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Cell intercalation during *Drosophila* germband extension and its regulation by pair-rule segmentation genes

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SUMMARY

After the onset of gastrulation, the *Drosophila* germband undergoes a morphological change in which its length along the anterior-posterior axis increases over two-and-a-half fold while its width along the dorsal-ventral axis simultaneously narrows. The behavior of individual cells during germband extension was investigated by epi-illumination and time-lapse video microscopy of living embryos. Cells intercalate between their dorsal and ventral neighbors during extension, increasing the number of cells along the anterior-posterior axis while decreasing the number of cells along the dorsal-ventral axis. Mutations that reduce segmental subdivision of the embryo along the anterior-posterior axis decrease both germband extension and its associated cell intercalation. In contrast, cell intercalation and germband extension are still detected in embryos that lack dorsal-ventral polarity. Characterization of germband extension and cell intercalation in mutant embryos with altered segmentation gene expression indicates that these processes are regionally autonomous and are dependent upon the establishment of striped expression patterns for certain pair-rule genes. Based on these observations, we propose a model for germband extension in which cell intercalation results from the establishment of adhesive differences between stripes of cells by pair-rule genes.

Key words: germband, extension, intercalation, *Drosophila*

INTRODUCTION

Morphogenesis involves changes in the shapes of populations of cells. Although these changes can occur by a number of mechanisms, one of the most common appears to be cell rearrangement (reviewed by Keller, 1987; Fristrom, 1988). Directed cell rearrangements that simultaneously narrow and lengthen tissues occur during gastrulation, neurulation, and at other stages of development in a variety of organisms, and can occur in both epithelial and non-epithelial cell layers (e.g. Jacobson and Gordon, 1976; Keller, 1978; Ettensohn, 1985; Warga and Kimmel, 1990; Keller et al., 1991). In some cases, cells appear to rearrange in response to external forces that pull on the extending tissue; this has been termed passive rearrangement. In other cases, however, cells rearrange in the absence of any external force, implying that the cell rearrangement is an active process (reviewed by Keller, 1987; Fristrom, 1988). Despite a number of suggestions, the mechanisms that drive active cell rearrangement remain unknown. Here, we describe epithelial cell rearrangements that occur during the extension of the *Drosophila* germband. The ability to apply the powerful genetic and molecular techniques available in *Drosophila* to the process of cell rearrangement should provide important insights into this fundamental process.

The germband is generally considered to be the part of the embryo that gives rise to the visibly segmented part of the animal, comprising the gnathal, thoracic, and abdominal segments (Campos-Ortega and Hartenstein, 1985). It includes the mesoderm, ventral ectoderm, and dorsal epidermis, but excludes the dorsal-most tissue in the embryo, the amnion-serosa. Germband extension begins shortly after the embryo starts to gastrulate, and it continues for over 2 hours, although most extension is completed within the first 45 minutes (Fig. 1; Campos-Ortega and Hartenstein, 1985). During extension, the length of the germband along the anterior-posterior axis increases over two-and-a-half fold while its width simultaneously narrows. The extension is oriented such that the germband extends around the posterior end of the embryo and then along the dorsal surface, effectively folding over on top of itself. Hartenstein and Campos-Ortega (1985) have examined fixed and sectioned embryos undergoing germband extension. This study revealed that in the ventral ectoderm much of the increase in the length of the germband occurs in the absence of cell division or changes in cell shape. Instead, the number of cells along the anterior-posterior axis increases and the number of cells along the dorsal-ventral axis decreases. Thus, they argued that at least some germband extension is accompanied by cell rearrangement.

Based on the orientations of the transient connections that cells retain with the yolk sac in the early gastrula, it has been proposed that germband extension could be mediated by a contraction of microfilaments underlying cells on the dorsal side of the embryo, which would then pull the germband onto the dorsal surface (Rickoll, 1976; Rickoll and Counce, 1980). This suggestion is supported by the behavior of the germ cell precursors, the pole cells, in mutant embryos lacking somatic cells. The pole cells form at the posterior end of the embryo and normally move to the dorsal surface and then anteriorly in

front of the advancing germband. In acellular embryos, the pole cells still move from the posterior end anteriorly along dorsal surface of the embryo (Rice and Garen, 1975; Rickoll and Counce, 1981). However, they move only a fraction of the distance that they traverse during normal germband extension, implying that other forces must also be involved.

Clues to such forces can be provided by genetic analysis. Although mutations that are specifically defective in germband extension have not yet been isolated, mutations in many of the genes involved in patterning along the anterior-posterior axis of the embryo also reduce germband extension. Germband extension is reduced by mutations in the maternal coordinate genes, which establish the anterior-posterior axis (Lehmann and Nüsslein-Volhard, 1986; Schüpbach and Wieschaus, 1986; Lehmann, 1988; Wieschaus et al., 1991), and by mutations in the zygotic gap and pair-rule segmentation genes, which further subdivide it (Nüsslein-Volhard and Wieschaus, 1980; Wieschaus et al., 1984; Lehmann and Nüsslein-Volhard, 1987; Lehmann, 1988; Wieschaus et al., 1991). Although the effects of these mutations on the process of germband extension have not previously been examined in detail, the observation that germband extension is affected by anterior-posterior patterning led to a model for germband extension in which cell rearrangement results from the establishment of adhesive differences between cells by segmentation genes (Gergen et al., 1986; Wieschaus et al., 1991).

In this work, we demonstrate and characterize cell rearrangements in living embryos; cells intercalate between their dorsal and ventral neighbors during germband extension. We then investigate the cause of these rearrangements by examining germband extension and cell intercalation in pattern formation mutants. Our results argue that cell intercalation provides a major force driving germband extension and support the proposal that cell intercalation is driven by adhesive differences between cells.

MATERIALS AND METHODS

Drosophila strains

Oregon-R was used as wild type. Stocks were maintained, and experiments were conducted, at room temperature (typically 23°C). The following mutant stocks were used to examine effects on germband extension: bcd^{E1}, nos^{L7}, tor^{PM51}, bcd^{E1} nos^{L7}, bcd^{E1} nos^{L7} tsl^{146}, $stau^{D3}$, *BB9+16* (6x bcd^{+}), Kr^{1}, kni^{7G75}, kni^{IID48} hb^{7M48}, gt^{YA82}, kni^{IID48} hb^{7M48} fkh^{E200} tll^{L10}, eve^{R13}, *Dfeve1.27*, ftz^{9H34}, run^{XD106}, h^{7h94}, $prd^{32.12}$, opa^{IIP32}, slp^{IIM105}, odd^{IIID36}, en^{IIB86}, wg^{CX2}, wg^{IG22} ptc^{6P43} en^{IM99}, nkd^{7H}, Ubx^{130}, *Df(3R)P9* (BX-C^{-}), stg^{7B}, *hs-eve19B*, dl^{1}, Tl^{10b}, $Toll^{rm10}$, gd^{2}; cac^{A2}, $snake^{rm4}$ Tl^{9Q}, snk^{229}.

Following cell movements

Individual cells were visualized using epi-illumination (Merrill et al., 1988): blastoderm embryos were placed in halocarbon 27 oil on a petriperm plate, flattened slightly with a coverslip, and illuminated with a fiber-optic light while time-lapse video recordings were made. Wild-type embryos treated in this way develop normally and hatch. Photographs were taken at intervals from the video monitor and cell rearrangements reconstructed by following individual cells during play-back of videotapes and marking their positions onto the photographs. Several epi-illumination videos were examined for each genotype. Detailed reconstructions such as those shown in figures were performed on five wild-type embryos and at least two of each mutant genotype.

Measuring and timing germband extension

Rates of germband extension were measured from time-lapse videos of embryos under bright-field illumination (Wieschaus and Nüsslein-Volhard, 1986). The ends of the germband were followed continuously and their positions marked at intervals on transparencies covering the video monitor. Germband length was determined by taping a shoelace to the transparency in the shape of the germband and marking off the lengths. The ends of the germband were defined according to morphological features that are visible in the gastrulating embryo. The posterior end was defined as the boundary between the region where the mesoderm invaginates and where the proctodeal cells form. The anterior end was defined as the cephalic furrow. The beginning of germband extension was defined as when the posterior boundary of the germband first shifts further posteriorly. Previous definitions have at times placed the beginning of germband extension several minutes later, after the posterior midgut invagination reaches the dorsal surface (Campos-Ortega and Hartenstein, 1985). Differences in how the germband and germband extension are defined probably account for most of the discrepancy between our measurements and those of Hartenstein and Campos-Ortega (1985), who described the total extension as only 220%. For mutants in which rates of germband extension were not quantified, qualitative determinations of their effects were made by examining time-lapse videotapes or populations of fixed embryos.

Epi-illumination videos were timed according to the formation of the cephalic furrow, because the initial posterior movement of the germband was not visible from this perspective. We estimate that this precedes germband extension by about 5 minutes. We also note that germband extension initiates at the posterior of the germband and spreads anteriorly; we presume this reflects the initiation of germband extension by the dorsal contraction. When the cephalic furrow was out of the field of view or in mutant embryos that do not make a cephalic furrow, embryos were timed according to the initial ventral movement of cells (Fig. 3). The location of the field of view was determined by first calibrating the screen field of view to the embryo (typically 33-34% egg length), and then using it as a ruler to measure in from the ends of the embryo.

Visualizing gene expression patterns

Embryos were stained for *eve* expression with a rabbit anti-Eve polyclonal antibody (Frasch et al., 1987) as described in Irvine et al. (1991), except that PBS buffer was substituted for Pipes and BSS buffers. Embryos were triply stained for *dpp*, *sim*, and *en* expresssion using anti-En monoclonal 4D9 (Patel et al., 1989) and cDNA fragments of *dpp* and *sim* according to the in situ/antibody staining procedure of Manoukian and Krause (1992) with the addition of 1.5 mM DTT to antibody incubation buffers to preserve RNasin activity.

RESULTS

Cells intercalate during germband extension

The behavior of cells during germband extension was examined in living embryos by using epi-illumination to visualize cells and time-lapse video microscopy to follow their movements. We concentrated on the anterior two-thirds of the germband because at the magnification needed to follow cells, more posterior cells rapidly move out of the field of view. We further restricted our analysis to the ventral ectoderm during the first 45 minutes of germband extension because of the absence of mitoses. During this time, the germband extends to almost two-and-a-half times its initial length, about 85% of its total elongation (Fig. 1). To characterize the movements of cells relative to their neighbors, rows of contiguous blastoderm cells extending from anterior to posterior were followed. In

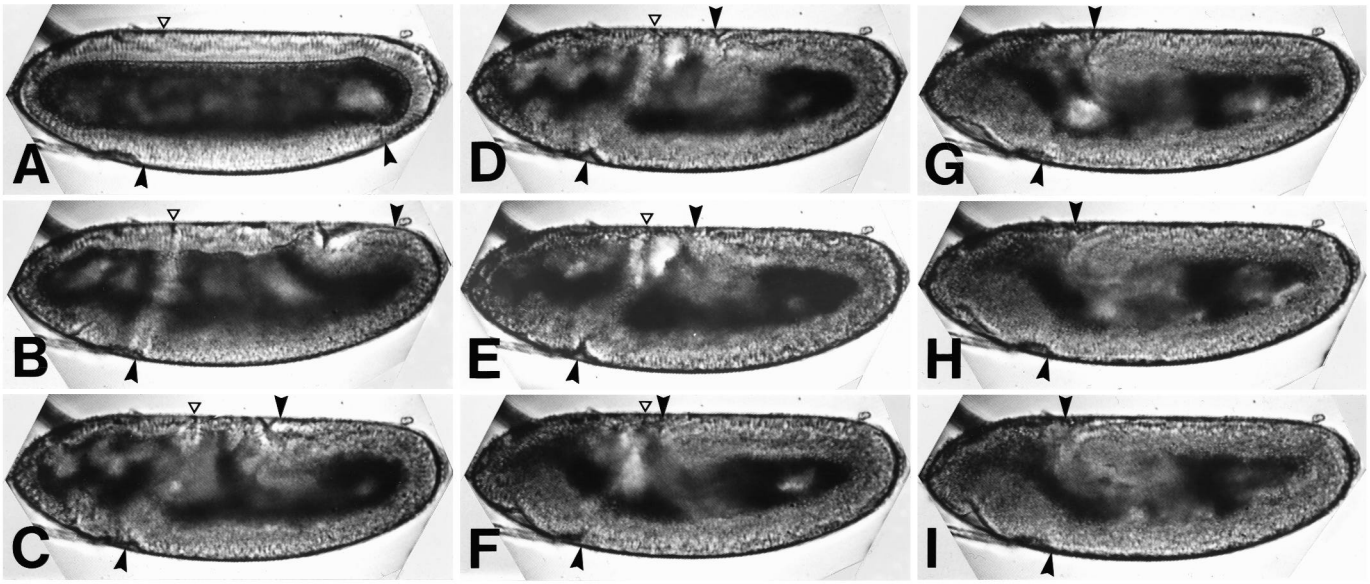

Fig. 1. Germband extension in wild-type embryos. The ends of the germband, which runs from approximately 15-70% egg length, are marked by arrowheads. The cephalic furrow is marked dorsally by an open arrowhead. In this and all subsequent figures, anterior is to the left and ventral is down. All panels show photographs of the same embryo taken from a video monitor. In A the embryo is about to begin germband extension; subsequent panels are 10 minutes (B), 20 minutes (C), 30 minutes (D), 40 minutes (E), 1 hour (F), 1.5 hours (G), 2 hours (H), and 2.5 hours (I) after beginning germband extension.

Fig. 2, the cells of three such rows are highlighted on photographs taken at 10-minute intervals during germband extension. Each gap that appears in these rows as germband extension proceeds indicates that another cell from a more dorsal or more ventral position has intercalated between two cells that were neighbors at the blastoderm stage. Cells first begin to intercalate between their dorsal and ventral neighbors about 10 minutes after the formation of the cephalic furrow (Fig. 2B), by 20 minutes most cells have separated from at least one of their blastoderm neighbors (Fig. 2C), and by 30 minutes most cells have separated from both of their blastoderm neighbors (Fig. 2D). Cell intercalation continues to separate blastoderm neighbors further from each other for at least the next 20 minutes (Fig. 2E,F). The intercalation is highly directional, as cells intercalate almost exclusively between dorsal and ventral neighbors and only rarely between anterior and posterior neighbors. Within cell rows in the region we analyzed, cell intercalation appears to be evenly distributed along the anterior-posterior axis. The relatively uniform decrease in the width of the germband during extension argues that this holds true throughout the entire germband (Hartenstein and Campos-Ortega, 1985). Although evenly distributed, cell intercalation does not occur in any precise pattern, as pairs of blastoderm neighbors may end up separated by zero, one, two, or three cells 45 minutes after the beginning of germband extension (Fig. 2F). Nonetheless, cells only intercalate between their nearest neighbors and do not migrate widely. This is easily seen by following columns of cells extending from dorsal to ventral. As the germband extends and cells intercalate, these columns of cells become shorter and wider, and while they collapse down into irregular shapes, the cells of a single column almost always remain together (Fig. 2G-I).

When the position of a germband cell in the ventral ectoderm is traced from the blastoderm stage onwards as it moves across a constant field of view, cells first shift slightly dorsally, then rapidly move ventrally, and finally move simultaneously ventrally and posteriorly (Fig. 3 and data not shown). Cells at different positions within the embryo consistently follow distinct trajectories. More dorsal cells move further ventrally than more ventral cells, and more posterior cells move further posteriorly than more anterior cells. Cells near the cephalic furrow first move anteriorly as they push the furrow forward, and then begin to move posteriorly (Figs 1, 3; Wieschaus et al., 1984). A cell's overall motion reflects three phenomena: its intercalation between neighboring cells, the invagination of the mesoderm on the ventral side of the embryo, and the shape changes of the extending epithelial sheet in which it resides. Along the anterior-posterior axis, intercalation typically causes a displacement of only one to two cells relative to immediate blastoderm neighbors, and along the dorsal-ventral axis it brings cells closer together (Fig. 2). Mesoderm invagination contributes only an early ventral motion that is common to all ectodermal cells. Thus, most of a cell's trajectory reflects the overall change in the shape of the epithelial sheet. This change, in turn, results largely from the summed intercalations of all cells (see Discussion).

Although cells appear to converge toward the ventral midline, the actual intercalation of cells is symmetrical, as dorsal cells come between their ventral neighbors and ventral cells come between their dorsal neighbors (Fig. 4). In addition to the ventral bias in the net movement of cells (Fig. 3), it appears that the number of cells along the anterior-posterior axis initially increases slightly more rapidly ventrally than dorsally. Although, because of the irregular nature of intercalation, this transient difference was not noticeable when rows of cells at different positions along the dorsal-ventral axis were

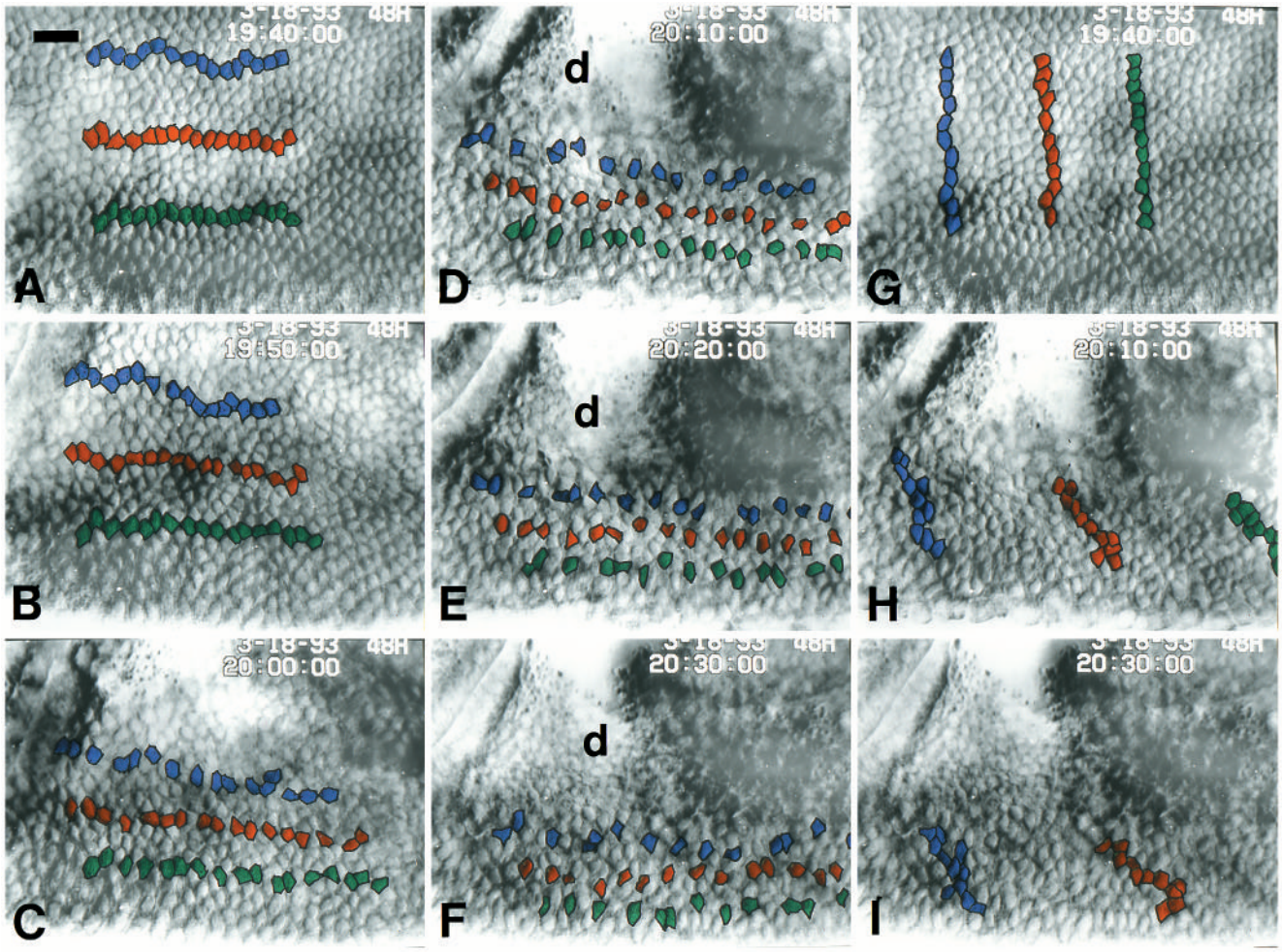

Fig. 2. Cell intercalation in wild-type embryos. All panels show photographs of the same embryo, viewed ventral-laterally. Scale bar (upper left in A) indicates 20 μm. In A-F rows of cells extending from anterior to posterior are highlighted; in G-I columns of cells extending from dorsal to ventral are highlighted. The initial field of view extends from approximately 36-69% egg length, thus comprising the anterior 60% of the germband. The highlighted cell rows extend for about five segments. The cephalic furrow is first visible at upper left in B. The initial field of view includes approximately the ventral 90% of the ventral ectoderm, which is 20-23 cells wide in the blastoderm embryo (Campos-Ortega and Hartenstein, 1985). The highlighted cell rows begin about four cells from the mesoderm and occupy, along the dorsal-ventral axis, approximately 55% of the ventral ectoderm. Flattened cells at the edge of the incipient ventral furrow are visible at bottom in A. The field of view was shifted two cells ventrally and one cell posteriorly 8 minutes after cephalic furrow formation so that cells would remain in the field of view longer. In A and G the embryo is 1 minute before cephalic furrow formation, about 6 minutes before germband extension; subsequent panels are 4 (B), 14 (C), 24 (D,H), 34 (E), and 44 (F,I) minutes after beginning germband extension. Visible but partially out of focus, dorsal epidermal cells (d) are in mitosis in D and reappear as smaller cells above the ventral ectoderm in E and F.

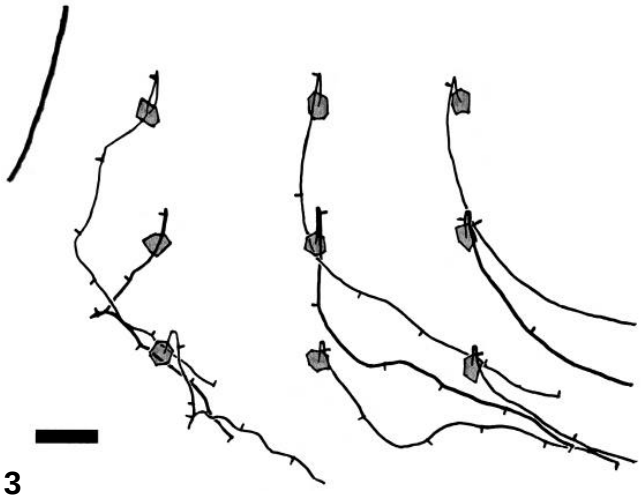

Fig. 3. Trajectories of cells in wild-type embryos. Scale bar indicates 20 μm. The paths of nine blastoderm cells (with initial positions shaded) during the 10 minutes preceding and 50 minutes following cephalic furrow formation are traced. Some cells moved out of the field of view during this time. The approximate initial locations of these cells are, from posterior to anterior, 44, 52 and 61% egg length, and, from ventral to dorsal, the second to third, ninth to tenth, and sixteenth to seventeenth cells of the ventral ectoderm. Hatch-marks denote 10-minute intervals, with the first mark at cephalic furrow formation. The early position of the cephalic furrow is indicated by the bold line at upper left. Note that cells first shift slightly dorsally before beginning to move ventrally around the time of cephalic furrow formation. Although the precise trajectories vary, the general features of cell movement illustrated by these nine cells were consistently observed among the few hundred cells whose routes we have traced.

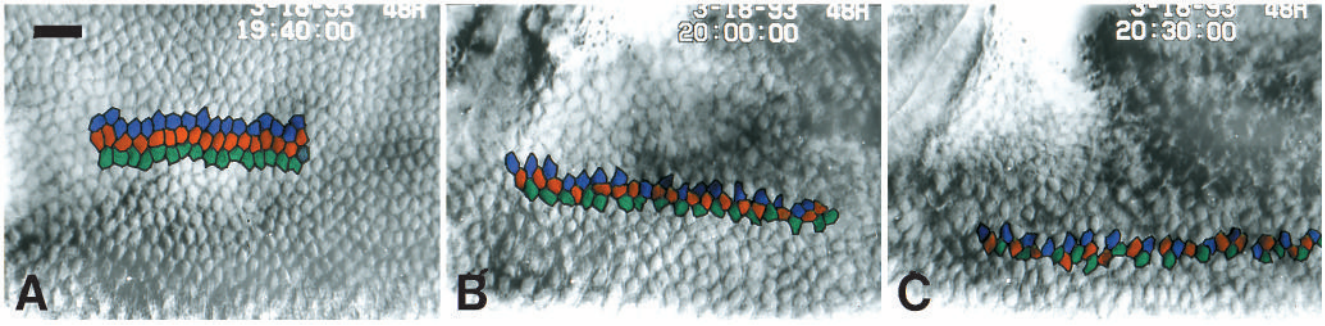

Fig. 4. Cells intercalate between both dorsal and ventral neighbors. Scale bar indicates 20 μm. Three adjacent rows of cells are highlighted in the same embryo as in Fig. 2. In A the embryo is 6 minutes before germband extension; subsequent panels are 14 (B) and 44 (C) minutes after beginning germband extension. In C, cells in each row have become separated both by cells that were initially more ventral and by cells that were initially more dorsal.

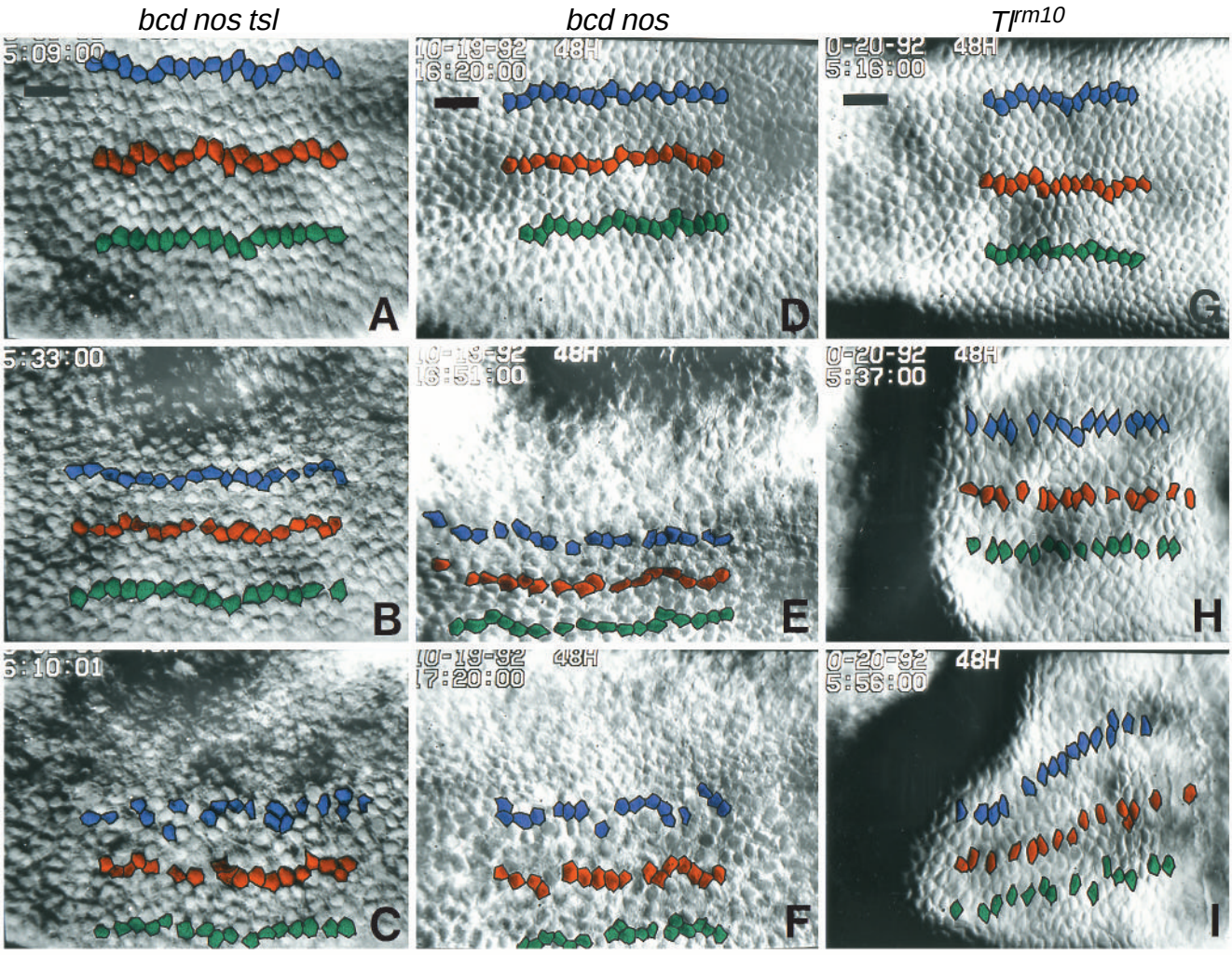

Fig. 5. Cell rearrangements in embryo polarity mutants. Scale bars (upper left) indicate 20 μm. A-C show an embryo from a *bcd nos tsl* mother 5 minutes before (A) and 15 (B) and 52 (C) minutes after beginning 'germband extension.' The initial field of view extends from approximately 34-67% egg length, and was shifted one-and-a-half cells ventrally between A and B. D-F show an embryo from a *bcd nos* mother 5 minutes before (D) and 26 (E) and 55 (F) minutes after beginning germband extension. The field of view extends from approximately 34-67% egg length. In E stretched cells were defined as those with axial ratios greater than or equal to 1.5:1 and with the long axis aligned preferentially along the anterior-posterior axis. Over 35% of cells fulfilled this definition, as compared with less than 2% of cells in wild-type embryos at this stage (Fig. 2D). (G-I) An embryo from a *Tl*^{rm10} mother 5 minutes before (G) and 16 (H) and 35 (I) minutes after beginning germband extension. The field of view extends from approximately 36-69% egg length. The dark shadow in H and I is caused by the abnormally deep cephalic furrow that forms in these embryos. In I cell rows have become distorted by the buckling of the germband.

compared (Fig. 2 and data not shown), it is implied by the earlier posterior movement of more ventral cells (Fig. 3), the tilting of columns of cells (Fig. 2I), and the observation that stripes of cells marked by pair-rule expression broaden ventrally during early germband extension (Carroll and Scott, 1985).

Positional differences along the anterior-posterior axis are required for germband extension

Positional information along the anterior-posterior axis in *Drosophila* is established by the action of three maternally provided patterning systems, anterior, posterior, and terminal (Schüpbach and Wieschaus, 1986; Nüsslein-Volhard et al., 1987). In the maternal triple mutant *bicoid nanos torso-like* (*bcd nos tsl*), anterior-posterior patterning within the embryo is completely disrupted (Nüsslein-Volhard et al., 1987). Dorsal-ventral patterning is, however, unaffected, and in addition to ectoderm these embryos differentiate along their entire anterior-posterior axis both amnion-serosa and mesoderm, which normally form only in the region of the embryo where the germband forms. Embryos derived from *bcd nos tsl* mothers fail to extend (Wieschaus et al., 1991). The zygotic quadruple mutant *knirps hunchback fork head tailless* also essentially lacks anterior-posterior patterning and fails to extend its germband (data not shown). These results indicate that the establishment of different positional values along the anterior-posterior axis is required for germband extension. When anterior-posterior rows of cells are followed in embryos from *bcd nos tsl* mothers, occasional gaps are detected 20-30 minutes after the time when germband extension would normally have begun (Fig. 5B-C). However, these gaps are minor compared to those detected in wild-type embryos (Fig. 5B, compare with 2C). In addition, cell rows become wider in places and retain essentially the same end-to-end length, suggesting that in contrast to wild type, cells are not undergoing directed intercalation but slight random movements relative to each other.

When considered separately, the terminal system and the anterior and posterior systems have distinct effects on germband extension. Terminal mutations such as *torso* or *trunk* have little effect on patterning within the germband, but they eliminate terminal cell fates. In embryos derived from *torso* or *trunk* mothers, the germband extends, but its posterior end remains near the posterior pole (Schüpbach and Wieschaus, 1986). Consequently, instead of extending along the dorsal surface of the embryo, much of the increased length of the germband is taken up in folds that develop along the ventral side. Similarily, when the invagination of posterior cells is blocked independently of changes in positional values, by mutations in *folded gastrulation* (*fog*), germband extension continues while the posterior end of the germband remains near the posterior pole, resulting in the formation of folds along the ventral side of the embryo (Zusman and Wieschaus, 1985). It appears then, that the failure of posterior cells to invaginate into the posterior midgut blocks the normal progression of the germband, but does prevent its elongation (Costa et al., 1993).

When both the anterior and posterior systems are disrupted while the terminal system is left intact, as in embryos from *bcd nos* mothers, segmentation is disrupted throughout the germband (Nüsslein-Volhard et al., 1987). In these embryos, the germband initially extends symmetrically in both anterior and posterior directions and at its normal rapid rate (Fig. 6A-C). However, after increasing to approximately 180% of its initial length, the germband stops extending and begins to contract back (Fig. 6D-F). By 2 hours after the beginning of germband extension, the germband has returned to its initial length and can even shorten further (Fig. 6F; data not shown). The zygotic double mutant *knirps hunchback* (*kni hb*) has similar effects on both segmentation and germband extension. The germband initially extends normally, but after about 15 minutes the rate of extension slows, and 15 minutes later extension stops and the germband starts to decrease in length (see below).

Some cell intercalation between dorsal and ventral neighbors is observed during the first 25 minutes of germband extension in embryos from *bcd nos* mothers (Fig. 5E). However, this intercalation is greatly reduced compared to that in wild-type embryos, and is associated with some stretching of cells along the anterior-posterior axis (Fig. 5E, compare with 2D). Such elongation of cells along the axis of extension, which does not occur here in wild-type embryos, is a characteristic feature of passive cell rearrangement in response to an external force (reviewed by Keller, 1987; Fristrom, 1988). The later contraction of the germband in embryos from *bcd nos* mothers is associated with a reversal of the limited cell intercalation that had occurred previously, as cells now intercalate between their neighbors along the anterior-posterior axis. As a consequence of this reverse intercalation, occasional pairs of cells that had separated come back together, and anterior-posterior rows of cells become both wider and shorter (Fig. 5F).

Subdivision of the embryo along the dorsal-ventral axis is not required for cell intercalation

It is striking that genes which establish patterning along the anterior-posterior axis are required for the intercalation of cells along the dorsal-ventral axis. By contrast, mutant embryos that lack all dorsal-ventral polarity still extend their germbands, provided they have ectodermal cell fates. Thus, both in apolar dorsalized embryos, which produce dorsal epidermis and amnion serosa, and apolar lateralized embryos, which produce only ventral ectoderm, the germband increases in length (Fig. 7; Nüsslein-Volhard, 1979; Costa et al., 1993). Similar degrees of germband extension were detected in embryos from three different maternal genotypes that result in the formation of ventral ectoderm around the entire embryo circumference: *Toll*^{rm10} (*Tl*^{rm10}), *cactus gastrulation defective*, and *snake Tl*^{9Q} (Fig. 7 and data not shown). The germband does not extend normally onto the dorsal surface in these lateralized embryos, because cells around the entire circumference of the embryo participate in germband extension. This often pushes the posterior pole into the interior of the embryo, and these embryos develop deep folds that accumulate the increased length of the germband (Fig. 7). Although these folds make it more difficult to follow individual cells during germband extension, it was nonetheless possible to detect cell intercalation in lateralized mutants (Fig. 5G-I). Interestingly, as the germband in these embryos attempts to extend and pushes against the posterior of the egg, cells appear to become compressed into elongated shapes that are perpendicular to the axis of extension (Fig. 5I compare with Fig. 2E).

Germband extension in zygotic segmentation mutants

The anterior, posterior, and terminal classes of maternal-effect genes establish patterning along the anterior-posterior axis by controlling the expression patterns of zygotic segmentation genes. Mutations in all of the earliest acting zygotic segmentation genes that affect patterning within the germband, the gap genes *hb*, *Krüppel* (*Kr*), *kni*, and *giant*, reduce germband extension (Fig. 8; Nüsslein-Volhard and Wieschaus, 1980; Wieschaus et al., 1984; Lehmann and Nüsslein-Volhard, 1987; Lehmann, 1988; data not shown). In order to better characterize their effects, rates of germband extension were measured from time-lapse videotapes for *Kr*, *kni*, and the double mutant *kni hb*. Notably, in each case, and in fact in all segmentation mutants that leave the posterior midgut intact, germband extension initially occurs at its normal rapid rate. However, after 15-25 minutes, germband extension slows or reverses in mutant embryos (Fig. 8). It appears then, that there is an initial phase of germband extension that occurs independently of segmentation gene action. Segmentation genes are, however, required both to maintain and to continue germband extension after this initial phase.

The gap genes establish the striped expression patterns of the segmentation genes at the next level of the segmentation gene hierarchy, the pair-rule genes. Pair-rule genes differ in their effects on germband extension. Among pair-rule mutants, *even-skipped* (*eve*) embryos have the greatest reduction of germband extension (Fig. 8E); both *runt*, and to a lesser extent *hairy*, also have significantly reduced germband extension (Fig. 8F,G; Nüsslein-Volhard and Wieschaus, 1980; Wieschaus et al., 1991). Mutations in *fushi tarazu* (*ftz*) cause a more subtle reduction of germband extension (Fig. 8H), and the effects of other pair-rule mutations are either similar to or weaker than those of *ftz* (data not shown). The segment polarity genes, which constitute the next level of the segmentation gene hierarchy, appear not to be required for germband extension, as all segment polarity mutants that have been examined extend their germbands normally (*dsh*, *wg*, *fu*, *arm*, *gb*, *hh*, *nkd*, *ptc*, *en*; Martinez Arias, 1985; Perrimon and Mahowald, 1987; Wieschaus et al., 1991; data not shown). To strengthen this conclusion, the segment polarity triple mutant *wingless patched engrailed* (Bejsovec and Wieschaus, 1993) was examined, and was found to have only a negligible reduction

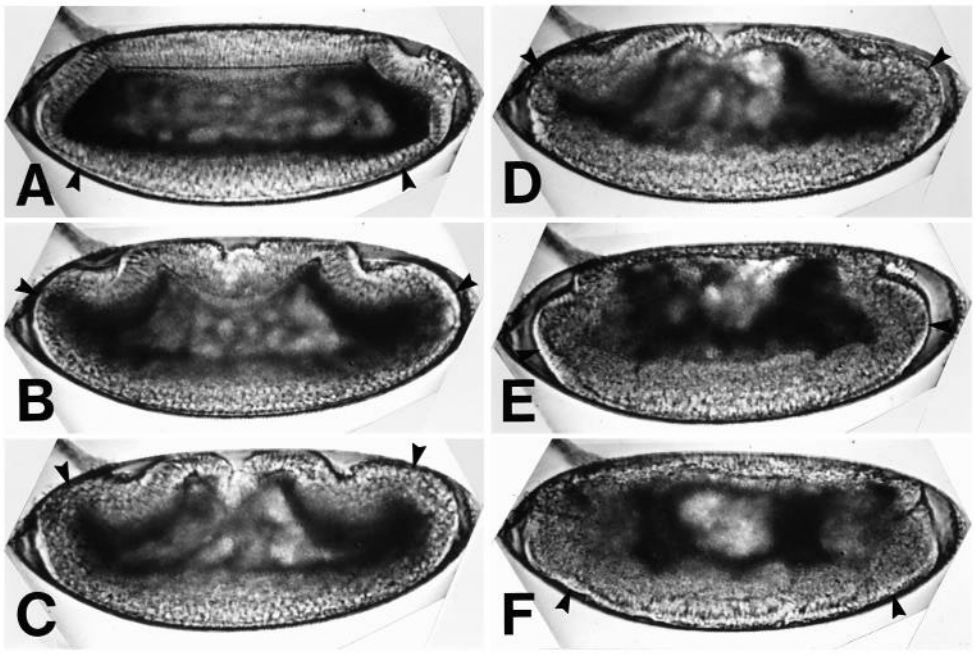

Fig. 6. Germband extension in *bcd nos*. The ends of the germband, defined as the region where mesoderm invaginates, are marked by arrowheads. In A the embryo is beginning germband extension; subsequent panels are 10 minutes (B), 20 minutes (C), 30 minutes (D) 1 hour (E), and 2 hours (F) after beginning germband extension.

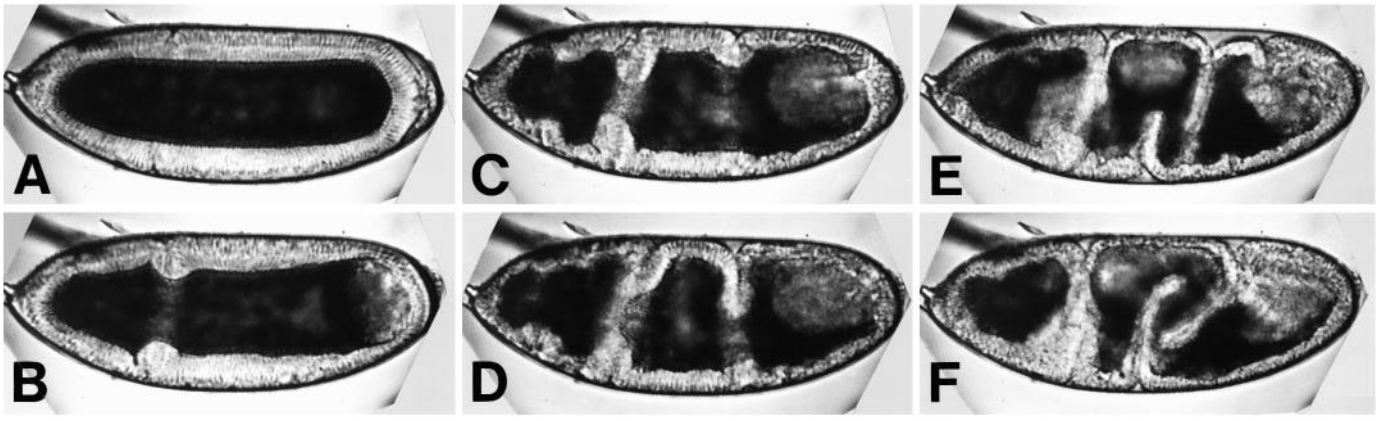

Fig. 7. Germband extension in Tl^{rm10}. In A the embryo is beginning germband extension; subsequent panels are 10 minutes (B), 20 minutes (C), 30 minutes (D), 1 hour (E), and 1.5 hours (F) after beginning germband extension.

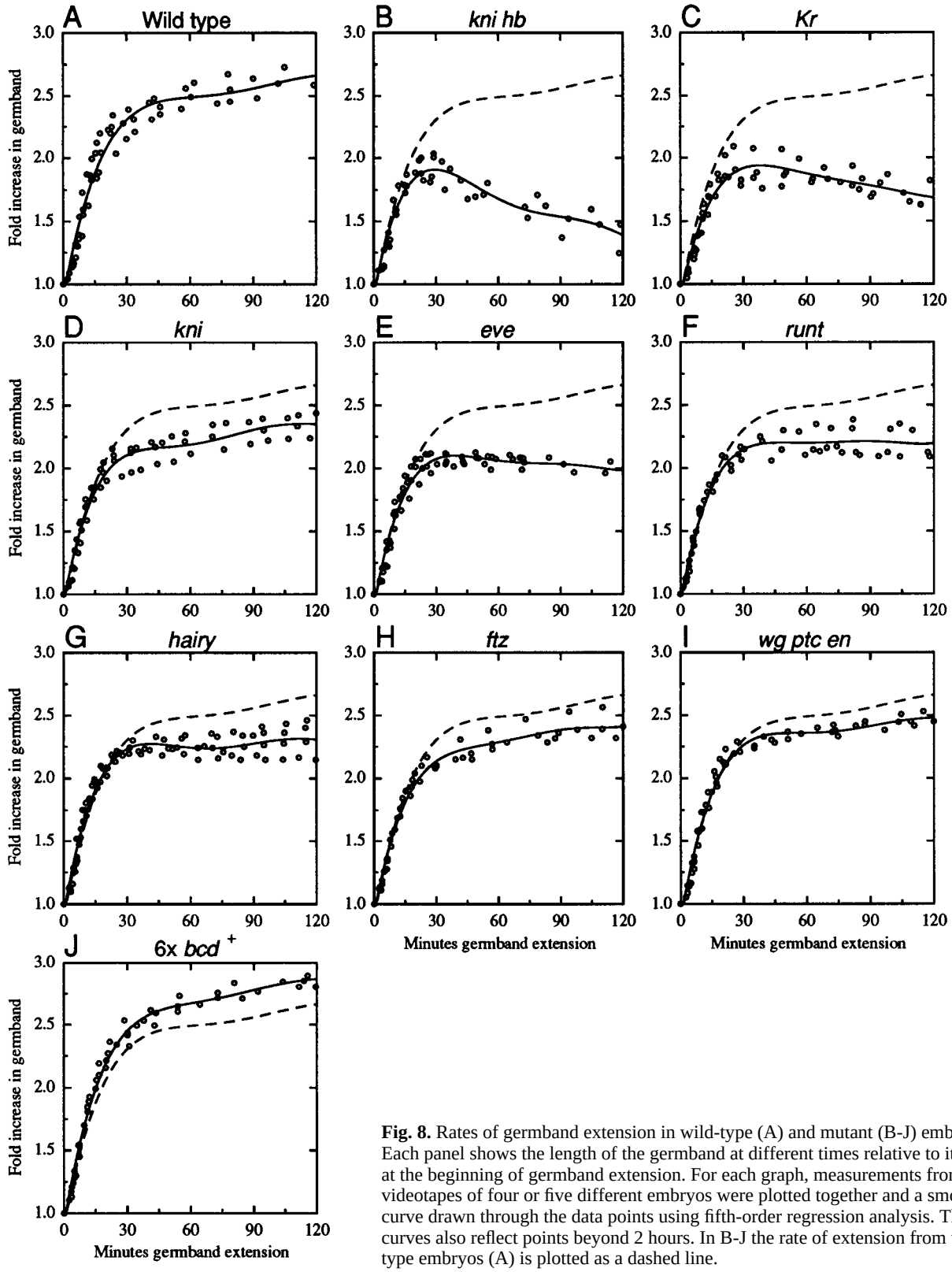

Fig. 8. Rates of germband extension in wild-type (A) and mutant (B-J) embryos. Each panel shows the length of the germband at different times relative to its length at the beginning of germband extension. For each graph, measurements from videotapes of four or five different embryos were plotted together and a smooth curve drawn through the data points using fifth-order regression analysis. These curves also reflect points beyond 2 hours. In B-J the rate of extension from wild-type embryos (A) is plotted as a dashed line.

of germband extension (Fig. 8I). The establishment of distinct identities for different segments is also not required for germband extension, as homeotic mutants extend their germbands normally (data not shown).

The segmentation genes form a transcriptional regulatory hierarchy (reviewed by Ingham, 1988). Hence, the effects of maternal coordinate and zygotic gap genes on germband extension may be accounted for by their regulation of pair-rule

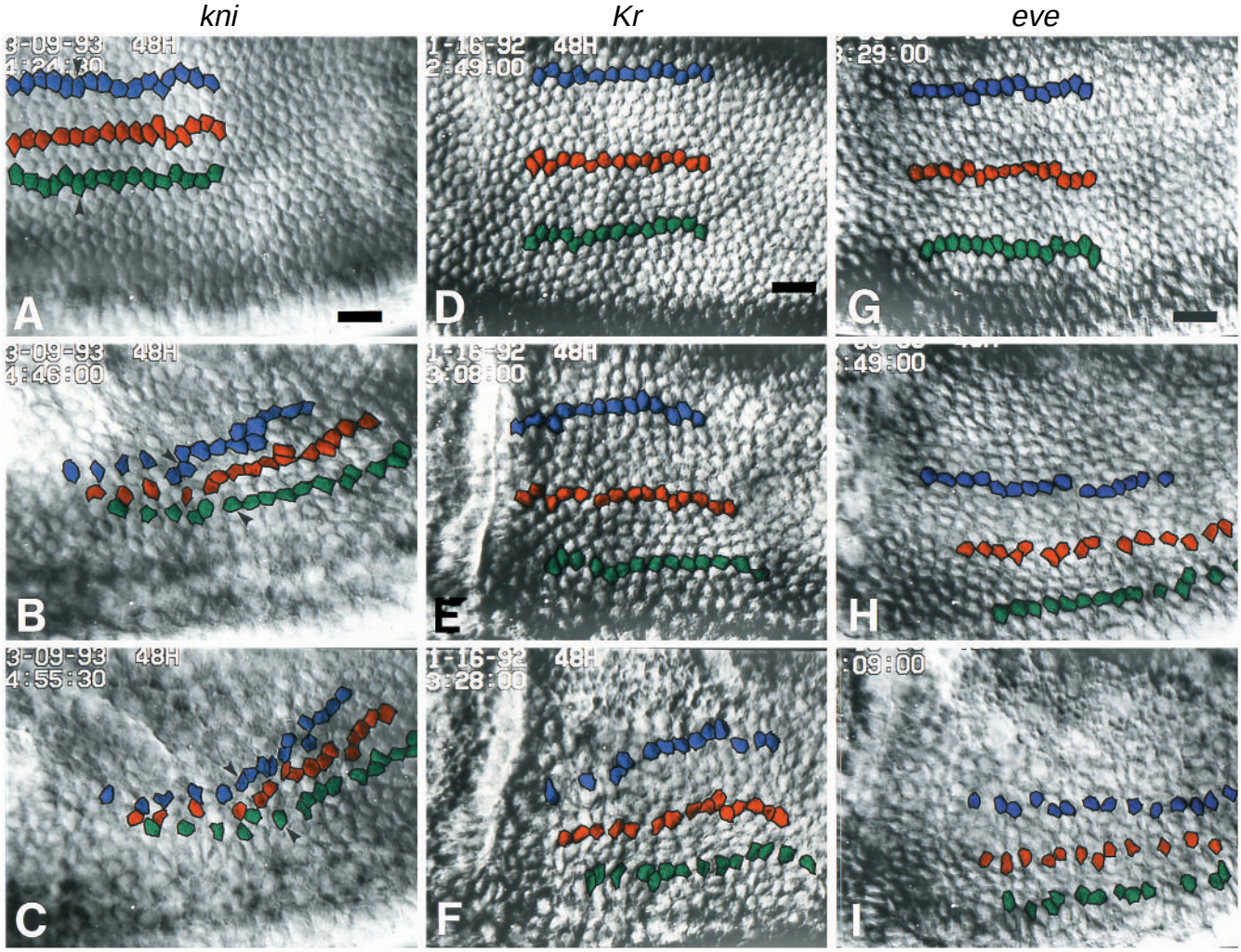

Fig. 9. Cell intercalation in *kni*$^{-}$, *Kr*$^{-}$ and *eve*$^{-}$ embryos. Scale bar (lower right) indicates 20 μm. (A-C) *kni*$^{-}$ embryo 7 minutes before (A), and 14.5 (B) and 24 (C) minutes after beginning germband extension. The initial field of view extends from approximately 22 to 56% egg length, and was shifted one cell posteriorly and ventrally between B and C. In C, the cell rows are curving around the posterior of the embryo. Arrowheads point to the boundary between normal and reduced cell intercalation, which occurs in this embryo at 49% and on average at 48% egg length. This approximates both the border of uniform *eve* expression (approx. 45% egg length; Fig. 11B; Frasch and Levine, 1987) and of uniform or absent *ftz*, *hairy*, or *runt* expression (approx. 50-51% egg length; Klingler and Gergen, 1993). (D-F) *Kr*$^{-}$ embryo 4 minutes before (D) and 15 (E) and 35 (F) minutes after beginning germband extension. The field of view extends from approximately 36-69% egg length. (G-I) *eve*$^{-}$ embryo 5 minutes before (G) and 15 (H) and 35 (I) minutes after beginning germband extension. The initial field of view extends from approximately 35-69% egg length, and was shifted one-and-a-half cells ventrally between G and H.

genes. Pair-rule genes also cross-regulate each other's expression (Carroll and Scott, 1986; Frasch and Levine, 1987; Ingham and Gergen, 1988; Carroll and Vavra, 1989), so it is possible that some of their effects on germband extension are mediated by this cross-regulation. However, since no single pair-rule mutant has as great a reduction of germband extension as *bcd nos* or *kni hb*, the extension detected in wild-type embryos cannot be attributed to the activity of any single pair-rule gene.

Cell intercalation and germband extension correlate with striping of *eve* expression

Because the analysis of germband extension in different segmentation mutants implicated pair-rule genes as playing key roles, and among these *eve* had the greatest reduction of germband extension, we focussed in greater detail on the relationship between *eve* expression, cell intercalation, and germband extension. In addition, as the effects of maternal coordinate and zygotic gap mutations on *eve* expression tend to parallel their effects on other pair-rule genes, *eve* expression can be considered a general marker for how these mutations affect the establishment of stripes of pair-rule gene expression (Carroll and Scott, 1986; Ingham and Gergen, 1988; Carroll and Vavra, 1989; Gaul and Jäckle, 1989; Warrior and Levine, 1990). In *eve* mutants, cell intercalation was detected but was reduced (Fig. 9G-I), consistent with the reduction of germband extension.

The gap and maternal mutants that reduce germband extension all alter *eve* expression. However, in many cases, rather than lacking *eve* expression, they have regions of

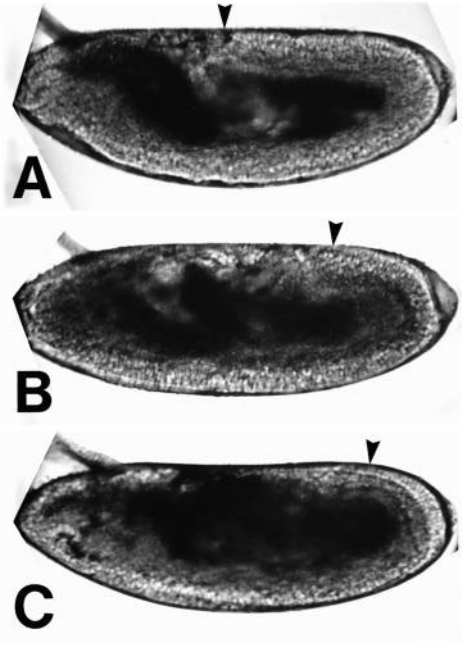

Fig. 10. Germband extension in *hs-eve* and *eve*$^{-}$ embryos. The posterior end of the germband is marked by an arrowhead. Embryos in A and B were heat shocked for 20 minutes during late cycle 14. (A) Oregon-R embryo after 2 hours of germband extension. Early heat shocks slow the initial stages of germband extension. (B) Embryo carrying *hs-eve19B* transgene after 2 hours of germband extension; extension is reduced compared to the control. More severe reductions of germband extension occur with earlier heat shocks (which must also be briefer to avoid cellularization defects). (C) *eve*$^{-}$ embryo after 2 hours of germband extension. The failure of some posterior cells to invaginate into the posterior midgut is typical in mutants that significantly reduce germband extension.

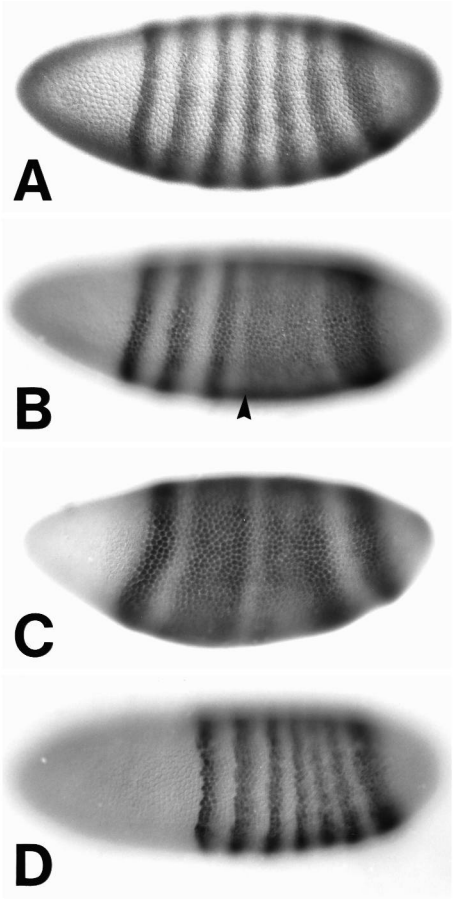

Fig. 11. *eve* expression in blastoderm embryos. (A) Wild-type embryo. (B) *kni*$^{-}$ embryo. The boundary between striped and uniform *eve* expression is marked by the arrowhead. In the ventral ectoderm this occurs at approximately 45% egg length. (C) *Kr*$^{-}$ embryo. (D) Embryo from a mother with six copies of *bcd*$^{+}$. The compression of *eve* stripes parallels the compression of the germband. In all four embryos, the first *eve* stripe marks the position of the cephalic furrow.

uniform *eve* expression. For example, in the absence of both the anterior and posterior maternal patterning systems, *eve* is expressed in a broad region extending from 20-80% egg length (Gaul and Jäckle, 1989). Uniform expression of *eve* can also be generated by inducing expression of a heat-shock promoter-*eve* fusion gene in transgenic flies. Induction of uniform *eve* expression during the end of cycle 14 caused a reduction of germband extension similar to that caused by *eve* mutations (Fig. 10B compare with 10C). With earlier heat shocks even greater reductions of germband extension were observed, possibly reflecting cross-regulation of other pair-rule genes by *eve* (Manoukian and Krause, 1992; data not shown). These observations indicate that it is not the expression per se of *eve* and other pair-rule genes that is required for germband extension, but rather the establishment of striped expression patterns for these genes.

The distinct alteration of pair-rule gene expression in *kni* mutants made it possible to confirm that the effects of segmentation genes on germband extension were mediated by their action within the germband cells undergoing intercalation, rather than on cells outside the germband, which would then indirectly affect extension. *kni* embryos have a region of normally striped *eve* expression adjacent to a broad region of uniform low level *eve* expression (Fig. 11B compare with 11A; Frasch and Levine, 1987). In *kni* embryos undergoing germband extension, the germband appears normal where *eve* stripes form, but is abnormally wide in the region corresponding to where expression of *eve* and other pair-rule genes is uniform (Fig. 12). The implication that intercalation would be reduced in the region where the germband failed to narrow during extension was confirmed by examining *kni* embryos with epi-illumination; a region of normal cell intercalation was detected adjacent to a region of greatly reduced intercalation (Fig. 9A-C). Although the boundary between normal and reduced intercalation in living embryos cannot be mapped precisely relative to the boundaries between striped and uniform pair-rule expression in fixed embryos, judging from their positions along the embryo axis, these do correspond at least approximately. We conclude, therefore, that the effects of segmentation genes on germband extension are at least regionally autonomous.

This correlation between *eve* striping and cell intercalation in *kni* mutants supports the proposal that the effects of maternal coordinate and zygotic gap genes on germband extension are mediated by their regulation of pair-rule genes. This proposal

was further supported by the effects of *Kr* mutations. *Kr* embryos have two large regions of uniform *eve* expression in the germband and lack a contiguous region with normal *eve* stripes (Fig. 11C; Frasch and Levine, 1987; Carroll and Vavra, 1989); the portion of the germband in which *eve* expression is not striped is considerably larger than the central region where *Kr* is expressed (Warrior and Levine, 1990). Consistent with the effect on *eve* expression, *Kr* embryos lack a region with normal germband extension and overall have a greater reduction of extension than *kni* embryos (Fig. 8C compare with D). As in *kni* embryos, the broad *eve* stripes in *Kr* embryos are regions of greatly reduced cell intercalation (Fig. 9D-F).

The segmentation gene mutations that reduce germband extension generate embryos with fewer and broader stripes of pair-rule gene expression. In embryos derived from mothers carrying extra copies of the anterior morphogen gene *bcd*, anterior fates are expanded posteriorly and the germband fate map is compressed (Driever and Nüsslein-Volhard, 1988; Struhl et al., 1989). Seven *eve* stripes and interstripes form, but they are narrower and occupy a smaller region of the embryo (Fig. 11D; Driever and Nüsslein-Volhard, 1988). Strikingly, this compressed germband has a relatively large increase in length during extension. The germband in wild-type embryos increases in length by 2.5-fold during the first hour of germband extension, while the germband in embryos from mothers with six copies of bcd^+ increases in length by 2.7-fold (Fig. 8J). Presumably, this increased extension is associated with increased cell intercalation.

DISCUSSION

Cell intercalation accompanies germband extension

By following cells with epi-illumination and time-lapse video microscopy, we have observed and characterized cell intercalation in the ventral ectoderm during germband extension. This intercalation is oriented such that it contributes to extension, with cells intercalating almost exclusively between their dorsal and ventral neighbors. During the first 45 minutes of germband extension, while the germband elongates to between 2.4 and 2.5 times its initial length, cells become separated from their blastoderm neighbors on average by approximately 1.3 intervening cells (Fig. 2 and data not shown). Thus, the observed cell intercalation accounts for most of the extension (1 cell +1.3 intervening cells = 2.3-fold increase in length). Although we have not yet investigated cell rearrangements during later stages of germband extension, the extension of the germband that occurs during this initial phase comprises about 85% of the total elongation (Fig. 8A). Hartenstein and Campos-Ortega (1985) have also argued that most germband extension in the ventral ectoderm can be accounted for by cell rearrangement, based on measurements and cell counts in fixed embryos.

In addition to accounting for most of the increased length of the germband, the change in the shape of the epithelial sheet that results from cell intercaltion can account for the net ventral and posterior movement of cells (Fig. 3), provided the germband is anchored at its anterior and ventral sides while it is free to contract dorsally and to extend posteriorly (Fig. 13). In fact, because cells in the anterior of the germband initially move anteriorly, it appears that instead of being anchored the germband actually pushes against the head region, which cannot then be displaced further anteriorly. Combined with the ability of the posterior end to move posteriorly and then along the dorsal surface, this effectively directs most of the extension posteriorly. The ventral side of the ectoderm is initially attached to the mesoderm, and then, after the mesoderm invaginates, to the ectoderm of the other side of the embryo. However, the cells dorsal to the germband, those of the amnion-serosa, flatten and spread during germband extension (Campos-Ortega and Hartenstein, 1985). They also appear not to intercalate, as this tissue widens instead of narrowing during extension. Thus, we suggest that cells move ventrally because amnion-serosa cells do not effectively resist the pull of the intercalating germband cells. Because cells remain contiguous, anchoring of ventral cells while intercalation occurs between cells along the dorsal-ventral axis might also bias cell movement during intercalation, accounting for the slight increase in cell number ventrally during early germband extension. This increase is a transient phenomenon, however, as the germband both begins and ends extension roughly rectangular in shape.

It has also been suggested that mitoses oriented along the anterior-posterior axis could contribute to germband extension (Hartenstein and Campos-Ortega, 1985). However, the observation that *string* embryos, which lack all post-blastoderm mitoses, extend their germbands nearly as far as wild-type embryos, indicates that the contribution of cell division to germband extension is slight (Edgar and O'Farrell, 1989). This conclusion is also supported by more recent studies in which the preferential orientation of mitoses along the anterior-posterior axis during germband extension was not observed (Foe, 1989). A small amount of germband extension could also be contributed by the slight increase in the lateral dimensions of ectodermal cells (Hartenstein and Campos-Ortega, 1985).

Distinct forces contribute to germband extension

At least two distinct mechanisms contribute to germband extension in *Drosophila*. We think the germband initially extends in response to a contraction, presumably microfilament mediated, on the dorsal side of the embryo (Rickoll and Counce, 1980). Indeed, the position and orientation of the cytoplasmic stalks, which connect the incipient cells to the yolk sac, argue that there are at least two contractile domains centered around the transient dorsal folds (Costa et al., 1993). In addition, the observation that the initial dorsal movement of the posterior pole precedes germband extension implies that the dorsal surface actively contracts. Finally, the observation of pole cell movement in acellular embryos that parallels their initial movement during germband extension in wild-type embryos implies that cell intercalation is not required for the initiation of germband extension (Rice and Garen, 1975; Rickoll and Counce, 1981).

Despite the pull that this contraction exerts on the germband, the effects of mutations that prevent the formation of a posterior midgut (e.g. *fog*, *torso*), and of mutations that result in the formation of only ventral ectodermal cell fates (e.g. Tl^{rm10}), indicate that cells can actively intercalate. Embryos from Tl^{rm10} mothers lack a dorsal contraction, so the cell intercalation and germband extension that occur cannot be attributed to its pull. Further, in embryos from both *torso* and Tl^{rm10} mothers the end of germband becomes fixed near the posterior pole; hence, there can be no effective external pull. Despite

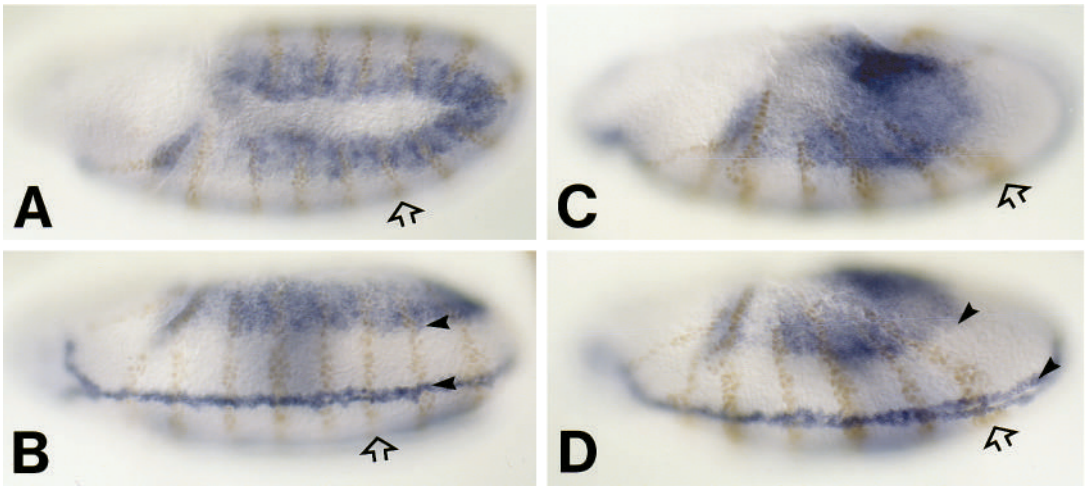

Fig. 12. Germband width in wild-type (A,B) and *kni*$^{-}$ (C,D) embryos. A and C are lateral views; B and D are ventral-lateral views. These stage 9 embryos (about 1 hour into germband extension) are stained for expression of *en* protein (orange stripes, marks posterior compartment of each segment; DiNardo et al., 1985), *decapentaplegic* (*dpp*; broad blue stripe, marks dorsal epidermis; St. Johnston and Gelbart, 1987), and *single-minded* (*sim*; thin blue stripe, marks ventral midline, not visible in A and C; Crews et al., 1988). The ventral ectoderm (delimited by arrowheads) includes the *sim*-expressing cells and the unstained region between *sim* and *dpp* expression. Germband width begins to increase in *kni*$^{-}$ at the seventh (parasegment 6) engrailed stripe (open arrow). In the embryo shown in D the width of the ventral ectoderm averaged 9 cells between the third and sixth *en* stripes, 10 cells in the seventh *en* stripe, 11-12 cells just posterior to the seventh *en* stripe, and 16-18 cells at its widest point. Based on the relationship between pair-rule and *en* stripes (Lawrence et al., 1987), the seventh *en* stripe corresponds to the beginning of absent *hairy* and uniform *ftz* and *runt* expression, and at blastoderm is about three cells anterior to the region of uniform *eve* expression.

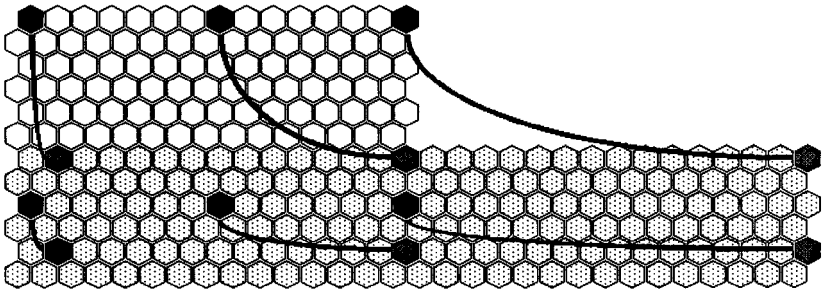

Fig. 13. Cartoon illustrating how cell intercalation could account for overall cell movements in the embryo. In this example, a cell sheet (initial position unshaded, final position lightly shaded and superimposed) doubles in length by evenly spaced intercalations while it is anchored anteriorly and ventrally. Under these conditions, a cell's net movement varies with its relative position within the sheet, with more dorsal cells moving further ventrally than more ventral cells, and more posterior cells moving further posteriorly than more anterior cells. For purposes of illustration, the initial (black) and final (darkly shaded) positions of six cells have been connected by elliptical curves.

this, germband extension and cell intercalation continue, indicating that a force driving extension is generated within the germband. These patterning mutations also indicate that extension does not require the presence of distinct cell fates either dorsal, ventral, or posterior to the ventral ectoderm. In wild-type embryos, the absence in much of the germband of cell stretching, which is typically associated with passive cell intercalation (Keller, 1987; Fristrom, 1988), also suggests that cells actively intercalate. The conclusion that germband cells actively intercalate is consistent with experiments performed on the silkworm, *Bombyx mori*, in which the germband still extends even when dissected away from the rest of the egg (Krause and Krause, 1964).

In contrast to the continued extension and cell intercalation observed in the absence of any external pull on the germband, there is no net germband extension or separation of cells along the anterior-posterior axis when segmentation gene expression is totally disrupted in the germband. Thus, although the germband initially extends in embryos from *bcd nos* mothers, the extension is only transient, and is also distinguished from that in wild type by greatly reduced cell intercalation and the appearance of some cell stretching. Two observations indicate that the reduction of germband extension and cell intercalation in segmentation mutants is not mediated by an effect on the dorsal contraction. First, the initial rate of germband extension, when the dorsal surface visibly contracts, is unaltered in segmentation mutants. Second, the reduction of cell intercalation and germband extension is regionally autonomous in *kni* mutants. If, by contrast, this reduction were due to an effect of segmentation genes on an external force, then the reduction would be expected to be distributed uniformly across the germband. Alternatively, segmentation genes could be required to allow cells to separate in response to an external pull. However, the observations that cells actively intercalate and that segmentation genes are required for normal and sustained cell intercalation argue against this. Instead, these observations together support the conclusion that segmentation genes effect active cell intercalation. This conclusion is strengthened by the observation that the active extension in embryos from terminal group mutants (e.g. *torso*, *tsl*) is eliminated if their mothers are additionally mutant for *bcd* and *nos*. Although the ultimate failure of germband extension in the absence of anterior-posterior patterning implies that intercalation mediated by segmentation genes is the key force driving

extension, in wild-type embryos both active intercalation and the dorsal contraction appear to act in concert, with the dorsal contraction serving to initiate, orient, and increase the rate of germband extension.

In addition to the ventral ectoderm, the germband comprises dorsal epidermis and mesoderm. Like lateralized embryos, apolar dorsalized embryos extend their germbands (Nüsslein-Volhard, 1979). This extension implies that dorsal epidermal cells can also undergo cell intercalation. Because dorsal epidermal cells go through mitosis early in germband extension and hence are difficult to follow with epi-illumination, we have not attempted to chart their movements. However, we have observed extensive cell intercalation in the dorsal epidermis of *string* mutant embryos (data not shown). In contrast to dorsalized and lateralized embryos, ventralized embryos, which make only mesoderm, fail to extend their germbands (Schüpbach, 1987; Roth et al., 1991). This suggests that the rearrangement of mesodermal cells that occurs during germband extension results from their adherence to ectodermal cells, rather than from active intercalation (Costa et al., 1993). Additionally, *twist* and *snail* mutant embryos, which lack mesoderm, extend their germbands almost normally (Simpson, 1983; Leptin and Grunewald, 1990).

A model for cell intercalation and germband extension

Why do cells intercalate? Our observations demonstrate that intercalation depends upon the establishment of positional differences along the anterior-posterior axis, not the dorsal-ventral axis, despite the fact that cells intercalate between dorsal and ventral neighbors. This rules out models in which cells migrate ventrally in response to a graded signal. Instead, intercalation depends upon the establishment of normal stripes of pair-rule gene products. When these stripes are widened or eliminated, either by pair-rule mutations, mutations in genes that regulate pair-rule gene expression, or ubiquitous expression of *eve*, then germband extension is reduced. The severity and location of the reduction correlates with how pair-rule gene striping is altered. When pair-rule stripes are narrowed, as in embryos from mothers with extra copies of bcd^+, then germband extension is increased. To explain these observations, we propose that pair-rule genes establish stripes of cells that differ in adhesiveness (Fig. 14). Pair-rule genes encode transcription factors, so these differences would be established by their regulation of other genes that affect cell adhesiveness.

The behavior of groups of adhesive cells has been modeled and investigated by Steinberg (1962, 1978). He noted that adhesive cells adopt configurations that obey thermodynamic rules. That is, cells rearrange until they are in the configuration of lowest energy, which is generally the configuration that maximizes contacts between cells of like adhesiveness. For a group of cells with identical adhesiveness, this configuration is a sphere, or in the case of an epithelium, a circle. With combinations of cells that differ in adhesiveness the situation is more complicated, but can still be predicted on thermodynamic grounds. If the pair-rule genes established alternating stripes of cells that differed in adhesiveness, then each stripe would contract to maximize contacts among cells of like adhesiveness (Fig. 14A). Provided the cells of each stripe also had some adhesiveness for cells of neighboring stripes, these stripes of cells would remain in contact. If cells of different stripes differed quantitatively in their strengths of adhesion, then less adhesive cells would encircle more adhesive cells (Steinberg, 1962, 1978). Therefore, this model also requires that cells of neighboring stripes differ qualitatively in their adhesiveness but that the relative strengths of adhesion among cells within different stripes be equal.

Although the postulated adhesive molecules remain to be identified, the ability of this model to explain the effects of embryonic patterning mutations on germband extension make it attractive. Because a group of adhesive cells will rearrange until it is in a configuration that is about as long as it is wide, the model predicts that the narrower pair-rule stripes observed in embryos derived from mothers with extra copies of bcd^+ will have increased intercalation and germband extension. Conversely, because they are already nearly in the configuration that maximizes contacts among cells of like adhesiveness, the wider pair-rule stripes observed in gap mutants like *kni* are predicted to have decreased intercalation (Fig. 14B). When the germband lacks positional differences but is stretched into an elongated configuration, as in embryos from *bcd nos* mothers, the model predicts that intercalation will occur between anterior and posterior neighbors as the germband contracts.

In its simplest form, this model for cell intercalation requires only two types of adhesive cells, which would be distributed in alternate segments. Although direct evidence for such a distribution of cell adhesiveness in the early *Drosophila* embryo is lacking, we find it intriguing that evidence in favor of just such a pattern of segmentally alternating differences in cell adhesiveness has been discovered in avian rhombomeres (Guthrie et al., 1993). Alternating stripes of adhesive cells

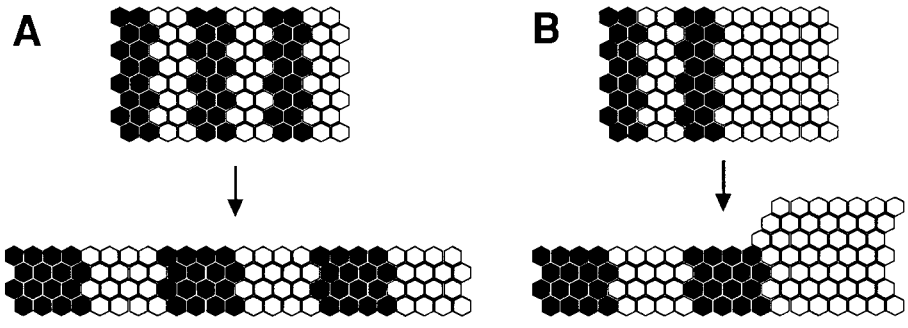

Fig. 14. Cartoon illustrating model for cell intercalation. (A) Pair-rule genes establish alternating stripes of cells with different adhesive properties. Cells then rearrange into the configuration that maximizes contacts among cells of like adhesiveness. If stripes of cells are constrained to remain in contact with their neighbors, this configuration is roughly square. (B) In kni^- embryos, cell intercalation occurs normally where pair-rule stripes form. The broad region of uniform *eve* expression establishes a group of adhesive cells that is already close to the optimal shape for maximizing cell contacts, so little intercalation takes place.

could explain many of our observations, but other distributions of adhesive molecules are also possible. Indeed, Gergen et al. (1986) and Wieschaus et al. (1991) have proposed a model for germband extension that relies on a graded distribution of adhesive differences. Regardless of how adhesive molecules are actually distributed, we think the dependence of cell intercalation on the establishment of pair-rule gene stripes, which are perpendicular to the direction of extension, supports models in which cells rearrange in response to adhesive differences.

Germband extension and convergent extension

Germband extension is an example of a process that in vertebrate gastrulation and neurulation has been termed convergent extension (reviewed by Keller et al., 1991). Similar processes, in which cells coordinately rearrange to increase the length and decrease the width of a tissue, occur in a variety of organisms (e.g. Jacobson and Gordon, 1976; Keller, 1978; Ettensohn, 1985; Warga and Kimmel, 1990), and a number of mechanisms have been proposed to explain why cells rearrange (reviewed by Keller, 1987; Fristrom, 1988). In fact, because there are significant differences between distinct examples of cell rearrangement, there must certainly be multiple mechanisms that can drive cell rearrangement. For example, cell rearrangements can occur among epithelial or non-epithelial cells, can be either active or passive, and can occur on time scales of seconds to days. Among described examples of cell rearrangement, germband extension has some similarities to archenteron elongation in sea urchins, as both involve active cell rearrangements in invertebrate epithelia and occur on similar time scales, with the archenteron undergoing a threefold extension in 3-4 hours (Ettensohn, 1985). Could the model we have proposed here for germband extension, in which segmental stripes of adhesive cells contract, be applied to any other examples of cell rearrangement? The lack of knowledge about the basic genetic and molecular requirements for these processes makes it impossible to be certain. However, it is intriguing that, as in the *Drosophila* germband, in some cases cell intercalation occurs in a tissue that is or will soon be obviously segmented.

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