Complementary patterns of *even-skipped* and *fushi tarazu* expression involve their differential regulation by a common set of segmentation genes in *Drosophila*

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We have examined the position-dependent expression of two segmentation genes that contain a homeo box, *even-skipped* (*eve*) and *fushi tarazu* (*ftz*). Products encoded by both genes accumulate in a series of seven transverse stripes along the anterior-posterior body axis of developing embryos. Double-staining experiments show that *eve* and *ftz* proteins accumulate in complementary sets of embryonic cells. A total of eight zygotically active segmentation genes are required for the establishment and refinement of the *eve* and *ftz* expression patterns during development. Mutations that affect one of the genes were always found to produce a reciprocal alteration in the other. These results suggest that the *eve* and *ftz* promoters independently "interpret" the same positional cues to give differential patterns of expression. We propose that a combination of segmentation gene products that activates the expression of *eve* represses the expression of *ftz*, and vice versa. This proposal is discussed in the context of a hierarchy of interactions among segmentation genes, whereby the gap genes establish asymmetric patterns of *eve* and *ftz* expression in blastoderm-stage embryos, and interactions with other pair-rule genes serve to refine further the complementarity of *eve* and *ftz* expression during gastrulation and germ band elongation.

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The nine pair-rule genes of *Drosophila* are required for the subdivision of the developing embryo into a repeating series of homologous body segments (Nüsslein-Volhard and Wieschaus 1980; Nüsslein-Volhard et al. 1985). Mutations in any one of these genes typically result in embryos that lack pattern elements in alternating segments. *eve* appears to be particularly important for the overall segmentation pattern since "strong" *eve* mutants lack all segmental subdivisions in the middle body region (Nüsslein-Volhard et al. 1985). In contrast, null mutations in any of the other eight pair-rule genes do not completely eliminate segmentation. There is a close correspondence between the sites of *eve* expression in wild-type embryos and the regions deleted in *eve* mutants (Harding et al. 1986; Macdonald et al. 1986; Frasch et al. 1987). Using an antibody raised against a β -galactosidase-*eve* fusion protein, it has been shown that the *eve* protein is distributed in a series of seven transverse stripes at the cellular blastoderm stage (Frasch et al. 1987). These sites of expression define the odd-numbered parasegments (a parasegment corresponds to the posterior compartment of one segment and the anterior compartment of the adjacent segment; Martinez-Arias and Lawrence 1985). The odd-numbered parasegments are deleted in weak *eve* mutants (i.e., hypomorphs), and

only the strongest *eve* mutants (i.e., null alleles) delete both the odd- and even-numbered parasegments. During earlier and later periods of wild-type development, the *eve* protein transiently accumulates in the even-numbered parasegments as well. For example, during gastrulation a 14-stripe pattern of expression occurs whereby high levels of the *eve* protein are observed in the original odd-numbered parasegments and lower levels are detected in the even-numbered parasegments. Thus, there is strong and persistent expression of the *eve* protein in those regions where there is a strong requirement for *eve* gene function (the odd-numbered parasegments), and weak expression in those regions where there is only a weak genetic requirement (the even-numbered parasegments).

It has been proposed that the establishment of selective patterns of pair-rule gene expression involves a network of hierarchical interactions among several classes of segmentation genes (Carroll and Scott 1986; Howard and Ingham 1986; Harding et al. 1986; Macdonald et al. 1986; Ingham and Martinez-Arias 1986). The first step in this process is thought to involve the activities of maternally expressed genes, which are responsible for establishing the basic features of the anterior-posterior organization of the embryo (Nüsslein-Volhard 1979;

Meinhardt 1986). Such maternal components appear to be required for the expression of zygotically active gap genes, which subdivide the embryo into broad regions that encompass several contiguous segment primordia. Gap mutants show deletions of large groups of adjacent segments. For example, mutations in the gap gene *knirps* (*kni*) result in deletions of the first through seventh abdominal segments (Nüsslein-Volhard and Wieschaus 1980).

Cross-regulatory interactions have been shown to occur among gap genes (Jäckle et al. 1986), and it has been proposed that combinations of gap gene products establish periodic patterns of pair-rule gene expression (Meinhardt 1986). The recent demonstration that gap mutants show strongly altered patterns of *fushi tarazu* (*ftz*) expression is consistent with a hierarchy of interactions whereby gap genes influence the segmentation pattern by regulating the expression of pair-rule genes (Carroll and Scott 1986; Ingham et al. 1986).

Here, we examine possible genetic mechanisms for the establishment of the periodic pattern of *eve* expression, as well as that of *ftz*, two genes that contain a homeo box-coding sequence (Scott and Weiner 1984; McGinnis et al. 1984a,b; Laughon and Scott 1984). Mutations in these two pair-rule genes produce reciprocal defects, in that *ftz*⁻ embryos show deletions of even-numbered parasegments (Wakimoto et al. 1984; Martinez-Arias and Lawrence 1985), and "weak" *eve* mutants show deletions of odd-numbered parasegments (Nüsslein-Volhard et al. 1985). Simultaneous localization of *eve*⁺ and *ftz*⁺ products in wild-type, gastrulating embryos shows that these genes are expressed in complementary sets of embryonic cells (Harding et al. 1986; see below), as predicted by previous genetic studies. We have examined the basis for these reciprocal patterns of expression by analyzing the distribution of *eve* and *ftz* proteins in various mutants that disrupt segmentation. These results suggest that the establishment of complementary patterns of *eve* and *ftz* expression involves the differential regulation of these genes by a common set of segmentation loci.

Results

Wild-type expression

The *eve* protein is first detected after the 11th nuclear cleavage, ~2 hr following fertilization (Frasch et al. 1987). At this time the protein is detected at uniform levels in each of the embryonic nuclei, including those located at the cortex as well as the yolk nuclei. A localized staining pattern is first detected during the 13th nuclear cleavage, whereby nuclei located in the posterior two-thirds of the embryo are more strongly stained as compared with those located more anteriorly (Fig. 1a). A rather sharp anterior boundary of staining is detected at ~70% egg length (where 100% egg length corresponds to the distance from the posterior pole to the anterior pole). Over the next ~20 min, in the absence of nuclear divisions or rearrangements, a periodic pattern of *eve* expression gradually unfolds, whereby *eve* protein is local-

Figure 1. (See facing page for legend.)

ized in a series of seven transverse stripes along the anterior–posterior axis (Fig. 1b). At this stage each expression stripe includes approximately four or five cells, and adjacent stripes are separated by approximately three to four cells that show lower levels of expression. Note that there is strong staining in the “inter-stripe” regions at this time, and the protein is actually distributed in a continuous, bell-shaped pattern. There is a refinement of this periodic pattern during gastrulation, such that each stripe gradually narrows and shows a progressively sharper anterior border. This sharpening appears to involve a gradual loss of staining from the more posterior cells of each stripe (see Fig. 10 for more details on this sharpening process). As germ band elongation begins (~4 hr after fertilization) seven new stripes of expression can be detected in addition to the original stripes (Frasch et al. 1987). Each of these new stripes is one to two cells wide and is much weaker than the original stripes. Both the original and new stripes gradually disappear during germ band elongation.

The *ftz* protein is distributed in a series of seven transverse stripes and shows a “four on, four off” pattern in cellular blastoderm-stage embryos (Carroll and Scott 1985). During cleavage cycle 14 the *eve* and *ftz* expression patterns overlap (Fig. 1c). This overlap largely results from the broad, bell-shaped distribution of each *eve* stripe (Fig. 1b,d). Between six and seven of the approximately eight nuclei that comprise a “double segment” are stained by the *eve* antibody (Fig. 1d). The *eve* and *ftz* stripes must overlap within at least two nuclei per double segment since each *ftz* stripe includes four nuclei at this stage (Carroll and Scott 1986). It is likely that the

overlap is even more extensive since the broad, composite stripes of *eve* + *ftz* staining are separated by a row of nuclei that show lower levels of staining (Fig. 1c and arrow, Fig. 1e). Detailed measurements of the relative positions of the *eve* and *ftz* stripes in embryos that have just completed cellularization indicate that the two expression patterns are “out of register” (see Fig. 10). Similar results were obtained by staining embryos with a mixture of mouse-anti-*ftz* and rabbit-anti-*eve* antibodies, which permitted the visualization of the two patterns with different fluorescent dyes. Non-overlapping patterns begin to emerge during gastrulation, and by the onset of germ band elongation the two genes show reciprocal patterns of expression (Fig. 1f). The establishment of these complementary patterns of *eve* and *ftz* expression was analyzed by examining the distribution of *eve* and *ftz* proteins in a variety of segmentation mutants, as described below.

Hunchback⁻

The gap gene *hunchback* (*hb*) is required for the establishment of both anterior and posterior body segments, including the labium and thorax as well as portions of the seventh and eighth abdominal segments (Lehmann 1985; Lehmann and Nüsslein-Volhard 1987; see Fig. 2). Parasegments 2–7 and parasegment 13 are either partially or completely deleted in *hb*⁻ mutants. These deleted regions correspond to *eve* expression stripes 2 (parasegment 3), 3 (parasegment 5), 4 (parasegment 7), and 7 (parasegment 13).

Embryos homozygous for a strong point mutation of *hb* show an abnormal pattern of *eve* expression, which closely corresponds to the segments deleted in advanced-stage *hb*⁻ embryos. This altered *eve* pattern is detected during the time when selective patterns of *eve* expression are first established, just prior to the completion of cellularization (Fig. 3a,b). The region from 73% to ~50% shows two broad bands of expression in place of the four narrower stripes seen in wild-type (Frasch et al. 1987). In addition, there is a reduction of the seventh *eve* stripe; the fifth and sixth stripes appear essentially normal. This *eve* pattern persists in gastrulating *hb*⁻ embryos (Fig. 3c), as well as those undergoing germ band elongation (data not shown). As for wild-type, *hb*⁻ embryos show a gradual “sharpening” of the *eve* expression stripes during gastrulation.

It has been shown that the *ftz* expression pattern is strongly altered in *hb*⁻ embryos (Carroll and Scott 1986). Comparison of the altered *eve* and *ftz* patterns reveals that they show reciprocal defects. Thus, regions in *hb*⁻ embryos where *eve* proteins are expressed coincide with regions where *ftz* proteins are not expressed, and vice versa. The simultaneous staining of *hb*⁻ embryos with anti-*eve* and anti-*ftz* antibodies clearly demonstrates the complementarity of the two expression patterns (Fig. 3d).

Krüppel⁻

The gap gene *Krüppel* (*Kr*) is required for the establishment of the thorax and anterior abdomen, including each of the three thoracic segments as well as the first

Figure 1. Distribution of *eve* and *ftz* proteins in wild-type embryos. Whole-mount preparations of fixed embryos were stained with affinity-purified anti-*eve* and anti-*ftz* antibodies, and visualized by indirect immunofluorescence using rhodamine-conjugated goat anti-rabbit antibodies. Embryos are oriented so that anterior is to the left and ventral is down. (a) Embryo undergoing the thirteenth nuclear cleavage that was stained with the anti-*eve* antibody. Staining is restricted to nuclei located from 70% egg length to the posterior pole. The strongest staining occurs from 70% to 50% egg length. (b) Cellular blastoderm embryo after staining with anti-*eve*. The *eve* protein is distributed in a series of seven transverse stripes along the anterior–posterior axis. Note the broad, bell-shaped pattern of *eve* expression. (c) Embryo just prior to the onset of gastrulation that was double-stained with anti-*eve* and anti-*ftz* antibodies. The *eve* expression stripes (strongly stained and designated e) have begun to sharpen at their anterior borders. The *ftz* stripes (more weakly stained and designated f) are broader, and each encompasses an average of four cells. The *eve* and *ftz* stripes partially overlap, and there are no unstained nuclei in the region from ~70% to 15% egg length. (d) High-magnification view of an embryo at the same stage as that shown in c; the fourth through the seventh *eve* stripes are shown. (e) High magnification of the embryo in c, which shows the second through fifth composite *eve* + *ftz* stripes. (f) Embryo at the onset of germ band elongation that was stained with anti-*eve* and anti-*ftz* antibodies. By this time *eve* and *ftz* show reciprocal patterns of expression, and there is a gap of ~1 cell in width that separates adjacent stripes. The *ftz* stripes coincide with the weak, transient *eve* stripes that are observed at this stage. (CF) Cephalic furrow.

Figure 2. Domains of gap gene function. This diagram shows the pattern elements that are deleted in advanced-stage embryos for each of the indicated gap mutants, as well as the regions deleted in "weak" *eve* mutants. For example, the labium, thorax, and portions of A8 are deleted in *hb*⁻ embryos. The top two lines relate the positions of segments with parasegments. For example, "weak" *eve* mutants lack portions of the labium and prothorax, which corresponds to parasegment 3. As can be seen, each gap gene has an overlapping, but unique domain of function. Lighter bars correspond to more weakly affected segments. For example, the eighth abdominal segment and telson are deleted in *tll*⁻, whereas the second through seventh abdominal segments are expanded progressively toward the posterior pole. The asterisk indicates the deletion of the malpighian tubules in *Kr*⁻ embryos. (A1–A8) First through eighth abdominal segments; (cl I) class I *hb*⁻ mutant, which corresponds to the null phenotype (Lehmann and Nüsslein-Volhard 1987); (Lb) labium; (Md) mandible; (Mx) maxilla; (PS) parasegments; (T1–T3), the first through third thoracic segments.

five abdominal segments (Gloor 1950; Nüsslein-Volhard and Wieschaus 1980; Wieschaus et al. 1984a; Knipple et al. 1985; see Fig. 2). *Kr*⁻ embryos show an abnormal pattern of *eve* expression, and as observed for *hb*, there is a close correspondence between the sites where the *eve* pattern is strongly altered, and the segments deleted in *Kr*⁻. An abnormal *eve* pattern is first detected during cellularization, when a prominent band of staining is detected at ~35% egg length, in addition to the normal first stripe. There is an abnormally low level of staining in the region posterior to the first stripe (Fig. 4a), where the second and third stripes normally arise in wild-type (Frasch et al. 1987). After cellularization, two broad bands of *eve* expression are observed in place of *eve* stripes 2–6 (Fig. 4b); the first and seventh *eve* stripes appear normal.

During gastrulation each of the *eve* bands sharpens, and nonuniform staining becomes evident along the dorsal–ventral axis (Fig. 4c). It has been shown that the *ftz* pattern is also strongly disrupted in *Kr*⁻ embryos (Carroll and Scott 1986; Ingham et al. 1986), and, as for *eve*, dorsal–ventral asymmetries were observed. These dorsal–ventral differences of *eve* and *ftz* expression are consistent with the observation that abnormalities in the segmentation pattern of *Kr*⁻ embryos are not equivalent in dorsal versus ventral tissues (Wieschaus et al. 1984a). To test the possibility that altered patterns of *eve* and *ftz* expression are reciprocal in *Kr*⁻, we simultaneously stained mutant embryos with anti-*eve* and anti-*ftz* antibodies (Fig. 4d). As for *hb* mutants, complementary *eve* and *ftz* patterns are observed. The extent to which these patterns are reciprocal is best illustrated by comparing *eve* and *ftz* staining in the broad anterior do-

main, where the dorsal–ventral asymmetries are most pronounced. For example, the ventral region where *eve* staining is split into two narrower stripes is "filled" by strong staining with the anti-*ftz* antibody (Fig. 4d).

Subtle alterations of the *eve* and *ftz* expression patterns were observed in approximately one-half of the embryos collected from *Kr*⁺/*Kr*⁻ adults, which probably correspond to *Kr*⁺/*Kr*⁻ heterozygotes (Fig. 4e). *Kr* heterozygotes are fully viable, although there is a dominant effect on the segmentation pattern, which includes subtle defects in the mesothorax and anterior meta-thorax, as well as in the second abdominal segment (Wieschaus et al. 1984a). Homeotic transformations are occasionally observed for several of the cuticular structures associated with these segments in adults. The *eve* and *ftz* expression patterns are "compressed" in *Kr* heterozygotes; this region includes the third *eve* stripe through the fourth *ftz* stripe. Normally this domain of *eve* and *ftz* expression extends from ~53% to 37% egg length, but in *Kr* heterozygotes extends from only ~52% to 42% (see arrows, Fig. 4e; summarized in Fig. 10). This region includes parasegments 5–8, which overlaps with the primary domain of *Kr*⁺ expression in wild-type (Knipple et al. 1985; Jäckle et al. 1987).

Knirps⁻

The gap gene *knirps* (*kni*) is required for the segmentation of the abdomen (Nüsslein-Volhard and Wieschaus 1980; see Fig. 2). *kni*⁻ embryos show abnormal *eve* and *ftz* patterns that closely correspond to the segments deleted in advanced-stage mutants. After cellularization, the fourth, fifth and sixth *eve* stripes are essentially ab-

sent, whereas stripes 1, 2, and 3 appear normal (Fig. 5a,b). The seventh stripe is shifted toward a more anterior position (see Fig. 10). The patterns of *eve* and *ftz* expression show reciprocal defects in *kni*⁻, as demonstrated by double-staining experiments with anti-*eve* and anti-*ftz* antibodies (Fig. 5c). As shown previously, the third through sixth *ftz* expression stripes are fused into a single broad band in *kni*⁻ (Carroll and Scott 1986), which coincides with the region where *eve* staining is absent.

A weak alteration of the *eve* expression pattern is also observed in heterozygous *kni*⁻/*kni*⁺ embryos. Such embryos are fully viable and occasionally show abnormalities of the fourth abdominal segment (Lehmann 1985). The fifth *eve* expression stripe is strongly reduced in *kni* heterozygotes, and in addition, the sixth and seventh stripes are shifted to more anterior positions as compared with wild-type (Fig. 5d; summarized in Fig. 10). In *kni* heterozygotes, the fifth and sixth *eve* stripes are confined to a narrower region along the anterior-posterior axis as compared with wild-type. Thus, the embryonic fate map is "compressed" within the normal domain of *kni*⁺ gene function (arrows, Fig. 5e), and there is a corresponding expansion of posterior regions of the fate map.

Hunchback⁻, Knirps⁻ double mutants

hb⁻, *Kr*⁻ embryos lack segment boundaries and show a continuous lawn of denticle hairs, which is a phenotype similar to strong *eve*⁻ embryos (Lehmann 1985). The altered patterns of *eve* and *ftz* expression that are observed in such mutants do not appear to represent a simple addition of the *eve* and *ftz* expression patterns seen in *hb*⁻ and *kni*⁻ embryos. In double mutants, *eve* and *ftz* proteins are not localized within stripes, but instead show continuous staining along the anterior-posterior axis (Fig. 6a,c). Both genes show approximately the same limits of expression, from ~74% to 13% egg length. Within this stained interval there are regions that show

somewhat reduced levels of expression (see arrows, Fig. 6a,c). The *eve* staining pattern rapidly diminishes during gastrulation (Fig. 6b), whereas *ftz* staining persists at a high level (Fig. 6d), similar to that observed in wild-type. During germ band elongation, discontinuities become more pronounced in the *eve* and *ftz* expression patterns, such that an anterior "stripe" can be discerned near the cephalic furrow (Fig. 6d). In addition, heterogeneities in the level of *ftz* staining become apparent, so that a mottling of the pattern can be seen in posterior regions of the germ band. Altered patterns of *eve* and *ftz* expression are also observed in *hb*⁻, *kni*⁻/*hb*⁺, *kni*⁺ heterozygotes

Figure 3. *eve* and *ftz* expression in *hunchback* mutants. The presentation of embryo whole mounts is described in the legend to Fig. 1. (a) Cleavage cycle 14 embryo just prior to the completion of cellularization, which was stained with anti-*eve* antibody. Two broad bands of staining are seen from 73% to 50% egg length. (b) Cellular blastoderm stage mutant. There is a broad domain of *eve* staining from 73% to 50% egg length. The two bands observed earlier (see a, above) continue to show the strongest staining within this region. In wild-type, this domain contains the first four *eve* stripes. The fifth and sixth stripes appear normal, whereas there is a marked reduction in the staining of the seventh stripe. (c) Gastrulating mutant oriented such that the ventral furrow can be seen. Each of the broad bands of staining observed in b have sharpened. Note that the seventh stripe persists at a reduced level of staining. (d) Similar to c, except that this embryo was double-stained with anti-*eve* and anti-*ftz* antibodies. The strong staining corresponds to *eve* (e), whereas the weaker staining corresponds to *ftz* (f). The region designated (e) corresponds to the reduced seventh *eve* stripe.

(summarized in Fig. 10), which roughly correspond to the addition of the defects seen in *hb⁻/hb⁺* embryos and *kni⁻/kni⁺* embryos (see Fig. 5e and summary in Fig. 10).

Giant⁻

Mutations in the gap gene *giant* (*gt*) result in anterior head defects, as well as disruptions in the fifth through seventh abdominal segments (Wieschaus et al. 1984b; Petschek et al. 1987; see Fig. 2). An abnormal *eve* expression pattern is first detected in *gt⁻* embryos after cellularization (Fig. 7a). At this time, the first and second *eve* stripes fail to resolve, as does the broad band of staining that normally yields the fifth and sixth stripes. Moreover, the seventh stripe is strongly reduced, and never reaches wild-type levels of expression. The first and second stripes separate during gastrulation, but shortly thereafter the second stripe rapidly decays and is reduced to a sharp line of cells that show unequal levels of staining (Fig. 7b). The posterior portion of the broad, composite 5/6 stripe gradually diminishes during gastrulation. By the onset of germ band elongation only the first, third, fourth, and fifth stripes appear normal, and show wild-type levels of staining.

The *ftz* expression pattern is also strongly altered in *gt⁻* embryos (Carroll and Scott 1986), and during germ band elongation, the *eve* and *ftz* expression patterns gradually become complementary (Fig. 7c). However, unlike the other gap mutants that were examined, extensive overlap in the *eve* and *ftz* staining patterns persists during gastrulation. In particular, many of the same embryonic cells show relatively high levels of *eve* and *ftz* expression within the region that contains the composite 5/6 *eve* band (Fig. 7c). Only after the onset of germ band elongation is there a loss of *eve* expression in this region and a persistence of *ftz* staining, thereby giving reciprocal patterns, as seen for the other gap mutants.

Figure 4. *eve* and *ftz* expression in *Krüppel* mutants. The presentation of embryo whole mounts is described in the legend to Fig. 1. (a) Anti-*eve* stained cleavage cycle 14 embryo undergoing cellularization. Two prominent bands occur at ~70% and 35% egg length. The region between these bands show abnormally low levels of staining. (b) Embryo after completion of cellularization. The anteriormost and posteriormost *eve* stripes appear normal. There are two broad bands of staining between these stripes. In wild-type, this region contains the second through sixth *eve* stripes. (c) A gastrulating embryo that was stained with anti-*eve* antibody. Each of the four bands of *eve* expression that were detected earlier (see b, above), persist during this stage. However, each has become narrower and pronounced dorsal-ventral differences in the staining pattern are observed for the second band. (d) Similar to c, except that this embryo was double-stained with anti-*eve* and anti-*ftz* antibodies. The stronger stripes correspond to *eve* (designated e), whereas the weaker stripes correspond to *ftz* (designated f). The complementarity of the two expression patterns is best illustrated by the second broad band of expression where dorsal-ventral asymmetries of expression are observed for both genes. (e) Cellularized *Kr⁺/Kr⁻* heterozygote after double-staining with anti-*eve* and anti-*ftz* antibodies. The arrows indicate a "compression" of *eve* and *ftz* expression. This region includes the third *eve* stripe through the fourth *ftz* stripe.

Figure 5. *eve* and *ftz* expression in *knirps* mutants. The presentation and antibody staining of the embryos is described in the legend to Fig. 1. (a) Embryo just after the completion of cellularization. The fourth, fifth, and sixth *eve* stripes fail to form. Instead, the 45–30% domain shows a reduced, uniform level of staining. (b) Gastrulating embryo stained with anti-*eve* antibody. The first, second, third, and seventh expression stripes sharpen during this time (note that the first stripe is obscured by the cephalic furrow). Weak staining above background levels is detected in the 45–30% domain. (c) Similar to b, but double-stained with both anti-*eve* and anti-*ftz* antibodies. The strong staining corresponds to *eve* (e), whereas *ftz* (f) shows weaker staining. The *eve* and *ftz* staining patterns are reciprocal. In particular, the 45–30% domain shows weak *eve* staining and strong *ftz* staining. (d) A cellular blastoderm-stage *kni*⁺/*kni*⁻ heterozygote after staining with the anti-*eve* antibody. The fifth stripe shows reduced levels of staining, and the arrows indicate a compression of the fate map.

posterior to ~40% egg length (summarized in Fig. 10), which results in strongly altered positions, and widths, for the fifth and sixth *eve* stripes (Fig. 8a). Correspondingly, there are abnormally large “gaps” of unstained cells separating the fourth and fifth stripes, as well as the fifth and sixth stripes (see arrows, Fig. 8a). By the onset of gastrulation, the altered patterns of *eve* and *ftz* (Mahoney and Lengyel 1987) expression observed in *tll*⁻ are complementary (Fig. 8b).

Pair-rule mutants

Pair-rule mutants were examined to determine whether any of these genes are required for the establishment or maintenance of the normal *eve* expression pattern. This study involved the use of the strongest available mutant alleles for all nine pair-rule genes. None of these mutants strongly alter the establishment of the wild-type *eve* expression pattern, and each shows a normal or near-normal pattern during cleavage cycle 14 development. In all cases, seven transverse expression stripes are established, and at most, only relatively mild alterations in the spacing and intensity of expression are detected in cellular blastoderm-stage embryos. However, two of the genes, *hairy* and *runt*, are essential for the normal refinement and maintenance of the *eve* expression pattern during gastrulation, as discussed below. The only other pair-rule genes that have consistent effects on the maintenance of the *eve* expression pattern are *eve* and *engrailed* (*en*), which will be described in a separate report (Warrior et al., in prep.). Mutations in the pair-rule genes *ftz*, *paired*, *odd-skipped*, *odd-paired*, and *sloppy paired* do not appear to affect the establishment or maintenance of the *eve* pattern.

In *runt*⁻ embryos an abnormal pattern of *eve* expression is first detected after cellularization, when the fifth expression stripe is narrower and weaker than in wild-type. Just prior to gastrulation, each of the *eve* stripes, except 5, broadens and encompasses an average of five cells. In addition, irregularities in the spacing of adjacent stripes can be observed. Normally at this stage in development the *eve* pattern shows a “four on/four off” arrangement, and the stripes begin to sharpen at their an-

Tailless⁻

Mutations in the gap gene *tailless* (*tll*) result in defects in the head, as well as the deletion of the eighth, ninth, and terminal abdominal segments, which is accompanied by a corresponding increase in the length of the remaining abdominal segments (Strecker et al. 1986; see Fig. 2). *tll*⁻ embryos show only 6, not 7, transverse stripes of *eve* staining (Fig. 8a). On occasion, a partial seventh stripe is detected on the dorsal surface near the posterior pole (data not shown). There is a progressive expansion of the six remaining stripes along the anterior–posterior axis; the most marked expansion occurs

Figure 6. *eve* and *ftz* expression in *hunchback*^{-/-}, *knirps*^{-/-} double mutants. The presentation of embryo whole mounts is described in the legend to Fig. 1. (a, c) Cellular blastoderm-stage double mutants stained with anti-*eve* (a), and anti-*ftz* (c). Both proteins are broadly distributed along the anterior-posterior axis and fail to show discrete stripes. However, some discontinuities in the staining patterns can be observed (arrows). (b, d) Gastrulating embryos stained with anti-*eve* (b), and anti-*ftz* (d). There is a premature reduction in the *eve* staining pattern, whereas *ftz* expression persists at a high level during this time.

terior borders (see Fig. 1b,c). In contrast, in *runt*^{-/-}, both the anterior and posterior margins of each expression stripe "sharpen" after cellularization (i.e., see arrows, Fig. 9a). During gastrulation, there are pair-wise fusions of adjacent stripes, particularly 6 and 7 (Fig. 9b), and irregularities in the spacing between stripes become more pronounced. For example, the gap separating the fourth and fifth stripes is significantly broader than normal.

An abnormal *eve* pattern is also detected in *hairy* mutants after cellularization. At this time the second and fifth *eve* stripes show reduced levels of staining, whereas the remaining stripes appear normal (Fig. 9c). During gastrulation there is a rapid decay of the overall *eve* expression pattern, and only weak staining of the first, third, and fourth stripes can be observed at the onset of germ band elongation (Fig. 9d). In contrast, wild-type embryos of similar age show strong expression of each of the seven stripes, which persist during germ band elongation (see Fig. 1c). Double staining of *hairy*^{-/-} and *runt*^{-/-} embryos with anti-*eve* and anti-*ftz* antibodies reveals complementary patterns of *eve* and *ftz* expression in these mutants (data not shown). Thus, in *runt*^{-/-} there is a premature narrowing, and loss, of the *ftz* expression stripes, whereas in *h*^{-/-} the *ftz* stripes are broad and persist during gastrulation and germ band elongation (Carroll and Scott 1986).

Discussion

We have examined the distribution of the *eve* protein in mutants for most of the known zygotically active seg-

mentation genes (Jürgens et al. 1984; Nüsslein-Volhard et al. 1984; Wieschaus et al. 1984b). Of the mutants analyzed (summarized in Table 1), less than half (9/19) have a discernible effect on *eve* expression: five disrupt the establishment of the *eve* pattern during cellularization, whereas an additional four alter the maintenance and/or "refinement" of the *eve* pattern during gastrulation (summarized in Fig. 10). The nature of the altered *eve* patterns observed in these mutants is consistent with the occurrence of a hierarchy of interactions among segmentation genes, whereby the gap genes control segmentation indirectly by influencing the expression of pair-rule genes such as *eve*.

Hierarchy of interactions

It has been proposed that the subdivision of the embryo into a repeating series of homologous body segments involves hierarchical interactions among several classes of early acting genes (Ingham and Martinez-Arias 1986; Meinhardt 1986). In particular, maternally contributed factors are thought to be responsible for the expression of gap genes, which in turn establish selective patterns of pair-rule gene expression. The pair-rule genes might implement segmentation by regulating the expression of *engrailed*, as well as segment polarity genes, which are required for the establishment of segment boundaries and the morphogenesis of other segmentally repeated pattern elements (Harding et al. 1986; Ingham et al. 1986). Consistent with this model is the demonstration that mutations in each of the known gap genes strongly

Figure 7. *eve* and *ftz* expression in *giant* mutants. The presentation of embryo whole mounts is described in the legend to Fig. 1. (a) Cellular blastoderm-stage embryo after staining with anti-*eve*. Composite bands are seen for stripes 1/2 and 5/6. (b) Gastrulating embryo stained with anti-*eve*. The first, third, fourth, and fifth stripes appear normal, whereas the second, sixth, and seventh show strongly reduced levels of staining. Note the sharp, heterogeneous staining of the second stripe. (c) Similar to b, except that the embryo was double-stained with anti-*eve* and anti-*ftz* antibodies. Nearly reciprocal staining patterns are observed, although the region between the fifth and seventh *eve* stripes shows relatively strong staining with both *eve* and *ftz* antibodies (labeled e,f). The arrow indicates a narrow gap in a broad band of *ftz* staining, which corresponds to the position where the sharp second *eve* stripe is observed at slightly earlier stages (see b, above).

perturb the *eve* expression pattern during cellularization, whereas mutations in pair-rule genes and segment polarity genes do not significantly alter the establishment of the *eve* pattern. Mutations in four of the nine known pair-rule genes affect the refinement of the *eve* pattern during gastrulation, and none of the segment polarity mutants that were examined have any detectable effect on *eve* expression. The effects of these different

Figure 8. *eve* and *ftz* expression in *tailless* mutants. The presentation of embryo whole mounts is described in the legend to Fig. 1. (a) Cellular blastoderm-stage embryo after staining with anti-*eve*. Only six stripes of expression are observed. These stripes, particularly 5 and 6, are shifted toward the posterior pole. Abnormally broad gaps occur between stripes 4 and 5, as well as 5 and 6. (b) Gastrulating embryo that was double-stained with anti-*eve* and anti-*ftz* antibodies. The embryo is oriented such that the ventral furrow can be seen. Reciprocal patterns of *eve* and *ftz* staining are observed.

classes of segmentation genes on the *eve* expression pattern suggest a "hierarchy of function" among the segmentation genes whereby the gap genes regulate the pair-rule genes, which in turn, might regulate the segment polarity genes. It is possible that this functional hierarchy involves a temporal sequence of interactions, such that the gap genes function prior to the pair-rule genes. Consistent with this possibility is the observation that, in general, gap mutants disrupt the *eve* expression pattern during cellularization, whereas pair-rule mutants show altered patterns of *eve* expression at later stages of development.

Differential regulation of *eve* and *ftz*

Although *eve* and *ftz* products accumulate in essentially complementary sets of embryonic cells, their overall patterns of expression are remarkably similar. *eve* and *ftz* transcripts are first detected during cleavage cycle 10, when they are both uniformly distributed along the anterior-posterior axis (Hafen et al. 1984; Harding et al. 1986; Macdonald et al. 1986). Localized transcript distribution patterns are not observed until the beginning of cleavage cycle 14, and periodic expression patterns gradually evolve during cellularization. Once established,

Figure 9. *eve* and *ftz* expression in pair-rule mutants. The presentation of embryo whole mounts is described in the legend to Fig. 1. (a, b) *runt*⁻ embryos after staining with the anti-*eve* antibody. (a) Cellular blastoderm-stage. The fifth *eve* stripe is narrower than in wild type, and each of the stripes shows a sharper posterior border than normal (i.e., arrows; compare with Fig. 1b). (b) Gastrulating embryo oriented so that the ventral furrow can be seen. Each of the *eve* stripes broadens during gastrulation, resulting in pair-wise fusions and irregularities in the spacing between adjacent stripes. The central regions of each stripe are less strongly stained than the margins (e.g., arrow). (c, d) *hairy*⁻ embryos stained with the anti-*eve* antibody. (c) Cellular blastoderm-stage embryo. The second and fifth expression stripes show slightly reduced levels of staining. (d) Ventral surface of a gastrulating embryo. There is a premature loss of *eve* staining. The arrows indicate the sites of expression seen in wild type. At this time only the first, third, and fourth stripes show residual nuclear staining.

the *eve* and *ftz* expression stripes become progressively more refined during gastrulation, and successively diminish during germ band elongation.

The overall similarity of the *eve* and *ftz* expression patterns suggests that the regulation of the two genes is closely coupled. A possible explanation for this coupling is that the two genes might directly regulate each others expression. For example, high levels of *eve*⁺ products might prevent the accumulation of *ftz*⁺ products, thereby restricting *ftz* expression to a complementary set of cells. Thus, mutations that perturb the expression of *eve* would result in a reciprocal alteration of the *ftz* pattern. This explanation seems unlikely since *eve* expression is normal in *ftz*⁻ embryos, and the establishment of the *ftz* pattern is essentially normal in *eve*⁻ (Harding et al. 1986). A second hypothesis is that a common set of segmentation genes differentially regulate *eve* and *ftz* expression such that a combination of gene products that activates *eve*, represses the expression of *ftz*, and vice versa. Based on the results presented in this study we favor the latter hypothesis.

The nine zygotically active genes required for the wild-type pattern of *eve* expression are the same genes that regulate *ftz* (Carroll and Scott 1986); none of the segmentation genes that were tested alter only one of the patterns. Mutations that affect one of the genes were always found to produce a reciprocal alteration in the

other. The only exception to this rule might be the apparent negative regulation of *eve* by *engrailed*, which occurs at the end of germ band elongation (Harding et al. 1986). This suggests that the *eve* and *ftz* promoters independently "interpret" the same positional cues to give complementary patterns of expression during development. We propose that the gap genes are responsible for establishing *eve* and *ftz* expression patterns that are "out of register" during cellularization (see summary Fig. 10); a combination of gap gene products that positively regulate *eve* negatively regulate *ftz*, and vice versa. The pair-rule genes do not appear to play a major role in the establishment of asymmetric *eve* and *ftz* expression patterns since mutations in pair-rule genes do not dramatically alter the *eve* pattern prior to gastrulation. Instead, the pair-rule genes, particularly *runt*, *hairy*, and *eve*, are involved in the refinement of the *eve* and *ftz* patterns so that they become completely complementary during gastrulation (summarized in Fig. 10).

Regulation by the gap genes

Gap genes clearly play a crucial role in establishing periodic domains of gene expression. A particularly striking example of this requirement is the finding that *eve* and *ftz* proteins are uniformly distributed along the anterior-posterior axis of cellular blastoderm stage *hb*⁻,

kni⁻ double mutants (Fig. 6a,b). It is possible that gap genes exert a relatively direct influence on pair-rule gene expression since there is only a short lag (less than 30 min) between the time when the gap genes *hb* and *Kr* are first expressed in wild-type (Knipple et al. 1985; Tautz et al. 1987), and when an altered pattern of *eve* expression is first observed in *hb*⁻ and *Kr*⁻ embryos.

The demonstration of cross-regulatory interactions among gap genes (Jäckle et al. 1986) complicates the interpretation of the altered patterns of *eve* and *ftz* expression seen in gap mutants. Thus, it is unclear to what extent an abnormal pattern of *eve* expression is the direct result of removing a given gap gene function, or an indirect consequence of altering the distribution of other gap gene products. The patterns of *eve* expression observed in the various gap mutants can be interpreted according to the occurrence of regulatory interactions among the gap genes. For example, the *eve* pattern observed in *tll*⁻ embryos might result not only from the absence of *tll*⁺ products, but also as an indirect consequence of an altered distribution of products encoded by the neighboring gap gene *kni* (see Fig. 2). Thus, *tll*⁺ products might be required for specifying the seventh *eve* stripe, and for repressing the expression of *kni* so that its expression is restricted to more anterior regions. In *tll*⁻ embryos this would result in the absence of the seventh *eve* stripe, and a posterior expansion of the *kni* domain of expression. The posterior shift of the fourth, fifth, and sixth *eve* stripes observed in *tll*⁻ embryos might result from such a posterior expansion of *kni*⁺ ac-

tivity. Such shifts in the positions of the "unaffected" *eve* stripes are also observed in each of the other gap mutants (see Fig. 10).

It is not known how gap genes might provide sufficient informational content to establish diverse, periodic patterns of pair-rule gene expression (e.g., Fig. 11). For example, the gap gene *kni* is required for the fourth through sixth *eve* stripes and the third through sixth *ftz* stripes (Fig. 5). The *eve* and *ftz* patterns that are observed in *kni*⁻ mutants suggest that *kni*⁺ product activates *eve* and represses *ftz*. It is not clear how such differential regulation generates an organized set of *eve* and *ftz* stripes. One possibility is that *kni* proteins are not uniformly distributed; perhaps gap proteins are themselves localized within crude or transient pair-rule boundaries. Transcripts encoded by *hb* and *Kr* do not display uniform distribution patterns (Knipple et al. 1985; Tautz et al. 1987), and it is possible that their proteins will show even more striking variations of expression.

It has been proposed that boundaries between adjacent sites of gap gene expression play an important role in organizing periodic domains of gene expression. A major drawback of this proposal is that the known gap genes do not appear to provide enough boundaries to establish periodic *eve* and *ftz* patterns that include a total of seven stripes. There are several explanations for this apparent discrepancy. One possibility is that the gap genes act in concert with maternal cues, such as *bicoid* (Frohnhofer et al. 1986), to regulate *eve* and *ftz*. Alternatively, additional zygotically active genes of unknown identity

Table 1. Summary of segmentation mutants analyzed

	Alteration of the <i>eve</i> pattern in	
	cellular blastoderm	gastrula
Gap mutants		
<i>hunchback</i> (<i>hb</i> ^{14F21})	strong	strong
<i>Krüppel</i> (<i>Kr</i> ¹)	strong	strong
<i>knirps</i> (<i>kni</i> ^{IID48})	strong	strong
<i>hb</i> , <i>kni</i> double mutant (<i>hb</i> ^{7M48} , <i>kni</i> ^{IID48})	strong	strong
<i>giant</i> (<i>gt</i> ^{YA82})	strong	strong
<i>tailless</i> (<i>tll</i> ¹)	strong	strong
Pair-rule mutants		
<i>even-skipped</i> (<i>eve</i> ^{3.77.17})	subtle	strong
<i>engrailed</i> (<i>en</i> ^{SFX31} and <i>en</i> ⁷)	mild and variable	mild
<i>fushi tarazu</i> (<i>Df</i> {3R}4 Scb)	subtle	none
<i>hairy</i> (<i>h</i> ^{K1})	mild	strong
<i>odd-skipped</i> (<i>odd</i> ^{IID36})	subtle	none
<i>odd-paired</i> (<i>opa</i> ^{5H57})	subtle	none
<i>paired</i> (<i>prd</i> ^{IIB42})	subtle	none
<i>runt</i> (<i>run</i> ^{III2})	mild	strong
<i>sloppy paired</i> (<i>slp</i> ^{7L48})	subtle	none
<i>h</i> , <i>opa</i> double mutant (<i>h</i> ^{IL79K} , <i>opa</i> ^{5H57})		like <i>h</i>
<i>h</i> , <i>ftz</i> double mutant (<i>h</i> ^{IL79K} , <i>ftz</i> ^{7B17})		like <i>h</i>
<i>odd</i> , <i>prd</i> double mutant (<i>odd</i> ^{IID36} , <i>prd</i> ^{32.12})	subtle	none
Segment polarity mutants		
<i>cubitus interruptus</i> (<i>Df</i> {4}G/ci ^D)	none	none
<i>gooseberry</i> (<i>gsb</i> ^{IIX62})	none	none
<i>hedgehog</i> (<i>hh</i> ^{IJ35})	none	none
<i>patched</i> (<i>ptc</i> ^{IN108})	none	none
<i>wingless</i> (<i>wg</i> ^{IG22})	none	none

might mediate the regulation of the pair-rule genes by the gap genes. And finally, the establishment of additional gap gene "boundaries" could involve a temporal and/or functional hierarchy of gap gene interactions. Additional studies of gap and pair-rule gene expression in various mutants and in mutant combinations will be required to distinguish among these possibilities.

Regulation by the pair-rule genes

As discussed above, the pair-rule genes appear to be involved in the refinement of the *eve* and *ftz* expression patterns during gastrulation. As for the gap genes, the

pair-rule genes *run*, *hairy*, and *eve* appear to exert opposite effects on *eve* and *ftz* expression. *run*⁺ products either directly or indirectly repress the expression of *eve*, and positively regulate *ftz*. Thus, *eve*⁺ products are over-expressed in gastrulating *run*⁻ embryos, whereas *ftz* products disappear prematurely. In contrast, *hairy* exerts a positive effect on *eve* expression and a negative effect on *ftz*, in that *eve* products prematurely disappear in *hairy*⁻, whereas *ftz* products are overexpressed. *hairy* and *run* mutations alter the *ftz* expression pattern upon cellularization (Carroll and Scott 1986), whereas the *eve* pattern is not strongly disrupted in these mutants until

Figure 10. (See facing page for legend.)

Figure 11. Summary of interactions among the segmentation genes. A combination of gap gene products that activates *eve*, represses *ftz*, and vice versa. In this way, asymmetric "out of register" patterns of *eve* and *ftz* expression are established during cellularization. Differential regulation of *eve* and *ftz* by the pair-rule genes *runt*, *hairy*, and *eve* are thought to be responsible for refining the *eve* and *ftz* patterns so that they become perfectly complementary during gastrulation.

gastrulation. The regulatory relationships between *eve*, *ftz*, *hairy*, and *runt* are summarized in Fig. 11.

It has been previously shown that there is a differential requirement for *runt*⁺ gene activity along the anterior-posterior axis of developing embryos (Gergen and Wieschaus 1986). There appears to be a close correspondence between the regions where *runt*⁺ gene function is most critically required, and the portions of the *eve* expression pattern that are most severely disrupted in *runt*⁻ embryos. For example, there is a strong requirement for *runt*⁺ activity in posterior regions of the embryo, including those regions that give rise to the fourth through seventh abdominal segments. This corresponds to the sixth and seventh *eve* stripes, which are fused and strongly overexpressed in *runt*⁻ embryos (Fig. 9a,b). Moreover, advanced-stage *runt*⁻ embryos show periodic deletions of cuticular structures, and mirror-image duplications of the remaining pattern elements (Gergen and Wieschaus 1986). In *runt*⁻, both the anterior and posterior borders of each *eve* stripe become progressively sharper during gastrulation (Fig. 9a,b), whereas in wild-type only the posterior border sharpens (Fig. 1b,c). The uniform sharpening of both borders in *runt*⁻ might cor-

respond to the mirror image duplications of cuticular structures seen in older embryos.

It has been previously shown that *hairy*⁺ products are required for the narrowing of the *ftz* expression stripes during gastrulation, and that *ftz*⁺ products are overexpressed in *hairy*⁻ embryos (Carroll and Scott 1986; Howard and Ingham 1986). On this basis it was proposed that *hairy* exerts a negative effect on *ftz* expression. It is possible that this negative regulation is cell-autonomous, since the wild-type *ftz* and *hairy* patterns overlap, but are not coincident. In gastrulating *hairy*⁻ embryos, *ftz* products persist at high levels in those cells that might normally express *hairy*. Here we have shown that *hairy* has the opposite effect on the *eve* pattern, in that there is a premature loss of *eve* products in gastrulating *hairy*⁻ embryos. Therefore it appears that *hairy* is required for the normal maintenance of the *eve* expression pattern during gastrulation.

Kr⁻ embryos show an altered pattern of *hairy* expression (Ingham et al. 1986), similar to the disruptions seen for *eve* and *ftz*. It is likely that mutations in the other gap genes also alter *hairy*, and that gap mutants disrupt the pattern of *runt* expression as well. Such alterations

Figure 10. Summary of *eve* and *ftz* expression patterns in segmentation mutants. *eve* or *ftz*-stained embryos were photographed in a central plane of focus, and the expression stripes were measured with respect to the anterior and posterior poles. An egg length of 100% corresponds to the anterior pole, and 0% corresponds to the posterior pole. The numbers (N) of each genotype and stage that were measured are shown in the right column. The indicated locations of each site of *eve* or *ftz* antibody staining are based on an average of the measurements. The standard deviations were an average of 1.6% egg length for the wild-type *eve* stripes, and an average of 0.8% egg length for the wild-type *ftz* stripes. The positions of staining during gastrulation and germ band elongation were inferred from the measurements of cellular blastoderm-stage embryos. The filled bars correspond to *eve* staining, and the striped bars to *ftz*. In cellular blastoderm-stage embryos the *ftz* pattern shows a "four on/four" arrangement, whereas the *eve* pattern shows a broader, bell-shaped distribution. At this time the two patterns overlap by one or two cells (compare Fig. 1d with Fig. 1e). The establishment of complementary *eve* and *ftz* stripes during gastrulation appears to involve a narrowing of the posterior borders for both the *eve* and *ftz* stripes. In addition, there appears to be an enhancement in the levels of *eve* staining in the anterior-most nuclei of each of the *eve* stripes, resulting in a slight anterior shift of the *eve* pattern. Only the *eve* pattern is presented for each of the indicated mutants. The first pattern shown for each mutant corresponds to embryos that are heterozygous for the mutant allele in question (het.). The other patterns correspond to homozygous mutants. Dorsal-ventral differences of *eve* staining is observed in *hb* and *Kr* mutants. Dorsal patterns of *eve* staining are shown above the midline of each bar, whereas ventral differences are shown below the midline. For *hb*, the numbers 1 and 2 correspond to two different mutant patterns that are observed in *hb*14F21 homozygotes. Number 1 corresponds to the pattern shown in Fig. 3c, d, and is essentially identical to the pattern obtained in homozygotes for a deficiency of *hb*. Increasing darkness of the bars corresponds to increasing levels of *eve* staining. (cell. bl.) cellular blastoderm-stage embryos; (gastr.) gastrulating embryos; (gb. el.) embryos undergoing germ band elongation; (het.) embryos heterozygous for the mutant allele in question; (N) number of embryos measured; (% el) percent egg length relative to the anterior and posterior poles.

in the distribution of *runt* and *hairy* products should nonetheless give reciprocal patterns of *eve* and *ftz* expression since these genes exert opposite regulatory effects on *eve* and *ftz* (summarized in Fig. 11).

Experimental procedures

Staged embryos from wild-type and balanced mutant stocks were fixed and stained with affinity-purified rabbit anti-*eve* antibodies, rabbit anti-*ftz* antibodies, or a mixture of both. This procedure was done exactly as described in Frasch et al. (1987). The identification of mutant genotypes was based on the fraction of embryos showing an altered *eve* expression pattern.

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Complementary patterns of even-skipped and fushi tarazu expression involve their differential regulation by a common set of segmentation genes in *Drosophila*.

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