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Integration of the head and trunk segmentation systems controls cephalic furrow formation in *Drosophila*

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SUMMARY

Genetic and molecular analyses of patterning of the *Drosophila* embryo have shown that the process of segmentation of the head is fundamentally different from the process of segmentation of the trunk. The cephalic furrow (CF), one of the first morphological manifestations of the patterning process, forms at the juxtaposition of these two patterning systems. We report here that the initial step in CF formation is a change in shape and apical positioning of a single row of cells. The anteroposterior position of these initiator cells may be defined by the overlapping expression of the head gap gene *buttonhead* (*btd*) and the primary pair-rule gene *even-skipped* (*eve*). Re-examination of the *btd* and *eve* phenotypes in live embryos indicated that both genes are required for CF formation. Further, Eve expression in initiator cells was found to be dependent upon *btd* activity. The control of *eve* expression by *btd* in these cells is the first indication of a new level of integrated regulation that interfaces the head and trunk segmentation systems. In conjunction with previous data on the *btd* and *eve* embryonic phenotypes, our results suggest that interaction between these two genes both controls initiation of a specific morphogenetic movement that separates two morphogenetic fields and contributes to patterning the hinge region that demarcates the procephalon from the segmented germ band.

Key words: cephalic furrow, *buttonhead*, *even-skipped*, segmentation, *Drosophila*, head, trunk

INTRODUCTION

During *Drosophila* gastrulation, the embryo is transformed from a regular array of morphologically identical cells, organized in a single layer, into a complex array of cell groups. These groups change cell shape and move together in a characteristic, predetermined manner (review by Costa et al., 1993). Two invaginations, the ventral and cephalic furrows, represent the first morphological manifestations of cell fate and differentiation programs. The ventral furrow (VF) invaginates along most of the ventral midline, bringing the mesoderm and possibly part of the anterior endoderm primordium into the interior of the embryo. Cephalic furrow (CF) invagination takes place laterally on both sides of the embryo, near its anterior end. Unlike VF formation, the CF is only transient. At the completion of germ-band extension, all cells of the CF slowly unfold back onto the surface of the embryo to contribute to the ectoderm (description in Campos-Ortega and Hartenstein, 1985; Foe, 1989; Costa et al., 1993).

Although the CF is a prominent morphological feature of the early gastrula, its developmental role remains enigmatic. No mutations have been isolated that affect only the CF and the mutations known to affect the VF and posterior midgut (PMG) invaginations leave the CF unaffected (Brönner et al., 1994; Costa et al., 1994; Reuter and Leptin, 1994). The cellular and genetic mechanisms that control its formation are also unknown. The absence of a CF in embryos derived from mothers mutant for *bicoid* (Frohnhöfer and Nüsslein-Volhard, 1986) and the reproducible shifts in its position and/or lateral extent observed in anteroposterior and dorsoventral pattern mutants (Zusman and Wieschaus, 1985; Driever and Nüsslein-Volhard, 1988; Struhl et al., 1989) indicates that the cell shape changes respond directly to positional information. However, these observations did not provide a specific clue to how that positional information is translated into specific changes in cellular morphology.

The CF arises in an interesting position in the embryo, at the juxtaposition of the patterning systems that define the head and trunk segments. These two systems involve separate groups of zygotically active genes and separate regulatory interactions (Ingham, 1988; Cohen and Jürgens, 1990; Jürgens and Hartenstein, 1993). Formation of the CF coincides with the anteriormost stripe of expression of the pair-rule gene *even-skipped* (*eve*), *eve* stripe 1; *eve* encodes a homeodomain protein that is essential for segmentation of the *Drosophila* embryo (Harding et al., 1986; McDonald et al., 1986). Just prior to cellularisation, the Eve protein is distributed in a series of 7 pair-rule stripes which foreshadow the position of odd-numbered parasegments PS 1 to 13 (Frasch and Levine 1987; Lawrence et al., 1987). The function of Eve stripe 1 in this process is unclear. Whereas Eve expression in stripes 2-7 has an instructive role in specifying expression patterns of the segment-polarity genes *engrailed* (*en*) and *wingless* (*wg*), *en* expression in PS1 occurs normally in *eve* mutant embryos (McDonald et

al., 1986; Ingham et al., 1988). An additional aspect that distinguishes Eve stripe 1 from other Eve stripes is the failure to identify *cis*-regulatory elements specific for that stripe, despite extensive studies on the mechanisms of regulation of *eve* expression (Goto et al., 1989; Harding et al., 1989).

In the experiments described below, we show that the CF is eliminated or abnormal in *eve* mutant embryos (see also Costa et al., 1993), suggesting a specific role of Eve stripe 1 in controlling this morphogenetic event. We further show that CF formation also depends upon activity of the head gap-like segmentation gene *buttonhead* (*btd*), which is required for formation of the antennal, intercalary and mandibular segments and part of the maxillary segment (Cohen and Jürgens, 1990; Wimmer et al., 1993, 1996). The transverse row of cells, which change shape and slip into the interior of the embryo during the first phase of CF invagination, express both *btd* and *eve* at the onset of gastrulation and *eve* expression in these cells is dependent upon *btd*. Our results suggest that the interaction between *btd* and *eve* integrates two systems of segmentation in controlling formation of the CF and patterning the border region that demarcates the head and the trunk.

MATERIALS AND METHODS

Fly stocks and observation of live embryos

The btd^{XG}, *svb* and the *hb>btd* transgenic lines were provided by Ernst Wimmer, the Rockefeller University, New York and described in Wimmer et al. (1997). The btd^{1}, btd^{XG} btd^{XA}, eve^{R13} and eve^{IIR59} mutant strains were obtained from the Tübingen Stock Center and the stocks carrying chromosomal translocations from the Bowling Green Stock Center (Bowling Green, OH). Blastoderm-stage embryos defective for CF formation were selected by observation of living embryos under Voltalef 3S halocarbon oil, using a stereomicroscope. The segregation of the translocation chromosomes in males was tested by mating them to *C(1)DXywf*; 1E females. The segregants can be easily identified by the presence or absence of the folded gastrulation phenotype (for reference, see Wieschaus and Sweeton, 1988; Müller and Wieschaus, 1996). Time-lapse recording of optical sections of individual embryos at higher magnification (×200 or ×400) was used to confirm the absence of CF formation in eve^{R13}, $btd^{XG}$and btd^{XA} mutant embryos.

In situ hybridisation and antibody staining

Whole-mount in situ hybridisation to embryos was performed with digoxigenin-labeled RNA prepared with the Genius kit from Boehringer Mannheim, using the procedure from Tautz and Pfeifle, 1989, except for the absence of proteinase K treatment. For Arm and Eve immunostaining, embryos were fixed using the heat-methanol and paraformaldehyde procedures, respectively (see Müller and Wieschaus, 1996). Staining of F-actin and Eve was performed on cellular blastoderm and early gastrula embryos devitellinized by hand-peeling according to Wieschaus and Sweeton (1988). The purified rabbit anti-Eve antibody (1/2000) was from Manfred Frasch (Mount Sinaï University, New York). The monoclonal anti-Sxl antibody was provided by Paul Schedl (Princeton University) and used 1/50. Goat anti-rabbit and anti-mouse biotin secondary antibodies (Vector Laboratories) were used 1/500. For immunofluorescence and rhodamine-phalloidin staining, embryos were mounted in Mowiol containing DABCO (1,4-diazabicyclo[2.2.2]octane) as an anti-bleaching agent and observed under an MRC 600 confocal microscope (BioRad Labs). Images were merged using Confocal Assistant (CAS™) and processed with Adobe Photoshop TM software.

nlsGFP recording in wild-type and *eve* mutant embryos

Transgenic lines expressing a nuclear Green Fluorescent fusion Protein (nlsGFP) were provided by Ilan Davis and P. O'Farrell (UCSF, San Francisco). The nlsGFP 34H; 34M strain (Davis et al., 1995) was used to establish the eve^{R13}; nlsGFP34M and btd^{XG} nlsGFP 34H; 34M strains. nlsGFP expression was observed on living embryos selected at the blastoderm stage and mounted in halocarbon oil on a permeable membrane with a glass coverslip. Images were collected every 3 minutes with the confocal microscope. Each image was obtained by merging four independent scans corresponding to sections separated by 2 µm (6 µm total depth).

RESULTS

The position of the cephalic furrow coincides with the second row of Even-skipped-expressing cell in stripe 1

Previous analyses of time-lapse cinematography and sectioned material suggested a sequence of cell shape changes and nuclear migrations underlying CF formation (Turner and Mahowald, 1977 and E. Wieschaus, unpublished data). First, cells in a single transverse row, designated henceforth as initiator cells, shift their nuclei basally and slip into the interior, forming a barely visible cleft in the embryo's surface. This movement seems to be associated with cell shortening along the apical-basal axis, as illustrated on confocal images of fixed embryos stained with fluorescent phalloidin to highlight cell shape (Fig. 1A). Subsequently, the apices of the cells immediately anterior and posterior to this initial cleft roll over its edge and follow the first row of cells into the interior (Fig. 1B,C). While a thorough description of CF formation will be reported elsewhere, we have focused our attention here on the antero posterior position of the CF relative to the segmentation process by double staining embryos with phalloidin and antibodies against the Eve protein. At the end of cellularisation when gastrulation is initiated, each Eve stripe is about three cells wide with a sharply defined anterior border. The cells that first undergo the change in shape that initialises CF formation were consistently found to be the second row of cells in stripe 1. During early stages of Eve expression, these cells express the highest levels of Eve in stripe 1. The position of the CF relative to Eve expression was maintained when the CF was shifted anteriorwards by gradually decreasing the amount of Bicoid gene product, using mutations either in *bicoid* (*bcd*) (Driever et al., 1988; Struhl et al., 1989) or *serendipity delta*, a *bcd* transcriptional activator (Payre et al., 1994; data not shown). In no instance did we observe formation of a cephalic fold in embryos when Eve stripe 1 was missing due to altered maternal genotypes.

Absence of CF in either *even-skipped* or *buttonhead* mutant embryos

The expression of Eve relative to the position of CF invagination raised the possibility that *eve* is specifically required for this morphogenetic event. CF defects were indeed recently noticed in *eve* mutant embryos (Costa et al., 1993). We therefore re-investigated the *eve* gastrulation phenotype in more detail by examining live embryos during gastrulation. Roughly one quarter of embryos from an eve^{R13} mutant stock (an amorphic *eve* allele, Nüsslein-Volhard et al., 1984) showed

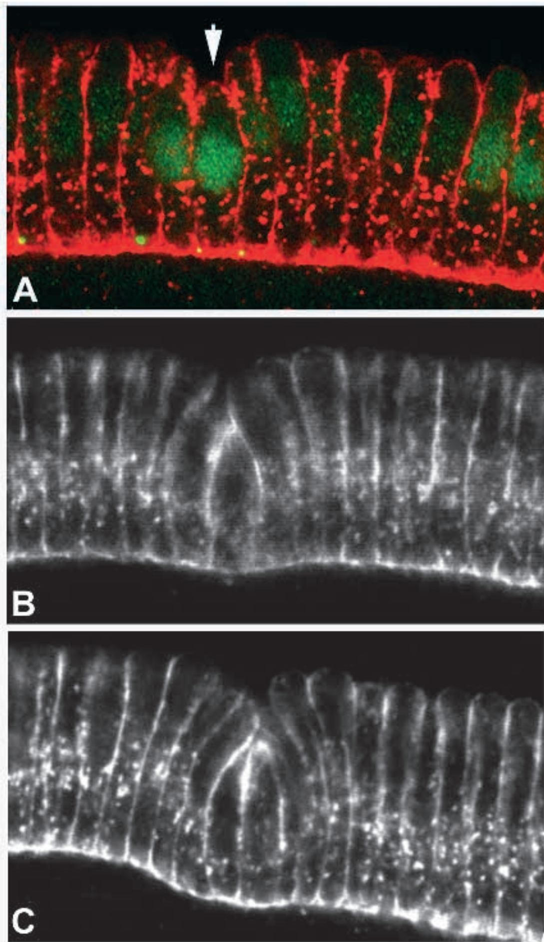

Fig. 1. Sequence of cell shape changes initiating CF formation. Sagittal views of wild-type embryos at the early gastrula stage, stained with rhodamine phalloidin to highlight cell morphology. Anterior is to the left. (A) First, a single cell, designated as initiator cell (arrow), undergoes an apical-basal constriction. (B,C) Cells immediately anterior and posterior then follow the initiator cell into the interior of the embryo. The initiator cell is the second Eve-expressing cell in stripe 1, as visualised in A by Eve antibody staining. Expression of Eve in the posterior-most cell of stripe 1 (behind, slightly out of focus) is already decreasing at this stage.

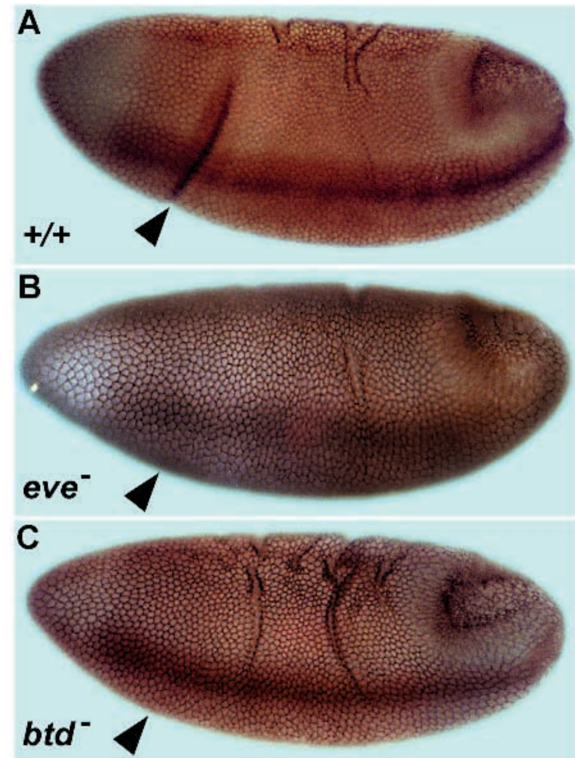

Fig. 2. Embryos mutant for either *eve* or *btd* fail to make a CF. Dorsolateral views of (A) wild-type, (B) eve^{R13} and (C) btd^{XA} embryos at the mid-gastrula stage, stained with anti-Arm to visualise the cell junctions. (B,C) Surface views; (A) the focus is deeper in the embryo to underline the progression of CF invagination that has already occurred in wt embryos at that stage. In neither *eve* nor *btd* embryos has a fold formed at the position of the CF (arrowhead), whereas the VF and PMG have progressed normally.

no CF formation at the expected time, i.e., concomitant with mesodermal invagination. Cuticle preparations of the CF-defective embryos hand-selected at stage 6 confirmed they were the *eve* mutant embryos. Fig. 2A and B show wild-type and *eve* embryos at the early gastrula stage (transition between stages 6 and 7), stained for Armadillo to highlight cell shape changes. *armadillo* encodes a *Drosophila* β-catenin homolog, which is a component of the adherens junction complex and accumulates at the sites of cell-cell contact (Peifer, 1993). At that stage, the cephalic furrow is clearly visible in wild-type embryos (Fig. 2A). In homozygous *eve* mutant embryos, there is no indication of CF formation while the other major morphogenetic movements, the VF and proctodeal invagination, have progressed normally (Fig. 2B). Time-lapse recording of live *eve* embryos (sagittal sections) between the beginning of cycle 14 and stage 7 did not show any signs of initiator cell shortening (data not shown).

In order to identify other genes involved in CF formation, we chose to search for CF defects in living embryos deficient for overlapping chromosomal regions. We started with the X-chromosome, using attached X-chromosomal females and males carrying XY translocations, a strategy previously successful in identifying cellularisation and cell adhesion genes (Wieschaus and Sweeton, 1988; Müller and Wieschaus, 1996). A single X-chromosomal region, 8F-9C, defined by the translocations *T(1;Y) B115* and *T(1;Y) B152*, was identified as being absolutely required for initiation of CF formation. Among the genes already known to map in this region, an immediate candidate for a ‘CF gene’ was *buttonhead* (*btd*, cytological position 9A), a head gap gene that encodes a zinc finger transcription activator and likely a direct Bcd regulatory target (Wimmer et al., 1993, 1995). Mutations in *btd* lead to an incomplete head involution (Wieschaus et al., 1984) with the absence of the antennal, intercalary, mandibular and part of the maxillary segments (Cohen and Jürgens, 1990; Wimmer et al., 1993, 1996). Although no defect in CF formation was previously ascribed to mutations in *btd*, we decided to re-investigate this possibility by examining mutant embryos carrying either an amorphic or a strong hypomorphic allele of *btd*, btd^{XA} and btd^{XG}, respectively (Wimmer et al., 1993). In both cases, examination of live and fixed *btd* embryos revealed the absence of CF (Fig. 2C). During early gastrulation, stages 6 and 7, the *eve* and *btd* mutant phenotypes

looked morphologically identical (compare Fig. 2B and C), i.e., they displayed a lack of initiator cell activity.

A late fold forms in the CF region of *eve* mutant embryos

Although no initiator cell behavior is observed during early gastrulation in *eve* embryos, an irregular anterior fold was frequently observed in later stage embryos, at a variable position within mitotic domain 2 (Fig. 3, see Foe, 1989 for description of mitotic domains). Although these folds arise in the region of the embryo that would normally form the CF, they do not appear to result from the normal CF program. Fig. 3 shows a time-lapse analysis of gastrulation in embryos expressing a nuclear form of the Green Fluorescent Protein (nlsGFP) (Davis et al., 1995). Whereas, in wild-type embryos, CF formation preceded mitosis 14 (Fig. 3A, see Foe et al., 1989), in *eve* embryos, the irregular anterior fold that forms arose only after cells in MD2 had entered mitosis (Fig. 3B,C). This fold eventually resolved into a complete transversal furrow (Fig. 3D). This late, deep fold observed in *eve* embryos was never seen in *btd* embryos. In *btd* embryos, cells in MD2 do not undergo mitosis, because expression of *string*, the *Drosophila* cdc25 homolog that drives the G_2/M transition during embryonic cycle 14, is dependent upon *btd* activity in this domain (Edgar et al., 1994). On the contrary, MD2 is expanded posteriorly in *eve* mutant embryos, to encompass more cells than in wild type (Fig. 3D). Thus, it is possible that the difference in late furrow formation between *eve* and *btd* embryos is due to their differential control of patterned mitosis. Finally, it should be noted that neither *btd* nor *eve* hypomorphic alleles (btd^1 and eve^{IIR59}, respectively) affect the cephalic fold (not shown), indicating that this morphogenetic movement is a low sensitivity phenotype contrary to the case in either head involution or trunk segmentation. This low sensitivity might explain why CF defects, which we only observed in amorphic *btd* or *eve* mutant embryos, have not been reported previously.

btd expression overlaps, and is required for, *eve* expression stripe 1

The observation that both *btd* and *eve* are required for CF formation suggested that the two were expressed in the CF initiator cells. In situ hybridization to *eve* and *btd* transcripts, or hybridisation to *btd* RNA and staining with Eve antibodies on embryos at early cycle 14, indicated an overlap of *btd* expression and *eve* stripe1 in one or two cells (Fig. 4 and data not shown). This overlap was clearly seen at high magnification (Fig. 4C), because the *btd* and *eve* transcripts display different subcellular localisations, basal and apical, respectively (Fig. 4A,B). The CF initiator cells, the cells at the center of Eve stripe 1 (see also Fig. 1A), are the posterior-most *btd*-expressing cells at that stage. Together with the CF phenotype of *btd* and *eve* mutant embryos, the *btd*/*eve* overlap in expression suggested at least two possible alternatives. Either Btd and Eve proteins act together in regulating the expression of a downstream target gene(s), or one may regulate the other, the two possibilities not being mutually exclusive. To test the two possibilities, we examined expression of *eve* and *btd* in mutant backgrounds. Consistent with the gap-like gene properties of *btd*, no change of its expression was seen in eve^- embryos. In contrast, Eve stripe 1 was found to be

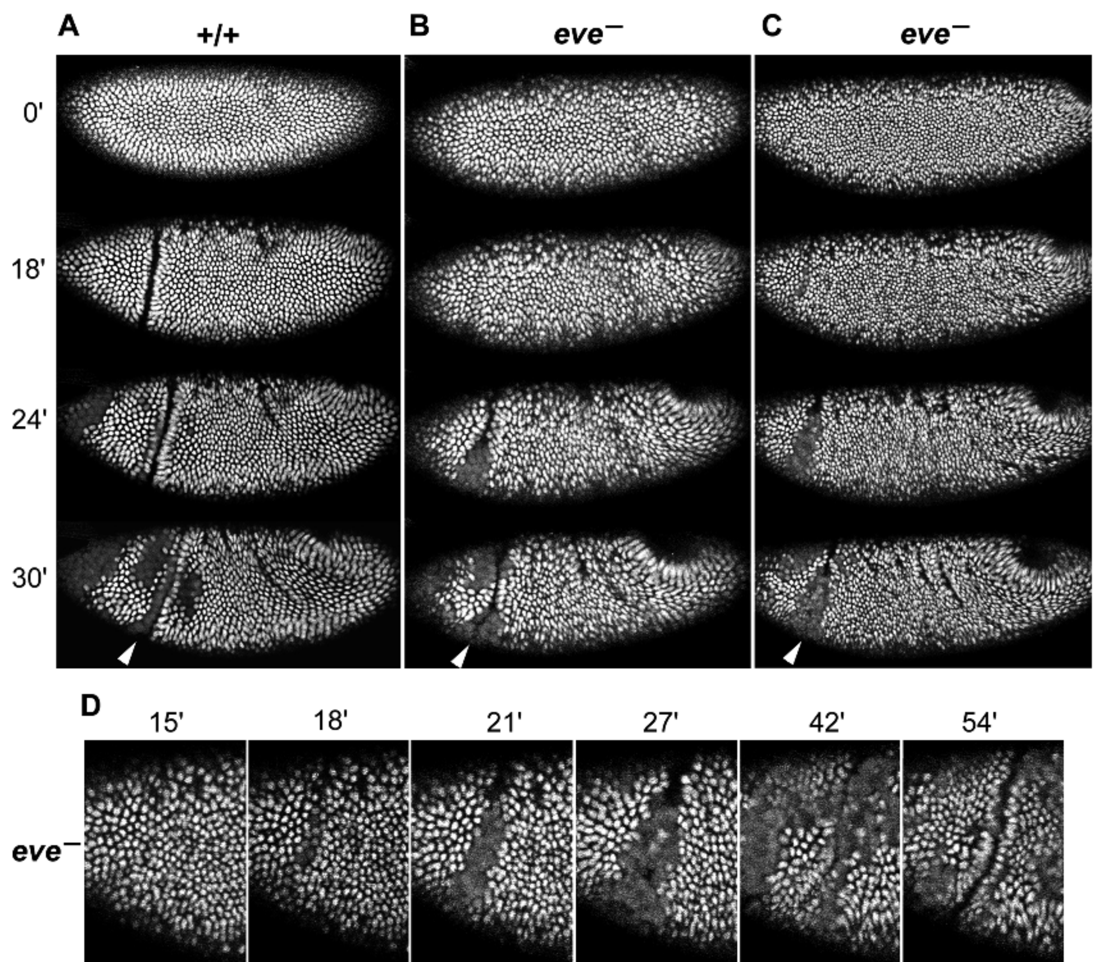

Fig. 3. Time-lapse analysis of CF formation and mitosis in wild-type and *eve* mutant embryos expressing a nuclear GFP. (A) Wild-type embryo; (B,C) eve^{R13} embryos. Position of the mitotic domain MD2 is indicated by an arrowhead. (D) Higher magnification of the embryo shown in C, to show the progression of the late fold in *eve* embryos, relative to the progression of mitosis 14.

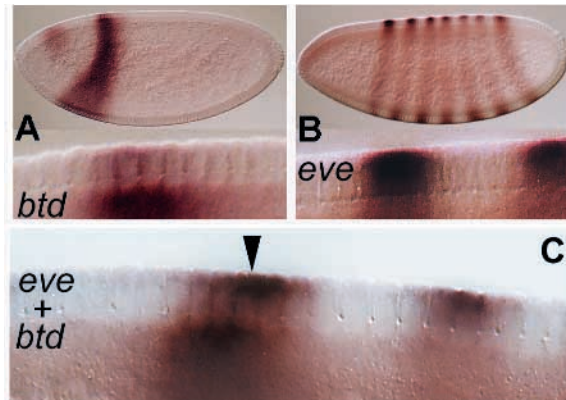

Fig. 4. *btd* and *eve* mRNA expression partly overlaps in wild-type embryos. *btd* and *eve* mRNAs were detected by whole-mount in situ hybridisation. Embryos are oriented with the anterior pole to the left. (A,B) Lateral views of an embryo at mid-cellularisation hybridised with *btd* and *eve*, respectively. The *btd* head stripe overlaps *eve* stripe 1, as can be seen better at higher magnification (C). *btd* and *eve* transcripts are localised basally and apically, respectively (see insets), allowing the nuclei to be counted in the overlap region. This overlap is two to three cells wide. The arrowhead indicates the position of the presumptive CF initiator cell.

dependent upon *btd* activity. In *btd* embryos at the early cellularisation stage, Eve stripe 1 was reduced to a single line of cells expressing low levels of Eve (Fig. 5A,B). The position of these cells relative to the stripe of *btd* expression or the anterior border of Eve stripe 2 indicated that they correspond to the posterior edge of stripe 1 (Fig. 5C,D). During early gastrulation (stage 6), Eve expression in wild-type embryos becomes restricted to a 2-cell-wide stripe laterally and about a 3-cell-wide stripe ventrally. In *btd* embryos, residual Eve expression was detected only in a few scattered cells mainly located on the ventral side of the embryo (Fig. 5D, see also Fig. 6D). Loss of the last row of Eve-expressing cells in the gastrula *btd* embryo presumably results from the normal sharpening of the Eve stripes by other pair-rule genes that occurs at this stage. Identical results were obtained by in situ hybridisation to *eve* RNA in place of Eve antibodies (not shown), indicating that *btd* is required, directly or indirectly, for *eve* transcription in parasegment 1 during cycle 14.

A hunchback>buttonhead transgene rescues the *btd* CF phenotype

A conditional *hunchback>buttonhead* (*hb>lacZ>btd*) transgene in which the *btd*-coding region is separated from the hunchback proximal promoter region by a *flp*-out cassette containing a *lacZ* gene has recently been constructed (Wimmer et al., 1997). When the *flp*-out cassette is removed during spermatogenesis, the *hb>btd* transgene allows *btd* expression in the anterior half of the embryo, both anteriorly and posteriorly to its normal expression domain (compare Fig. 6A and 4A). *hb>btd* expression completely overlaps the position of Eve stripe 1 and most of the stripe 1-stripe 2 interstripe. Expression of this transgene rescues the *btd* mutant head phenotype to wild-type (Wimmer et al., 1997). We tested whether it also was able to restore normal CF formation by examining live embryos at the early gastrulation stage. In the control btd^{XG81}, *svb/FM7* inter se cross, roughly 25% of the embryos (over 200 embryos individually examined) lacked a cephalic furrow and showed a typical *btd, svb* double mutant phenotype in cuticular preparations. When *btd, svb* females were mated to males carrying both the *hb>lacZ>btd* and *b2-tubulin>FLP* transgenes, the fraction of progeny lacking a cephalic furrow lowered to around 15% (48 embryos out of a total 333 individual embryos examined). This result, consistent with an excision of the *lacZ* cassette in more than 80% of fertile sperm (Wimmer et al., 1997), indicated that the *hb>btd* transgene is able to rescue formation of the CF. This was confirmed by examining the larval cuticles of CF^+ embryos hand-selected at the early gastrulation stage. The expected fraction showed both normal head skeleton structures and a *svb* phenotype, indicating that they were rescued *btd*, *svb* mutant embryos (data not shown). Finally, we looked at Eve stripe 1 in this class of embryos. The different embryonic phenotypes were distinguished by either triple immunostaining for Eve, Sxl and β-Gal or in situ with a *lacZ* probe followed by immunostaining for Sxl and Eve (Fig. 6 B-D and data not shown). In *btd*, *hb>btd* embryos, the anteroposterior position and width of Eve stripe 1 and its placement in relation to CF position were restored to normal (Fig. 6B,C). This suggests that, while *eve* stripe 1 is dependent upon *btd* activity, its position involves repressor elements acting independently of *btd*.

DISCUSSION

Genetic and molecular analyses of the biological process of patterning during *Drosophila* development have defined elaborate cascades of gene interactions that sequentially subdivide the embryo into an array of different types of tissues and specialised segments (review by St Johnson and Nüsslein-Volhard, 1992). Among the earliest manifestations of this patterning are the movements of gastrulation, which involve successive changes

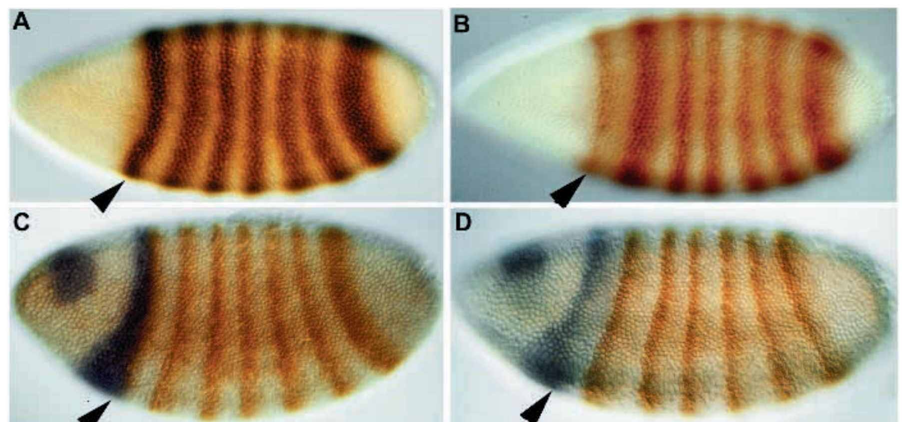

Fig. 5. *btd* is required for expression of Eve stripe 1. Anti-Eve immunostaining of whole-mount (A,C) wild-type or (B,D) btd^{XA} embryos. (A,B) mid-cellularisation embryos (C,D) embryos at the onset of gastrulation stained also for *btd* mRNA (blue). The position of CF initiator cells is indicated by an arrowhead. In *btd* mutant embryos, Eve stripe 1 is reduced to a single row of cells.

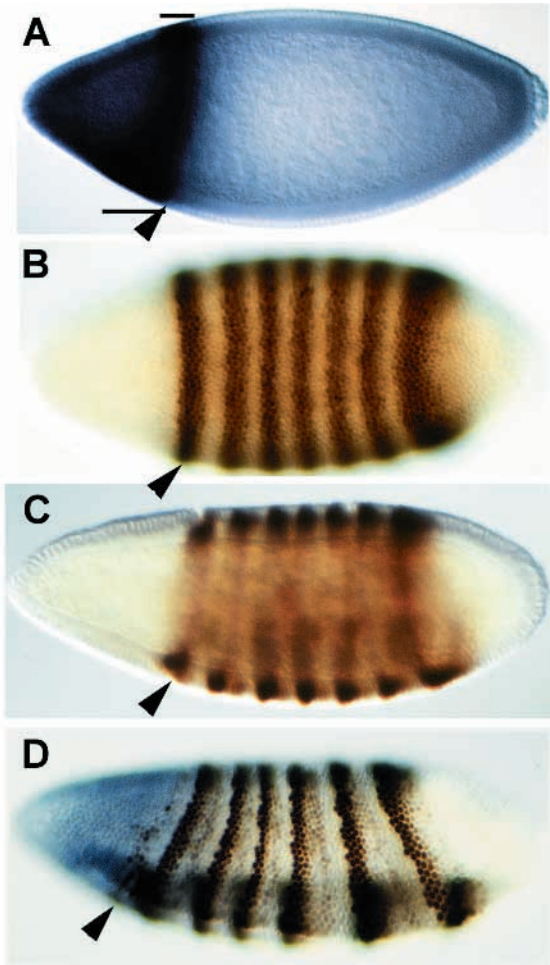

Fig. 6. Expression of the *hb>btd* transgene restores normal CF formation. (A,B) Embryos at the early cellularisation stage and (C,D) embryos during early gastrulation (stage 6); (A) *btd* mRNA expression in a wild-type embryo after *flp*-out and activation of the *hb>btd* transgene: ectopic *btd* expression can be detected in addition to the endogeneous *btd* expression pattern indicated by horizontal black bars; (B-D) *btd* mutant embryos after *flp*-out and activation of *hb>btd* (B,C) or (D) carrying the uninduced *hb>lacZ>btd* transgene, were stained for *lacZ* mRNA (blue) and Eve protein (brown). Expression of *hb>btd* restores a wild-type Eve pattern and CF formation. In D, note the residual Eve stripe 1 expression in a few scattered cells, typical of *btd* mutant embryos.

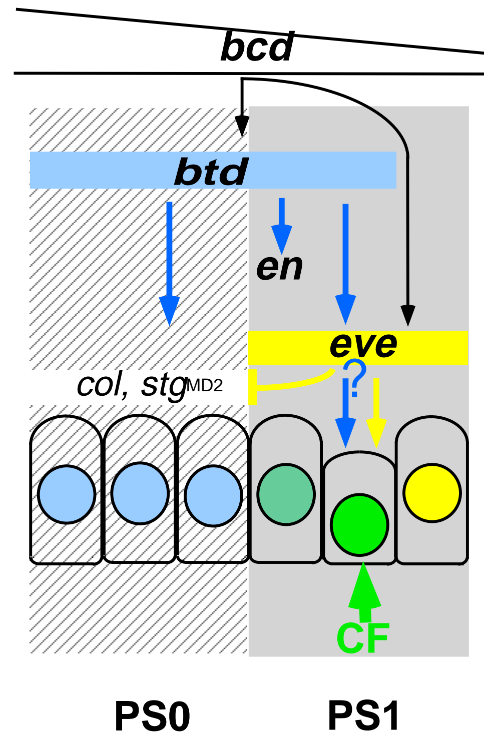

Fig. 7. Interaction between *btd* and *eve* contributes to patterning the head/trunk boundary region. Data reviewed in Ingham (1988) and Jürgens and Hartenstein (1993) and from experiments performed in our laboratories have been combined and are diagramatically represented. The gradient of the Bcd protein product is schematically represented at the top of the figure. The relative positions of the presumptive parasegments PS0 (hatched) and PS1 (shaded) of the blastoderm stage embryo are indicated at the bottom. Although the number of embryonic cells that express *btd* (blue nuclei), *eve* (yellow) and *btd+eve* (green) gradually increases from dorsal to ventral, five *btd*-expressing cells are shown here, two of which overlap the *eve* expression domain. The position of the CF initiator cell is indicated by an arrowhead. Positive regulation (direct or indirect) is indicated by an arrow and negative regulation by a line ending with a bar. Positive regulation of *stg* and *col* expression by *btd* in PS0/MD2 has previously been reported (Edgar et al., 1994; Crozatier et al., 1996).

in the shape of epithelial cells at defined positions (Costa et al., 1993; Leptin, 1994). The initial step in CF formation is a change in shape and apical positioning of a single row of cells. The position of these initiator cells may be defined by the overlapping expression of the segmentation genes *btd* and *eve*, both of which are required for the initiator cells to form. The control of Eve expression by *btd* in these cells reveals a new level of integrated regulation, which interfaces the head and trunk segmentation systems. We propose that the CF in *Drosophila* transiently separates two morphogenetic fields and possibly reflects the existence of an early organiser region analogous to the mid-hindbrain border in vertebrates (see Bally-Cuif and Wassef, 1995 and Joyner, 1996, for reviews).

Initiator cells in cephalic furrow formation

The best known process of *Drosophila* gastrulation is the formation of the VF, which involves the apical flattening of cells in the ventral plate, a domain about 18 cells wide, followed by the apical constriction of cells in the central region. This ultimately results in the formation of a shallow groove or furrow along the ventral midline (Sweeton et al., 1991). A different sequence of cell shape changes occurs during CF formation. First, a single row of lateral cells shorten along the apicobasal axis. We define these cells as the initiator cells. The presence of a single row of initiator cells distinguishes the invagination of CF from that of VF and PMG invagination, in which a sheet of cells undergo a coordinated cell shape change. Control of the timing and extent of mesodermal invagination by the transcription regulators *twist*, *snail* and *huckebein*, now well established, involves the expression of at least one gene, *folded gastrula-*

tion, in a region prefiguring the VF (Costa et al., 1994; Reuter and Leptin, 1994). Neither the upstream regulators nor the downstream effectors of VF play a role in CF formation. Further understanding of CF formation at the cellular level will therefore require the identification of such putative effectors.

Cephalic furrow position and Eve stripe 1

The Bicoid gradient specifies a unique positional identity to each cell along the anteroposterior axis by activating target genes, including *btd* and *eve* (stripes 1 and 2) in a concentration-dependent manner (Driever and Nüsslein-Volhard, 1988; Small et al., 1991; Wimmer et al., 1995). The CF is either missing or shifted anteriorwards in embryos laid by *bcd* mutant females. The same relative shift is observed for *btd* expression and *eve* stripe1. Together with the observation that both *btd* and *eve* are required for CF formation, it suggests that the trigger driving the shape change of initiator cells is the combinatorial action of Bcd, Eve and Btd in these cells. It was recently shown that early Eve stripes act as morphogenetic gradients at the single-cell level (Fujioka et al., 1995). Since the cell with the highest level of Eve expression at the early cellularisation stage corresponds to the second cell of Eve stripe 1, it is possible that a high level of Eve expression prefigures the initiator cell. However, this behavior cannot be purely quantitative as hypomorphic alleles of *eve* still form a CF even though the initiator cell would presumably have a lower level of Eve activity than the non-initiator cells in Eve stripe 1 of wild-type embryos. Thus, it may be that, within the stripe, relative levels of Eve expression, rather than absolute values, determine which cell displays initiator activity.

The finding that *eve* stripe 1 expression itself requires *btd* raises the possibility that absence of CF in *btd* mutant embryos is solely a consequence of the lack of Eve stripe 1. Since a CF forms only at the position of stripe 1, some additional factor must distinguish this stripe of *eve* expression from the other six. Although a direct role of Bcd concentration in the process of demarcating Eve stripe 1 from the rest cannot be formally excluded, we suspect that a combination of zygotically active genes may provide a more precise designation of CF cells. One candidate for this function might be Btd itself. However, a *hb>btd* chimaeric gene can rescue the embryonic *btd* phenotype (Wimmer et al., 1997) despite a domain of *btd* expression shifted both anteriorly and posteriorly, therefore completely overlapping Eve stripe 1 and possibly extending into Eve stripe 2. In rescued embryos, Eve stripe 1 expression and CF formation are at a normal position. These results indicate that the position and size of Eve stripe 1 is determined by repressor elements acting downstream of Bcd and overriding *btd*-dependent activation. It strengthens the conclusion that *btd* might be a ‘generic transcriptional activator’ required for transcriptional activation of specific target genes, such as *eve* and *collier* (*col*) (this report and Crozatier et al., 1996) but whose limits of expression are not instructive for head development (Wimmer et al., 1997).

Previous promoter fusion studies have established that at least some of the Eve stripes are regulated by separate enhancers present in the *eve* promoter (Goto et al., 1989; Harding et al., 1989), but the Eve stripe 1 enhancer has not been characterised. Our results suggest that activation of Eve stripe 1 is similar to that of Eve stripe 2 (Small et al., 1992; Arnosti et al., 1996), with *bcd*, possibly *hb*, and *btd* representing ‘generic’ activators working synergistically. A striking difference, however, is the mosaic nature of the stripe 1 enhancer since the posterior-most row of Eve stripe 1 cells is still present in *btd* mutant embryos. This correlates with the observations that *btd* is not expressed at significant levels in this row of cells and that part of the maxillary anterior compartment (posterior PS1) remains in *btd* mutants (Gonzales-Reyes and Morata, 1991; Wimmer et al., 1996).

Functions of btd and eve in patterning the head/trunk boundary region

Recent genetic and molecular data from *Drosophila* support the view that the process of segmentation of the head is fundamentally different from that of the trunk (Cohen and Jürgens, 1990; Finkelstein and Perrimon, 1991; Wimmer et al., 1997). The questions of the interface between the two segmentation systems and the potential role of the CF have remained, however, largely unaddressed. These are important issues since the cephalic furrow marks the boundary between the procephalon and the segmented germ band (Gonzales-Reyes and Morata, 1991), although it does not correspond to any boundary within the later head. The control of *eve* by *btd* in anterior cells of PS1 is the first indication of an hierarchical relation between head gap genes and trunk pair-rule genes. Interestingly, Engrailed expression in PS1 requires *btd* but does not require *eve*, contrary to the situation in parasegments 2 to 14 (McDonald et al., 1986; Cohen and Jürgens, 1990). Despite having no effect upon En expression in PS1, *eve* may have a role in patterning this parasegment, as its expression and placement in relation to En is conserved between long germ-band and short germ-band insects (Patel et al., 1994). Moreover, recent data on the activation of *col*, a novel transcription factor, in PS0/MD2 (Crozatier et al., 1996), suggest a possible mechanism by which *btd* and *eve* cooperate to pattern PS1. Activation of *col* requires *btd.* Conversely, in the absence of *eve, col* expression is expanded posteriorly to overlap a region roughly corresponding to PS1, indicating that Eve acts as a repressor of *col* in this parasegment (Crozatier, M. and Vincent, A. unpublished). Likewise*,* expression of *string* in MD2, which also requires *btd* (Edgar et al., 1994), is expanded posteriorly in *eve* mutant embryos (Fig. 3 and data not shown). Our current working model is that the activation of *eve* by *btd* in anterior PS1 cells allows for differential gene expression between PS0 and PS1 (Fig. 7). In addition to the control of CF formation, the *btd*/*eve* interaction may thus assign separate gene expression and mitotic programs to cells on either side of the procephalon/posterior head border. It remains to be understood what possible function the CF plays in this demarcation process. In embryos laid by *bcd* females, the anterior part of the germ band on the ventral side extends further anteriorly, suggesting that the CF may serve to anchor anterior (ventral) cells such that the germ band extends only posteriorly (Costa et al., 1993). The CF may therefore be viewed as transiently separating two morphogenetic fields. Whether the foremost expression of Engrailed in PS1 in *Drosophila* and at the midbrain-hindbrain boundary in vertebrates reflects the existence of an ancestral organiser region, of which the CF might constitute a specialised evocation, is an interesting question for future consideration.

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