transgenic lines. In that respect our result remains inconclusive.

To test whether nuclear localization might allow eGFP to form a stable gradient similar to Bcd we made a second construct in which a nuclear localization signal (NLS) coding sequence was inserted in frame at the end of the eGFP sequence. A stop codon was included between eGFP-NLS and the first codon of *bcd* (Figure S1). The attachment of the NLS to eGFP appeared to increase the levels of the protein and its nuclear localization made it easier to detect. Again the eGFP-NLS transcripts were anteriorly localized in all lines tested (Figure S6). In contrast to the uniform levels of protein observed with eGFP alone, embryos expressing eGFP-NLS did produce a shallow gradient extending the entire length of the embryo (Fig. 5).

However, unlike eGFP-Bcd, the level of eGFP-NLS continued to rise during late cleavage cycles and persisted until gastrulation. This apparently greater stability allowed significant accumulation of the eGFP-NLS protein at the posterior pole (Fig. 5). Although this distribution differs from the Bcd gradients in normal developing embryos, both the stability and accumulation of the eGFP-NLS protein and its shallower distribution are observed when *Drosophila* eGFP-Bcd is expressed in unfertilized eggs that lack nuclei (Gregor et al., 2007a). Moreover, the shape of the observed eGFP-NLS gradient fits very closely the shape of a gradient obtained from a numerical solution of the diffusion equation (see Materials and methods and Gregor et al., 2005) with a diffusion constant of 6 ± 1 $\mu m^2/s$. In this simulation we omitted protein degradation, which leads both to a more shallow final gradient shape and to an overall rise of protein concentration within the embryo. Note that a diffusion

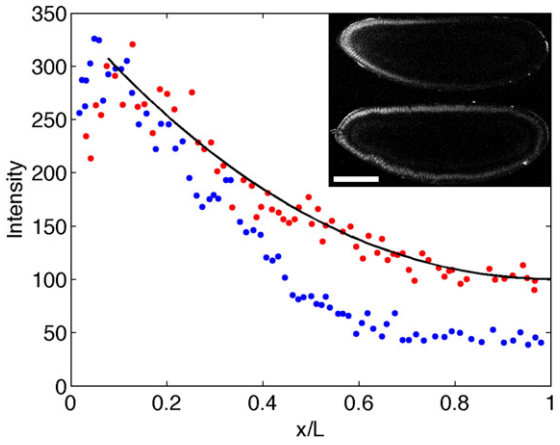

Fig. 5. Nuclear eGFP fluorescence intensity profiles in late cell cycle 14 *Drosophila* embryos expressing the *Drosophila* eGFP-Bcd-fusion protein (blue) and an eGFP-NLS fusion protein (red) (see text and Figure S1). Black line represents a fit of a numerical solution of the diffusion equation (no degradation) with a source at the anterior pole. Sole fitting parameter is the diffusion constant $D=6\pm1$ $\mu m^2/s$. Abscissa is relative to egg-length. Inset shows corresponding *Drosophila* embryos expressing the *Drosophila* eGFP-Bcd fusion protein (upper) and an eGFP-NLS fusion protein (lower). (Scale bar: 100 μm.)

constant of 6 ± 1 $\mu m^2/s$ would be large enough to establish a Bicoid gradient within the available developmental time by a passive diffusion-based mechanism (Gregor et al., 2007a).

These combined experiments argue that while nuclear localization per se is not completely sufficient for any anteriorly localized RNA to form a Bcd-like gradient, the presence of nuclei is required.

Discussion

Embryo length and scaling

Despite differences of up to 5-fold in embryo length, the expression patterns of segmentation genes appear to scale with embryo length in higher dipteran embryos (this work; Gregor et al., 2005; Lott et al., 2007; Sommer and Tautz, 1991). However we did observe a slight anterior-ward shift in expression patterns in the larger embryos of *Calliphora* and *Lucilia* compared with *Drosophila*. Indeed there is evidence that scaling need not be perfect and that other mechanisms can compensate. While the perturbation of normal scaling in *Drosophila* embryos from females containing up to 6 copies of Bcd resulted in a posterior shift in patterning and an expanded head region, these embryos developed into apparently normal larvae and flies (Berleth et al., 1988; Frohnhöfer and Nüsslein-Volhard, 1986). It has been shown that this is due to a fate map repair system involving increased cell death in expanded regions (Namba et al., 1997). This suggests that some variation in the scaling of embryonic pattern can be permitted and this may explain how differences in scaling with respect to embryo length evolved in sibling species of the *D. melanogaster* complex (Lott et al., 2007).

Species with large embryos do not have more stable Bcd proteins

It has been shown that the scaling of patterning with embryo length is controlled by maternal factors, which must have evolved in concert with embryo length in dipteran embryos (Gregor et al., 2005; Lott et al., 2007). Therefore we tested if Bcd from large embryos could travel further along the A–P axis than Bcd from smaller embryos. However, we found that the *Calliphora* and *Lucilia* Bcd proteins actually adopted the profile of the *Drosophila* Bcd gradient in *Drosophila* embryos. Although we cannot rule out the possibility that the protein degradation machinery is less potent in *Calliphora* and *Lucilia*, our results indicate that some feature of the embryo in which the protein finds itself controls its distribution and not species-specific Bcd stability. This also suggests that the coordinated evolution of embryo length and scaled patterning is independent of the Bcd protein at least between distantly related dipterans.

Co-evolution and incompatibility in Bcd regulatory interactions

We found that while *Drosophila* eGFP-Bcd was able to fully rescue *Drosophila bcd* mutant embryos, Ls-eGFP-Bcd and Cv-eGFP-Bcd gave little or no rescue of *bcd* mutants or shifts in morphological markers in normal embryos, supporting the results of cytoplasm transfer experiments by Schröder and Sander (1993). These results are somewhat surprising given the gradients made by Ls-eGFP-Bcd and Cv-eGFP-Bcd in *Drosophila* embryos and the conservation of functional protein domains. Indeed, it has been shown that even a *Drosophila bcd* transgene expressing only the first 246 amino acids can rescue *bcd* mutants (Schaeffer et al., 1999). However, previous in vivo and in vitro experiments of binding affinity and transcriptional output indicated that the protein–DNA interactions between Bcd and Bcd-binding sites in the enhancers of target genes have co-evolved in dipterans (McGregor et al., 2001; Shaw et al., 2002).

We also found that *Calliphora* Bcd protein failed to activate the *Calliphora hb* target in *Drosophila* embryos (although it is possible that our reporter constructs may not contain the complete *Calliphora hb* enhancer and/or activation of this enhancer requires other *Calliphora* transcription factors such as Hb (Simpson-Brose et al., 1994)). This suggests that the protein–protein interactions between Bcd and transcription co-factors have also co-evolved as has been described between other systems (Ruvinsky and Ruvkun, 2003). Therefore, while foreign Bcd proteins can produce distributions characteristic of *Drosophila* Bcd when introduced into *Drosophila* embryos, productive interactions may no longer be possible in the *Drosophila* cytoplasm.

Nuclei are required but not sufficient for gradient formation

Experiments by Crauk and Dostatni (2005) indirectly demonstrated that the synthetic transcription factor GAL4-3GCN4 was able to make an anterior–posterior gradient when anteriorly localized in *Drosophila* embryos. While this protein is able to localize to the nucleus and activate transcription, it has no obvious similarities to Bcd at the amino acid level. This suggests that while transcriptional activity per se is not required for gradient formation, as demonstrated by our Cv-eGFP-Bcd construct, transcriptional activation domains in combination with nuclear localization (see below) are required. Bcd has at least 4 transcriptional activation domains, and while it has been shown that there is some redundancy in their function (Fig. 3; Schaeffer et al., 1999) each of these domains could be involved in gradient formation.

Here we also tested whether any anteriorly localized source generates a gradient and the effect of nuclear localization on gradients. We found a remarkable absence of any anterior to posterior graded protein distribution in embryos that express a localized source of eGFP at the anterior pole. However, if these anteriorly emanated eGFP molecules are tagged with a NLS we do see a gradient resembling the Bcd gradient seen in unfertilized eggs (Gregor et al., 2007a): it peaks at the anterior pole, falls off more shallowly than a Bcd gradient in fertilized eggs, and it rises along the entire A–P axis over time. This result suggests that nuclear localization sequences are needed for gradient

formation, but are not sufficient to recover the shape of the wild type Bcd gradient. Some other Bcd-specific property, such as degradation rate, perhaps mediated by its transcriptional activation and/or PEST domains, seems to be relevant to distinguish its shape from gradients of both unfertilized eggs and eGFP-NLS expressing embryos.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.01.039.

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