Cross-Regulatory Interactions Among Pair-Rule Genes in *Drosophila*

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The pair-rule genes of *Drosophila* are required for the subdivision of the developing embryo into a repeating series of homologous body segments. One of the pair-rule genes, *even-skipped* (*eve*), appears to be particularly important for the overall segmentation pattern since *eve*$^-$ embryos lack all segmental subdivisions in the middle body region. On the basis of homeo box cross-homology we have isolated a gene, S72, which probably corresponds to *eve*. In embryo tissue sections S72 transcripts show a periodic distribution pattern. The *eve*$^-$ phenotype appears to involve altered patterns of *fushi tarazu* and *engrailed* expression. These and other findings suggest that pair-rule gene expression might involve hierarchical cross-regulatory interactions.

ELABORATION OF POSITIONAL IDENTITY ALONG THE anterior-posterior axis of the *Drosophila* embryo requires the activities of segmentation genes and homeotic genes (*1–6*). Segmentation genes divide the embryo into a repeating series of homologous segment primordia (*3–6*). Homeotic genes establish the diverse pathways by which each embryonic segment develops a distinct adult phenotype (*1, 7*). Mutations in segmentation genes usually cause a reduction in segment number or an alteration in segment polarity (*3*). Mutations in homeotic genes do not alter segment number or polarity, but instead result in the partial or complete transformation of one segment into the homologous tissues of another (*1, 2, 7, 8*).

The expression patterns of segmentation and homeotic genes are stringently regulated. The tissue distributions of transcripts encoded by many of these genes have been previously determined by in situ hybridization. Each of the six homeotic and five segmentation genes that has been examined displays a distinctive pattern of expression (*9–19*). Disruptions of the embryonic segmentation pattern are often associated with altered distributions of products encoded by one or more segmentation or homeotic genes (*14, 20–22*). A central problem in the control of positional information is how these different genes come to be expressed in specific regions along the body axis of the developing embryo.

The mechanisms responsible for the selective patterns of homeotic gene expression have been examined in detail (*1, 20–26*). Many homeotic loci reside within one of two gene clusters in the *Drosophila* genome, the bithorax complex (BX-C) (*1, 27, 28*) or the Antennapedia complex (ANT-C) (*2, 29, 30*). There is evidence that hierarchical cross-regulatory interactions among at least some of the ANT-C and BX-C homeotic loci play a role in maintaining their spatially restricted patterns of expression (*14, 20, 21, 23, 24*). The molecular basis for these interactions is not known. However, it has been shown that each of the six known ANT-C and BX-C homeotic lethal complementation groups contains a similar 180-bp protein-coding region designated the homeo box (*31–34*). Homeo box protein domains appear to contain sequence-specific DNA binding activities (*35–37*). It has been proposed that each homeo box–containing homeotic gene autoregulates its own expression, and, because of homeo box homologies, cross-regulates the expression of other homeotic genes (*14, 20, 38*).

The regulation of segmentation gene expression appears to be complex. In this article we examine the expression of three genes that belong to a specific class of segmentation genes called the pair-rule genes (*3*). Mutations in any of the nine known pair-rule genes usually result in embryos that lack alternating segmental pattern elements (*3–6*). Analyses of the transcript distribution patterns of two pair-rule genes, *fushi tarazu* (*ftz*) and *hairy* (*h*), reveal rather general patterns of expression at early blastoderm stages. However, during later blastoderm stages, there is a gradual unfolding of the *ftz* and *h* periodic (zebra) patterns of expression (*11, 15, 16*). These expression patterns are then maintained throughout gastrulation. Proteins specified by one or more pair-rule genes may serve as specific factors for the regulated expression of other pair-rule genes. For example, mutations in three of the pair-rule genes result in abnormal distribution patterns of *ftz* protein (*39*). These results suggest that pair-rule gene expression might involve a complicated network of cross-regulatory interactions among pair-rule genes. Since several pair-rule genes contain a homeo box, it is possible that some of these interactions are similar to those proposed for the homeotic genes.

Here we describe the pattern of expression of a newly isolated pair-rule gene, called S72, that contains a homeo box. The S72 gene maps within the *even-skipped* (*eve*) genetic region (*40*) and shows a zebra pattern of expression from approximately 69 to 19 percent egg length (100 percent egg length corresponds to the distance from the posterior to anterior pole of the embryo). The zebra pattern of S72 expression is complementary to that of *ftz*. These results suggest that S72 corresponds to *eve*. Here we show that the maintenance, and not the initiation or evolution, of the *ftz* expression pattern requires *eve*$^+$ function. Further evidence for possible cross-regulatory interactions among pair-rule genes that contain a homeo box is the finding that *engrailed* (*en*) is not expressed from approximately 69 to 15 percent egg length in *eve*$^-$ embryos, and that S72 shows 15 stripes of expression in *en*$^-$.

Isolation of the S72 gene. The importance of *eve* in the establishment of the overall segmentation pattern is suggested by

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the phenotype of embryos homozygous for strong *eve*$^-$ mutant alleles (*40*) (Fig. 1b). Such *eve*$^-$ embryos lack all segmental subdivisions in the middle body region, whereas null mutations in the other pair-rule genes do not completely eliminate segmentation (*3, 40*). For example, the mutant embryo shown in Fig. 1e is homozygous for a deletion of *ftz*, yet retains one-half the normal number of middle body segments.

Since *ftz* has been shown to contain a homeo box (*31–33, 35, 41*), we attempted to isolate *eve* on the basis of homeo box cross-homology. In a search for previously uncharacterized homeo box sequences, we screened a *Drosophila* DNA library for clones that cross-hybridize with a homeo box probe from the homeotic gene, *Sex combs reduced* (*Scr*) (*38*). Here we present the spatial and temporal limits of expression for one of the newly isolated clones, S72. On the basis of in situ hybridization to salivary gland chromosome spreads, S72 was found to map to the 46C region of the second chromosome, within the limits of a small *eve* deficiency [*Df(2R)eve*$^{1.27}$] (*40*). S72 transcripts are not detected in embryos homozygous for this deficiency.

The homeo box region of the S72 clone was used to screen a phage λgt10 complementary DNA (cDNA) library prepared from wild-type embryos at 3 to 12 hours (after fertilization) (*42*). One of the cDNA clones obtained, pS72-6, is approximately 1.4 kilobases (kb) in length and contains most of the S72 protein coding region. The S72 homeo box shares approximately 50 percent amino acid identity with the *Antennapedia* and *en* homeo boxes. A single 1.4-kb transcript was detected in Northern blot hybridizations with pS72-6 as a probe. Peak levels of S72 transcript are found 2 to 3 hours after fertilization (*43*).

Distribution of S72 transcripts in embryo tissue sections. Since *eve* is a pair-rule gene, *eve* transcripts might display a zebra distribution pattern similar to those shown for *ftz* and *hairy* (*11, 16*). In order to obtain further evidence that S72 corresponds to *eve*, S72 transcripts were localized within embryo tissue sections by in situ hybridization.

After fertilization, the zygotic nucleus undergoes 13 divisions before the cellular blastoderm is formed. After the eighth division, the nuclei migrate to the periphery of the embryo, during which time they undergo a ninth division. This migration results in a syncytial blastoderm, about 90 minutes after fertilization. After syncytial blastoderm formation, there are four additional nuclear divisions. Once the 13th nuclear division is complete, the nuclei elongate and cell membranes are laid down to form a cellular blastoderm. Cellularization occurs over a period of about 30 minutes during cleavage stage 14 and is immediately followed by the onset of gastrulation (*44–47*).

Specific hybridization signals with the S72 cDNA probe were first detected during cleavage stage 10. At this time, a rather general labeling pattern is observed. By cleavage stage 11, most of the detectable S72 hybridization signals are localized in a broad band that has a well-defined anterior margin at approximately 69 percent egg length. During stage 14 there is a gradual evolution of the S72 expression pattern, which results in the elaboration of seven zebra stripes of labeling (Fig. 2, a to e). Based on measurements of 20

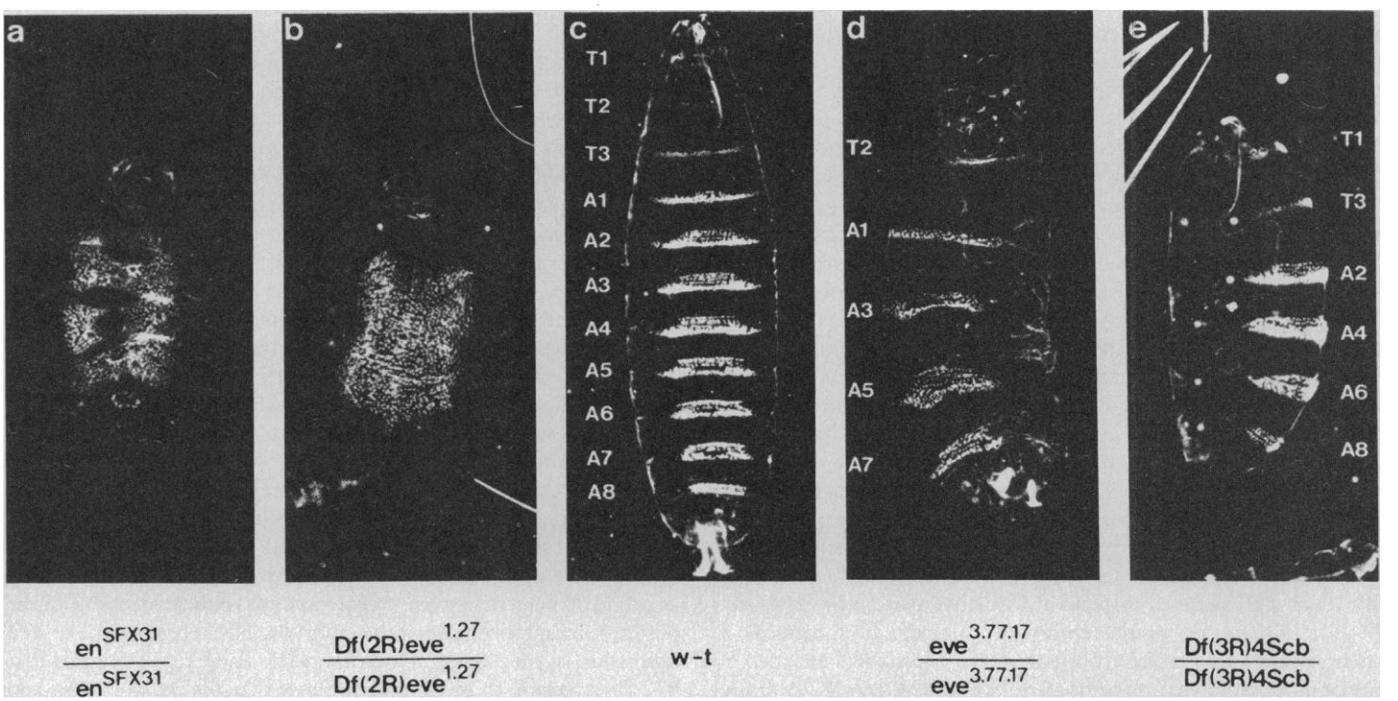

Fig. 1. Cuticular phenotypes of wild-type, *en*$^-$, *eve*$^-$, and *ftz*$^-$ embryos. All embryos are oriented so that anterior is up and the ventral surface is displayed. (a) *en*^{SFX31} homozygote. This deficiency uncovers both the *engrailed* and *invected* loci (*42*). Mutant embryos show an almost complete fusion of denticle belts; however, some naked cuticle between pair-wise fusions can be detected (*3*). (b) *Df(2R)eve*$^{1.27}$ homozygote. This deficiency uncovers the *even-skipped* (*eve*) locus. Embryos homozygous for the deletion show no overt segmentation of the middle body region (*40*); an uninterrupted lawn of denticle hairs over the thorax and the abdomen can be seen. (c) Wild-type embryo. The denticle belts associated with the anterior portion of each of the three thoracic and eight abdominal segments can be seen. (d) An *eve*$^{3.77.17}$ homozygote, which displays the "weak" *eve* phenotype (*40*). The denticle belts of T2 (T2A) are fused with naked cuticle of T3 (T3P), thereby resulting in a composite T2A-T3P segment. Similar fusions in the abdominal region result in the following composite segments: A1A-A2P, A3A-A4P, A5A-A6P, A7A-A8P. (e) Ventral-lateral aspect of a *Df(3R)4Scb* homozygote. This deficiency uncovers the *ftz*, *Scr*, and *Antp* loci (*6*). Embryos homozygous for this deficiency display the *ftz*$^-$ phenotype, which appears to be reciprocal to that observed for weak *eve* mutants. *ftz*$^-$ Embryos show pair-rule fusions that result in the following composite segments: T1A-T2P, T3A-A1P, A2A-A3P, A4A-A5P, A6A-A7P, A8A-A9P. Cuticles were prepared as described in (*59*). The preparations were photographed with dark-field optics. Abbreviations: T1 through T3, first through third thoracic segments; A1 through A8, first through eighth abdominal segments (for example A1, first abdominal segment; A2, second abdominal segment, and so forth).

tissue sections from different embryos, the S72 expression pattern covers approximately 69 to 19 percent egg length. By late cleavage stage 13, a broad band of expression is detected from approximately 69 to 55 percent egg length (Fig. 2a). Soon after completion of the 13th cleavage, two broad bands of labeling are seen (Fig. 2b) and several minutes later a third band emerges near the posterior end of the embryo (Fig. 2c). By the middle of stage 14, seven narrower bands can be discerned; each band encompasses approximately six nuclei and adjacent bands are separated by approximately two nuclei that show lower levels of expression. There is a gradual sharpening of each of these seven bands during embryogenesis such that by cellularization, each encompasses about four nuclei (Fig. 2e). During gastrulation, each stripe covers approximately three cells and is separated from adjacent stripes by approximately five unlabeled cells. The anterior-most hybridization stripe is located over the cephalic furrow (Fig. 2f). This expression pattern persists throughout gastrulation, but gradually diminishes during germ band elongation (Fig. 2, g to i). By the completion of this process (at 5 to 6 hours after fertilization), the seven original expression stripes are no longer detectable. However, a new expression pattern begins to emerge by 4.5 to 5 hours after fertilization. Initially, a band of hybridization is detected over the posterior-most portion of the germ band (Fig. 2, h and i, arrowheads), which is gradually drawn into the invaginating posterior midgut rudiment. This portion of the germ band appears to correspond to the presumptive malpighian tubules. Subsequently, during neurogenesis (7 to 10 hours after fertilization), S72 transcripts are detected in discrete subsets of neurons in the developing ventral cord (Fig. 2j, arrows).

The *ftz* and S72 expression patterns are complementary. Embryos homozygous for weak *eve* mutant alleles show deletions in alternating middle body segments (*3, 4, 40, 48*) (compare Fig. 1d with Fig. 1c). The regions deleted in these *eve* embryos are complementary to those absent in *ftz*$^-$ embryos (*3, 29, 40, 48, 49*) (Fig. 1e). If S72 corresponds to *eve*, then the zebra pattern of S72 expression should be complementary to the *ftz* pattern. We tested this by simultaneously hybridizing embryo tissue sections with a mixture of pS72-6 cDNA probe and a *ftz* genomic DNA probe. The hybridization results suggest that S72 derives from the *eve* locus, as described below.

During cleavage stages 12 and 13, composite *ftz* + S72 hybridization signals uniformly label the cortex of the embryo, from approximately 69 to 15 percent egg length. Over the course of stage 14 development, there is a gradual resolution of the composite hybridization signal into 14 evenly spaced stripes. At early periods of stage 14 development, the combined hybridization signals extend continuously from approximately 69 to 15 percent egg length (Fig. 3, a and b). However, in slightly older embryos, alternating weak and strong hybridization signals can be discerned (Fig. 3c). Parallel

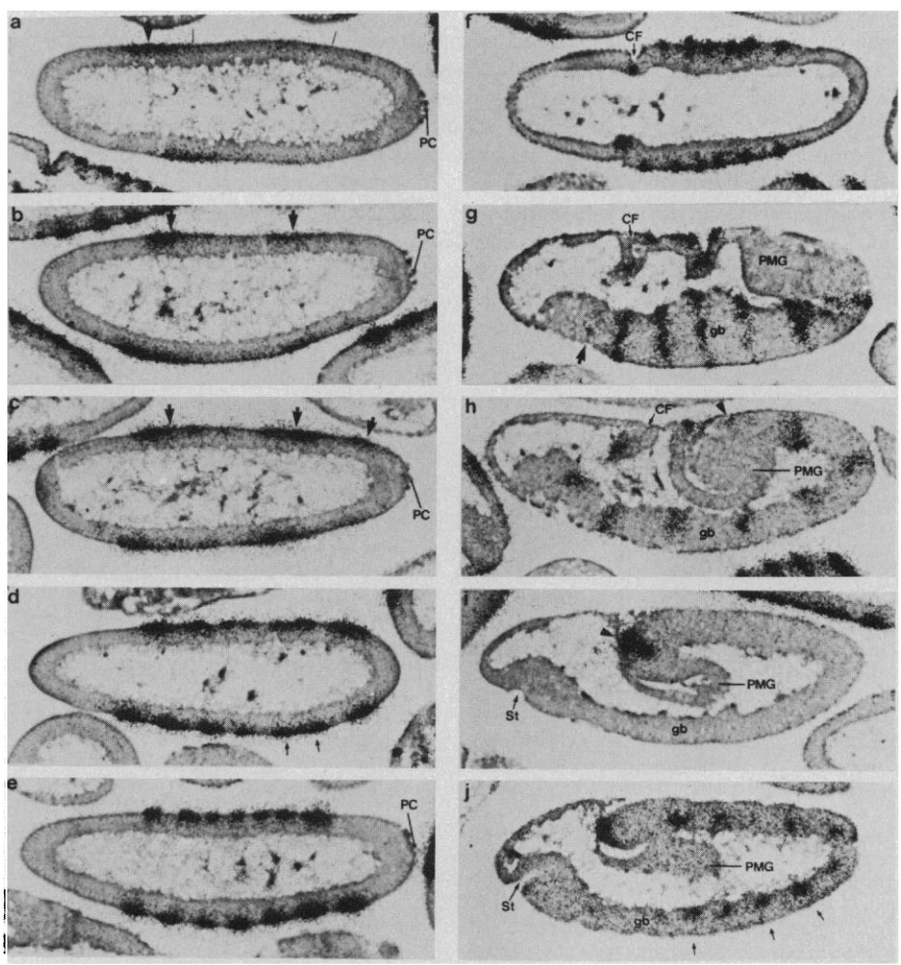

Fig. 2. Distribution of S72 transcripts in wild-type embryos. All sections are oriented so that anterior is to the left; sagittal sections are oriented so that dorsal is up. (a to e) Distribution of S72 transcripts in precellular wild-type embryos. (f to j) Distribution of S72 transcripts during gastrulation and germ band elongation. (a) Horizontal section through a cleavage stage 13 to 14 embryo. Strong S72 hybridization signals are detected in a broad anterior band from approximately 69 to 55 percent egg length (arrow); weaker signals extend more posteriorly (brackets). (b) Sagittal section through an early stage 14 embryo. The strongest S72 hybridization signals are detected over two broad bands (arrows). (c) Horizontal section through an embryo 5 to 10 minutes older than that shown in (b). Three strong bands of hybridization are detected (arrows). (d) Sagittal section through an embryo at the midpoint of stage 14 development. A total of seven evenly spaced stripes of labeling is detected. However, the fifth and sixth stripes have not yet completely separated (arrows). (e) Sagittal section through a stage 14 embryo, just before cellularization. Seven regularly spaced stripes of hybridization are seen. (f) Horizontal section through an embryo during early gastrulation. The anterior-most of the seven stripes is located within the cephalic furrow. (g) Sagittal section through an embryo during early germ band elongation. The anterior-most S72 hybridization stripe (arrow) shows a lower level of labeling than the six more posterior stripes. (h) Sagittal section through an embryo undergoing germ band elongation. Eight sites of hybridization are seen; the posterior-most site (arrowhead) is not detected in younger embryos. (i) Sagittal section of an embryo nearing the completion of germ band elongation. By this time, the original seven hybridization stripes are no longer detected, and only a single site of labeling is observed (arrowhead). This corresponds to the posterior-most signal seen in (h) and probably includes the presumptive malpighian tubules. (j) Sagittal section of an embryo after completion of germ band elongation. At this stage, S72 hybridization signals can be detected within the neurogenic region of most of the segments along the germ band (for example, arrows). All tissue sections were prepared as described in (*60*) and ^{35}S-labeled single-stranded pS72-6 RNA probes were prepared as described in (*16*). Abbreviations: PC, pole cells; CF, cephalic furrow; PMG, posterior midgut invagination; gb, germ band; St, stomodeum.

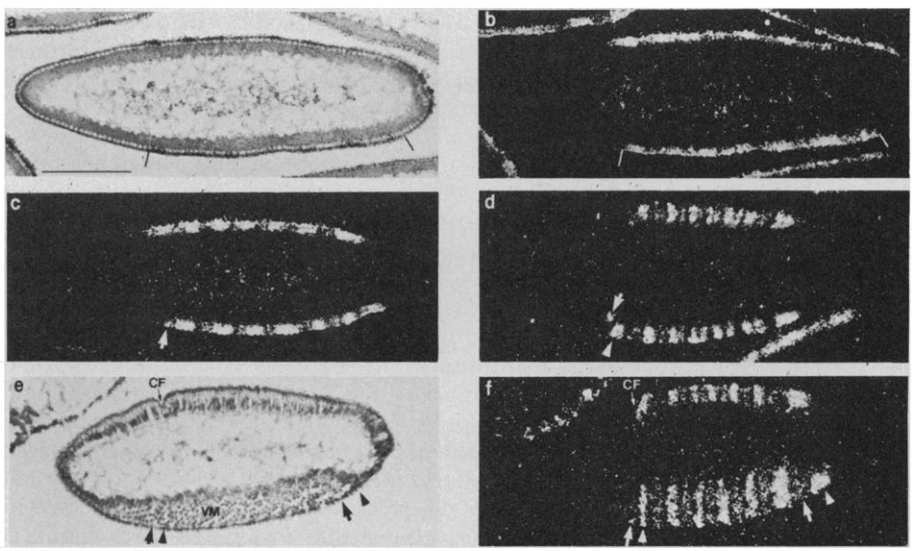

Fig. 3. Double labeling of embryo tissue sections with the *ftz* and S72 probes. All embryos are oriented so that anterior is to the left. Sagittal sections are oriented so that dorsal is up. Bar in (a) represents 0.1 mm. Embryos were simultaneously hybridized with *ftz* and S72 probes. The weaker signals correspond to S72 transcripts, whereas the stronger signals correspond to *ftz*. Arrows indicate the anterior- and posterior-most S72 bands, arrowheads indicate the anterior and posterior-most *ftz* bands. (a and b) Bright- and dark-field photomicrographs of a sagittal section through an embryo at the midpoint of stage 14 development. A band of continuous labeling is detected from approximately 69 to 15 percent egg length. Brackets delineate the anterior and posterior boundaries of expression. (c) Dark-field photomicrograph of a horizontal section through an embryo just before cellularization. By this stage, the *ftz* and S72 labeling patterns begin to separate and the S72 signals (arrow) can be discerned. (d) Dark-field photomicrograph of a horizontal section through a gastrulating embryo. Specific labeling by the S72 probe (arrow) can be clearly distinguished from *ftz* label (arrowhead). The anterior-most S72 stripe is located over the cephalic furrow, whereas the anterior-most *ftz* stripe is just posterior to it. (e and f) Bright- and dark-field photomicrographs of a sagittal section through a gastrulating embryo. The anterior-most S72 stripe shows a lower level of hybridization than do the more posterior S72 stripes. At this stage, S72 and *ftz* stripes are each about three cells wide and are separated from neighboring stripes by a gap of about one cell. Tissue sections were prepared as described (*60*). The *ftz* hybridization probe was prepared from the genomic DNA fragment p523B (*32*). The p523B and pS72-6 DNA probes were ^{3}H-labeled by nick-translation (*10*). Abbreviations: oCF, cephalic furrow; VM, ventral mesoderm.

sections through the same embryo were hybridized with the S72 probe alone, which showed that the weak signals correspond to S72 transcripts. Alternating S72 and *ftz* hybridization signals are more clearly seen just before cellularization. By gastrulation, each of the 14 hybridization stripes encompasses about three cells; a gap approximately one cell wide separates adjacent stripes (Fig. 3, d to f).

These results show that the overall patterns of S72 and *ftz* expression are similar. Transcripts encoded by each gene are detected at approximately the same time during development, and each shows a gradual evolution to a zebra pattern of expression during cleavage stage 14. The domains of expression for these genes do not encompass the entire length of the embryo; each is expressed only along about 50 percent of the anterior-posterior axis (from approximately 69 to 19 percent for S72 and from 65 to 15 percent for *ftz*). Finally, S72 and *ftz* transcripts gradually diminish during the latter stages of germ band elongation (5 to 6 hours after fertilization) and reappear during neurogenesis (*39*). The most obvious difference between the *ftz* and S72 expression patterns is that they do not correspond to the same cells. The seven S72 zebra stripes are anterior to the corresponding seven *ftz* stripes.

Maintenance of the *ftz* expression pattern requires *eve*$^{+}$ activity. The S72 cytogenetic map position and expression pattern suggest that it corresponds to *eve*. If this is so, then the S72 transcript distribution profiles shown in Fig. 2 provide an explana-

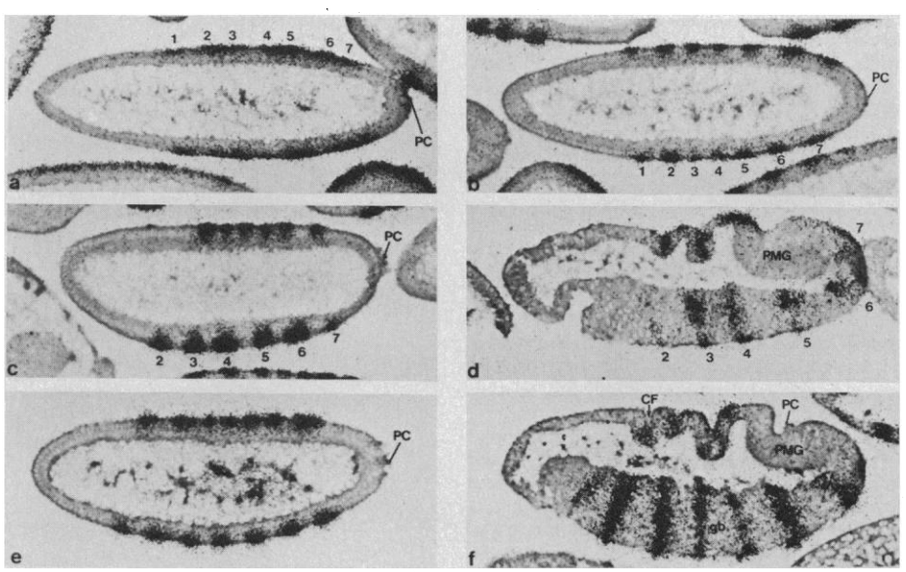

Fig. 4. Localization of *ftz* transcripts in *eve*$^{-}$ embryos and S72 transcripts in *ftz*$^{-}$. All sections are oriented with anterior to the left; sagittal sections are oriented so that dorsal is up. (a to d) *Df(2R)eve*$^{1.27}$ homozygotes (*eve*$^{-}$) after hybridization with the *ftz* probe. (e and f) *Df(3R)4Scb* homozygotes (*ftz*$^{-}$) after hybridization with the pS72-6 cDNA probe. (a) Horizontal section through an early stage 14 *eve*$^{-}$ embryo. Four bands of hybridization with the *ftz* probe are detected. The anterior-most band is half the width of the three more posterior bands. (b) Horizontal section through an *eve*$^{-}$ embryo at the midpoint of stage 14 development. Seven regularly spaced stripes of hybridization with the *ftz* probe are seen. (c) Sagittal section through a late stage 14 *eve*$^{-}$ embryo undergoing cellularization. Only six stripes of hybridization are seen. (d) Sagittal section through an *eve*$^{-}$ embryo at the start of germ band elongation. Six bands of hybridization with the *ftz* probe are observed. The intensity of band 2 is less than that of the more posterior bands. (e) Sagittal section through a *ftz*$^{-}$ cellular blastoderm after hybridization with the pS72-6 cDNA probe. Seven regularly spaced bands of hybridization are detected. (f) Sagittal section through a *ftz*$^{-}$ embryo undergoing germ band elongation. Seven S72 hybridization stripes are seen. Tissue sections were prepared as described in (*60*). Single-stranded ^{35}S-labeled p523B RNA probes (*ftz*) were used for (a to d); single-stranded ^{35}S-labeled pS72-6 RNA probes were used for (e) and (f). Abbreviations: PC, pole cells; CF, cephalic furrow; PMG, posterior midgut invagination; gb, germ band.

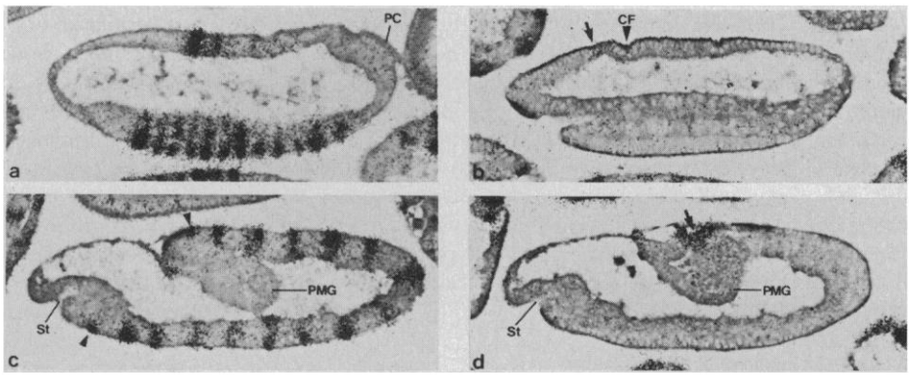

Fig. 5. Distribution of *en* transcripts in wild-type and *eve*$^-$ embryos. All sections are sagittal and are oriented so that anterior is to the left and dorsal is up. (a) Gastrulating wild-type embryo after hybridization with an *en* cDNA probe. In this plane of cutting only 12 of the 14 *en* expression stripes are seen. (b) A gastrulating *eve*$^-$ embryo after hybridization with the *en* probe. Only a single site of hybridization is detected (arrow), which is just anterior to the cephalic furrow. (c) Distribution of *en* transcripts in a wild-type embryo during germ band elongation. Sixteen hybridization stripes are detected; the anterior-most and the posterior-most of these stripes appear after gastrulation (arrowheads). (d) Expression of *en* in an *eve*$^-$ embryo during germ band elongation. In this plane of cutting, only one site of hybridization is detected, near the posterior-most portion of the germ band (arrow). This site of labeling appears to correspond to the posterior-most stripe seen in (c). In other planes of cutting, the anterior-most *en* hybridization stripes can also be seen at this time. Tissue sections were prepared as described in (*60*). An *en* cDNA was used as a probe (*12, 42*) after labeling with ^{35}S as described in (*16*). Abbreviations: CF, cephalic furrow; PC, pole cells; PMG, posterior midgut invagination; St, stomodeum.

tion for the weak *eve* mutant phenotype (Fig. 1d). It has been suggested that there is an approximate correspondence between the regions deleted in *ftz*$^-$ embryos and the sites of *ftz* transcript accumulation in wild-type embryos (*11*). On the basis of relatively crude measurements, there may be a similar relation between the sites of S72 expression and the regions absent in weak *eve* mutants. It is possible that *eve* mutants have reduced levels of the S72 product, resulting in a failure of proper segment morphogenesis within the regions of the embryo that would normally express high levels of *eve*$^+$ product. However, the S72 expression pattern does not provide a simple explanation for the extreme *eve*$^-$ phenotype (Fig. 1b). It is possible that this phenotype involves altered expression patterns of other pair-rule genes. It has been shown that there is an abnormal distribution of *ftz* proteins in *eve*$^-$ embryos (*39*). The *eve*$^+$ products might be required for the initiation, the elaboration, or the maintenance of the wild-type *ftz* expression pattern. To test these possibilities, *ftz* transcripts were localized within tissue sections of *eve*$^-$ embryos.

During wild-type stage 14 development, four broad *ftz* expression bands are gradually resolved into seven narrower stripes (*11, 15, 50*). In *eve*$^-$ embryos the initiation and evolution of the *ftz* expression pattern appears to be normal. In early stage 14 *eve*$^-$ embryos, four broad bands of *ftz* expression can be seen. As for wild type, the anterior-most band encompasses six to seven nuclei and each of the three more posterior bands encompasses 12 to 14 nuclei (Fig. 4a). In *eve*$^-$ embryos at the mid point of stage 14 development, seven equally spaced *ftz* stripes are detected, as for wild type (Fig. 4b). However, soon after the *ftz* zebra pattern is established in *eve*$^-$ embryos, the anterior-most stripe shows reduced labeling. During cellularization, there is a further reduction of *ftz* transcripts in this region (Fig. 4c). After cellularization is complete, there are additional abnormalities in the *ftz* expression pattern in *eve*$^-$; irregularities in the width and spacing of successive hybridization stripes are observed. Finally, the overall *ftz* expression pattern is less stable in *eve*$^-$ as compared with wild type. Normally, the seven *ftz* expression stripes persist throughout gastrulation and during the initial periods of germ band elongation. However, in *eve*$^-$, *ftz* transcripts rapidly diminish in abundance during gastrulation, such that by the onset of germ band elongation there is at least a two- to fourfold reduction as compared with wild type (Fig. 4d). These results suggest that *eve*$^+$ product is in some way required for the maintenance of the *ftz* expression pattern, but not for its initiation or evolution. Although *eve*$^+$ products appear to be required for the normal *ftz* expression pattern, *eve* expression does not appear to require *ftz*$^+$ activity (compare Fig. 4, e and f, with Fig. 2, e to g).

The initiation of the *en* expression pattern requires *eve*$^+$ activity. The expression pattern of *en*, another pair-rule gene that contains a homeo box, is also altered in *eve*$^-$ embryos. The *en* gene is required for the specification of posterior compartments in every segment (*51–56*). Much of the naked cuticle associated with each segment (Fig. 1c) appears to derive from the epidermal cells of the posterior compartment. Embryos homozygous for strong *en* mutant alleles show deletions of naked cuticle and fusions of adjacent denticle belts (*3, 52, 53, 56*) (Fig. 1a). The cuticular phenotype of this *en*$^-$ mutant is similar to that observed for *eve*$^-$ (Fig. 1, a and b). It is possible that the strong *eve*$^-$ phenotype results from the absence of *en*$^+$ products. In order to test this possibility, we hybridized an *en* cDNA probe (*12, 42*) to tissue sections of *Df(2R)eve*$^{1.27}$ homozygous embryos.

In gastrulating wild-type embryos, *en* transcripts are detected in the posterior compartments of each of the 14 middle body segments (*12, 15, 18*) (Fig. 5a). In contrast, only one *en* hybridization stripe is detected in an *eve*$^-$ embryo of similar age (Fig. 5b). When germ band elongation is complete, 17 *en* stripes are detected in wild type (*15, 18, 57*) (Fig. 5c), whereas only three stripes are seen in *eve*$^-$ (Fig. 5d). These three stripes occur outside the domain of *eve* gene function and correspond to *en* stripes 1, 2, and 17 observed in wild type. These results suggest that *eve*$^+$ products are somehow required for the initiation or maintenance of the normal *en* expression pattern.

Altered pattern of S72 expression in *en*$^-$ embryos. Within 30 minutes after *en* products reach high steady-state levels during gastrulation, there is a gradual decline in the abundance of S72 and *ftz* transcripts. It is possible that *en* is somehow responsible for the normal suppression of the S72 or *ftz* expression patterns. To test this possibility, we examined the distribution of S72 and *ftz* transcripts within tissue sections of *en*$^-$ embryos.

The S72 hybridization pattern is altered in *en*$^-$ embryos. There are 15 S72 hybridization stripes in *en*$^-$ embryos undergoing germ band elongation (Fig. 6a). In contrast, during a similar stage of wild-type development, only a single site of S72 labeling is observed (Fig. 2i). The S72 hybridization pattern is not altered in *en*$^-$ embryos before gastrulation. These results suggest that *en* products somehow negatively regulate S72 expression. Although *en*$^+$ activity is required for the normal suppression of the S72 zebra pattern, the *ftz* pattern does not appear to require *en*$^+$ activity (Fig. 6b).

Cross-regulatory interactions between *ftz*, *eve*, and *en*. Our results suggest that S72 corresponds to *eve*. However, definitive proof will require P-mediated gene transfer studies. Nonetheless, the wild-type pattern of S72 expression does not provide a simple

explanation for the unsegmented phenotype of strong *eve*$^-$ mutants (*40*) (Fig. 1b). The genetic domain of *eve*$^+$ function includes embryonic regions where S72 transcripts are not normally detected. It appears that the strong *eve*$^-$ phenotype involves altered expression patterns of other pair-rule genes. This possibility is supported by recent studies suggesting that the selective patterns of pair-rule gene expression depend on regulatory interactions among these genes (*39, 50*). Since null mutations in other pair-rule genes do not completely eliminate segmentation (*40*), it would appear that *eve* plays a critical role in a complex network of pair-rule gene interactions. The observation that the *ftz* and *en* expression patterns are altered in *eve*$^-$ supports this idea. A tentative hierarchy for possible interactions among *ftz*, *eve*, and *en* is summarized in Fig. 7.

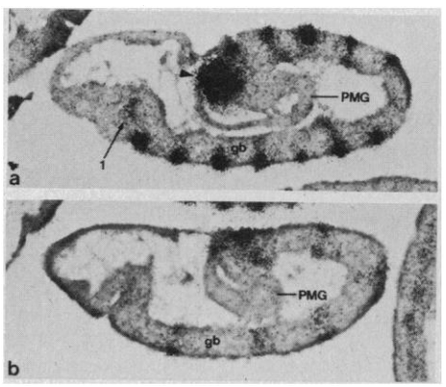

Fig. 6. Distribution of S72 and *ftz* transcripts in *en*$^-$ embryos. Both sections are from embryos undergoing germ band elongation and are oriented so that anterior is to the left and dorsal is up. (a) Expression of S72 in an embryo homozygous for the deficiency *Df(2R)en*^{SFX31}. This deletion uncovers both *en* and *invected*. Fifteen sites of hybridization are detected. The posterior-most site of labeling (arrowhead) corresponds to that seen in wild-type embryos at a similar stage (see Fig. 2i). (b) Expression of *ftz* in an embryo that is homozygous for the *Df(2R)en*^{SFX31} deletion. Seven regularly spaced stripes of hybridization are detected. This pattern appears to be identical to that observed for a wild-type embryo of similar age. Single-stranded ^{35}S-labeled RNA probes were used for hybridization (*16*). The pS72-6 cDNA probe was used in (a) and the p523B genomic fragment was used in (b). Abbreviations: PMG, posterior midgut invagination; gb, germ band.

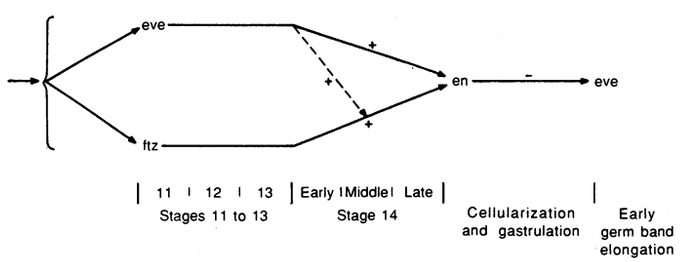

Fig. 7. Summary of interactions between *ftz*, *eve*, and *en*. The upper portion shows possible interactions between *ftz*, *eve*, and *en* during wild-type development. The lower portion shows the time during development when these interactions appear to occur. During cleavage stages 11 to 13, *ftz* and *eve* are each expressed over a broad region along the anterior-posterior axis. By the middle of stage 14 each is expressed as a series of seven zebra stripes. Once the *ftz* zebra pattern has been established, *eve*$^+$ activity is required for its maintenance. After cellularization, *en* is expressed as a series of 14 stripes (*15, 18, 57*). Both *eve* and *ftz* are required for the establishment of this *en* expression pattern. The absence of *en* transcripts in *eve*$^-$ might result from the combined effects of removing *eve*$^+$ products and altering the *ftz* expression pattern. In wild-type, *eve*$^+$ products might be required for initiating the odd-numbered *en* expression stripes, whereas *ftz* might be required for the even-numbered *en* stripes. In *eve*$^-$, there is a premature termination of *ftz* expression that could, in turn, result in the failure to initiate the even-numbered *en* expression stripes. The *ftz* and *eve* expression patterns gradually diminish in wild-type embryos undergoing germ band elongation. The decline in *eve* expression might result from negative regulation by *en*$^+$ products.

It has been shown that *en* is expressed in the posterior compartment of each segment (*12, 15, 18, 57*), and is required for the maintenance of segment boundaries (*52*). Embryos homozygous for strong *en* mutant alleles show fusions of adjacent segments. In this study we have shown that *en* is not expressed in the middle body region of *eve*$^-$ embryos. Thus, the absence of segmental subdivisions in *eve*$^-$ might result, at least in part, from the lack of *en* products. We propose that *ftz* and *eve* primarily affect the segmentation pattern by regulating the expression of *en*. In this model, *eve* and *ftz* products positively regulate *en* expression in alternating regions along the anterior-posterior body axis of gastrulating embryos; *eve* is required for the odd-numbered *en* stripes, whereas *ftz* regulates the even-numbered stripes. The absence of *en* products in *eve*$^-$ embryos might result from the combined lack of *eve*$^+$ products and the premature decay of the *ftz* expression pattern (Fig. 4). We do not intend to imply that *ftz* and *eve* affect pattern formation solely through the regulation of *en*.

If the absence of *en* products within the middle body region of *eve*$^-$ embryos were the sole cause of the lack of segmental subdivisions, the *en*$^-$ and *eve*$^-$ cuticular phenotypes would be identical. Since these phenotypes are somewhat different (Fig. 1, a and b), it appears that *eve* products can specify pattern information independently of *en* gene function. In the absence of *en*, S72 appears to be expressed in each of the 14 middle body segments during gastrulation and germ band elongation. Persistence of S72 transcripts in *en*$^-$ embryos might result in the formation of some segment boundaries despite the lack of *en* product. Therefore, it is possible that in wild type, *eve* and *en* are independently required for the formation of the normal segmentation pattern. If this is the case, the complete lack of segmentation in *eve*$^-$ embryos might result from the absence of both *en* and *eve* products, whereas *en*$^-$ embryos might retain some segment boundaries since *eve* products are present.

The interactions between *ftz*, *eve*, and *en* could be either direct or indirect. An example of an indirect interaction would be that the removal of *eve* gene function causes such a gross disruption of positional information that the expression patterns of many genes are indiscriminately altered. However, the timing, specificity, and patterns of the alterations seen in *eve*$^-$ are not consistent with such an indirect effect. In *eve*$^-$, the initiation and establishment of the *ftz* zebra pattern is normal. Moreover, the expression patterns of at least three homeotic genes (*Sex combs reduced*, *Antennapedia*, and *Abdominal-B*) are not obviously altered in *eve*$^-$ embryos at the cellular blastoderm stage (*58*). Finally, *en* expression is affected only within the middle body regions of *eve*$^-$ and appears normal near the anterior and posterior poles of the embryo, which are outside the limits of *eve* gene function. These observations are consistent with the possibility that the interactions that we examined are relatively direct.

Although one or more of the interactions between *ftz*, *eve*, and *en* may be direct, they are not sufficient to explain how each of these genes achieves its selective pattern of expression. For example, although *ftz* appears to be a positive regulator of *en*, *en* is not expressed in every cell that contains *ftz* product. In addition, S72 expression is not altered in *ftz*$^-$ embryos even though *en* appears to be a negative regulator of S72 and shows an altered expression pattern in *ftz*$^-$ (*50*). A possible explanation for these discrepancies is that a combination of pair-rule gene products acts in concert to regulate the expression of a given pair-rule gene. In this article we have analyzed the expression of only three of the nine pair-rule genes that have been identified. Further studies that include additional pair-rule genes are required to sort out the complex network of interactions that appear to occur.

The molecular basis for the interactions observed among *ftz*, *eve*, and *en* is not known. However, it is possible that some or all are mediated by the homeo box. The protein domain encoded by the *en* homeo box (the *en* homeo domain) includes a sequence-specific DNA binding activity. The *en* homeo domain binds to regions upstream from its 5′ terminus and possibly to upstream regions of other homeo box–containing genes (*37*). It is possible that homeo box proteins function as specific transcription factors for the expression of other homeo box genes. For example, *en* proteins might negatively regulate *eve* expression by directly binding to *cis* regulatory sequences associated with the *eve* gene.

REFERENCES AND NOTES

1. E. B. Lewis, *Nature (London)* **276**, 565 (1978).
2. T. C. Kaufman, R. Lewis, B. Wakimoto, *Genetics* **94**, 115 (1980).
3. C. Nusslein-Volhard and E. Wieschaus, *Nature (London)* **287**, 795 (1980).
4. ———, H. Kluding, *Arch. Entwicklungs Mech. Org.* **193**, 267 (1984).
5. E. Wieschaus, C. Nusslein-Volhard, G. Jurgens, *ibid.*, p. 283.
6. G. Jurgens, E. Wieschaus, C. Nusslein-Volhard, H. Kluding, *ibid.*, p. 296.
7. W. H. Ouweneel, *Adv. Genet.* **18**, 179 (1976).
8. R. E. Denell, *Genetics* **75**, 279 (1973).
9. M. Akam, *EMBO J.* **2**, 2075 (1983).
10. M. Levine, E. Hafen, E. R. Garber, W. J. Gehring, *ibid.*, p. 2037.
11. E. Hafen, A. Kuroiwa, W. J. Gehring, *Cell* **37**, 833 (1984).
12. T. Kornberg, I. Siden, P. O'Farrell, M. Simon, *ibid.* **40**, 45 (1985).
13. M. Akam and A. Martinez-Arias, *EMBO J.* **4**, 1689 (1985).
14. K. Harding, C. Wedeen, W. McGinnis, M. Levine, *Science* **229**, 1236 (1985).
15. M. P. Weir and T. Kornberg, *Nature (London)* **318**, 433 (1985).
16. P. W. Ingham, K. R. Howard, D. Ish-Horowicz, *ibid.*, p. 439.
17. D. C. Knipple, E. Seifert, U. B. Rosenberg, A. Preiss, H. Jaeckle, *ibid.* **317**, 40 (1985).
18. A. Fjose, W. McGinnis, W. J. Gehring, *ibid.* **313**, 284 (1985).
19. F. Kilchherr, S. Baumgartner, D. Bopp, E. Frei, M. Noll, *ibid.* **321**, 493 (1986).
20. E. Hafen, M. Levine, W. J. Gehring, *ibid.* **307**, 287 (1984).
21. G. Struhl and R. A. H. White, *Cell* **43**, 507 (1985).
22. P. A. Beachy, S. L. Helfand, D. S. Hogness, *Nature (London)* **313**, 545 (1985).
23. C. Wedeen, K. Harding, M. Levine, *Cell* **44**, 739 (1986).
24. G. Struhl, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 7380 (1982).
25. ——— and D. Brower, *Cell* **31**, 285 (1982).
26. I. Duncan and E. B. Lewis, in *Developmental Order: Its Origin and Regulation*, S. Subtelney, Ed. (Liss, New York, 1982), p. 533.
27. F. Karch *et al.*, *Cell* **43**, 81 (1985).
28. E. Sanchez-Herrero, I. Vernos, R. Marco, G. Morata, *Nature (London)* **313**, 108 (1985).
29. B. T. Wakimoto and T. C. Kaufman, *Dev. Biol.* **81**, 51 (1981).
30. T. Hazelrigg and T. C. Kaufman, *Genetics* **105**, 581 (1983).
31. M. P. Scott and A. J. Weiner, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4115 (1984).
32. W. McGinnis, M. Levine, E. Hafen, A. Kuroiwa, W. J. Gehring, *Nature (London)* **308**, 428 (1984).
33. W. McGinnis, R. L. Garber, J. Wirz, A. Kuroiwa, W. J. Gehring, *Cell* **37**, 403 (1984).
34. M. Regulski *et al.*, *ibid.* **43**, 71 (1985).
35. A. Laughon and M. P. Scott, *Nature (London)* **310**, 25 (1984).
36. J. C. W. Shepherd, W. McGinnis, A. E. Carrasco, E. M. DeRobertis, W. J. Gehring, *ibid.*, p. 70.
37. C. DesPlan, J. Theis, P. H. O'Farrell, *ibid.* **318**, 630 (1985).
38. M. Levine *et al.*, *Cold Spring Harbor Symp. Quant. Biol.* **50**, 209 (1985).
39. S. B. Carroll and M. P. Scott, *Cell* **45**, 113 (1986).
40. C. Nusslein-Volhard, H. Kluding, G. Jurgens, *Cold Spring Harbor Symp. Quant. Biol.* **50**, 145 (1985).
41. A. Kuroiwa, E. Hafen, W. J. Gehring, *Cell* **37**, 825 (1984).
42. S. J. Poole, L. M. Kauvar, B. Drees, T. Kornberg, *ibid.* **40**, 37 (1985).
43. T. Hoey, C. A. Rushlow, H. J. Doyle, M. Levine, in preparation.
44. B. P. Sonnenblick, in *Biology of* Drosophila, M. Demerec, Ed. (Wiley, New York, 1950), pp. 162–167.
45. D. Poulson, *ibid.*, pp. 168–274.
46. V. E. Foe and B. Alberts, *J. Cell Sci.* **61**, 31 (1983).
47. J. A. Campos-Ortega and I. Hartenstein, in *The Embryonic Development of* Drosophila melanogaster (Springer-Verlag, New York, 1985), pp. 9–25.
48. C. Nusslein-Volhard, E. Wieschaus, G. Jurgens, *Verh. Deutsch. Zool. Ges.* **91** (1982).
49. B. T. Wakimoto, F. R. Turner, T. C. Kaufman, *Dev. Biol.* **102**, 147 (1984).
50. K. R. Howard and P. Ingham, *Cell* **44**, 949 (1986).
51. P. Lawrence and G. Morata, *Dev. Biol.* **50**, 321 (1976).
52. T. Kornberg, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 1095 (1981).
53. ———, *Dev. Biol.* **86**, 363 (1981).
54. P. Lawrence and G. Struhl, *EMBO J.* **1**, 827 (1982).
55. A. Garcia-Bellido, P. Ripoll, G. Morata, *Nature* (*London*) *New Biol.* **245**, 251 (1973).
56. D. Gubb, *Arch. Entwicklungs Mech. Org.* **194**, 236 (1985).
57. S. DiNardo, J. M. Kuner, J. Theis, P. H. O'Farrell, *Cell* **43**, 59 (1985).
58. K. H. and C. Wedeen, unpublished results.
59. Dechorionated embryos were fixed at 60°C for 30 to 60 minutes in a solution of glycerol and acetic acid (1:4). After fixation, the embryos were mounted in Hoyer's medium (*4*) diluted 1:1 with lactic acid and allowed to clear at 60°C for 1 hour to overnight. We removed the vitelline membranes by gently pressing down on the cover slip before the mountant hardened.
60. Tissue sections were prepared (*16*) with the following modification: Eggs were dechorionated in freshly diluted 50 percent Clorox for 3 minutes. Fixation conditions varied with the age of the embryos, as follows. Embryos aged 0 to 3 hours after fertilization were first fixed in a mixture of 5 ml of heptane and 5 ml of 4 percent paraformaldehyde and then agitated for 20 minutes. They were postfixed as described (*16*). Embryos aged 2 to 4 hours after fertilization were first fixed in a mixture of 5 ml of heptane, 2.5 ml of 8 percent paraformaldehyde, 1.5 ml of 1× phosphate-buffered saline (PBS) and 1 ml of dimethyl sulfoxide (DMSO) and agitated for 20 minutes. They were postfixed as described (*16*). Embryos aged 3 to 6 hours after fertilization were first fixed in a mixture of 5 ml of heptane, 2.5 ml of 8 percent paraformaldehyde, 0.5 ml of 1× PBS and 2 ml of DMSO and agitated for 20 minutes. They were postfixed as described (*16*).

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