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# Control Elements of the Drosophila Segmentation Gene *fushi tarazu*

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## Summary

***Fushi tarazu* (*ftz*) is one of the Drosophila homeo-box-containing genes required to establish the segmental pattern. *Ftz*⁺ transcripts accumulate in cells that form seven equally spaced bands at the blastoderm stage. We have analyzed *cis*-acting controlling sequences of the *ftz* gene by germ line transformation. The functional *ftz* gene includes a transcription unit of 1.9 kb and ∼6.1 kb of the 5′-flanking sequences. Fusion of the 5′-flanking sequences to the E. coli *lacZ* gene directs expression of β-galactosidase in a "zebra" pattern in transformed embryos. The fusion gene is also expressed in certain neuroblasts that contribute to ganglion cells of all segments. Various morphogenetic controlling elements have been identified within the 5′-flanking sequences.**

## Introduction

Extensive genetic analyses in Drosophila have revealed that two classes of genes expressed in the zygotic genome are responsible for forming the metameric units, the segments. Segmentation genes are necessary to establish the correct segment number and polarity (Nüsslein-Volhard and Wieschaus, 1980), whereas homeotic genes are required to assign the proper identity of each segment (reviewed by Ouweneel, 1976; Lewis, 1978; Lawrence and Morata, 1983). Analysis of double mutants suggests that the establishment of segment identity is independent of the process of establishing the individual segments (Nüsslein-Volhard and Wieschaus, 1980).

The *fushi tarazu* (*ftz*) locus is one of the segmentation genes belonging to the "pair rule" class. Mutations in the *ftz* locus are embryonic lethal: homozygous *ftz*⁻ embryos lack parts of alternating segments and die as larvae possessing half the number of segments of the wild-type embryos (Wakimoto and Kaufman, 1981; Nüsslein-Volhard et al., 1982). *Ftz* is located within one of the homeotic gene clusters, the *Antennapedia* complex (ANT-C), on the right arm of the third chromosome (reviewed by Kaufman, 1983).

Recent molecular cloning of the *ftz* gene (Scott et al., 1983; Kuroiwa et al., 1984; Weiner et al., 1984) has opened a way to elucidate the mechanism by which the *ftz* product functions in the establishment of the segmental pattern. The *ftz* gene contains a copy of the homeo box, a conserved DNA sequence present in a number of homeotic and segmentation genes (McGinnis et al., 1984a; 1984b; Kuroiwa et al., 1984; Laughon and Scott, 1984; Fjose et al., 1985; Poole et al., 1984). Since the homeo domain, the protein domain encoded by the homeo box, contains many basic amino acids and includes a region exhibiting homology to regulatory proteins that bind DNA, it has been proposed that genes containing a homeo box encode regulatory proteins that control the activity of other genes (Shepherd et al., 1984; Laughon and Scott, 1984). The nuclear localization of the protein product of the homeotic gene *Ubx* is consistent with this hypothesis (White and Wilcox, 1984; Beachy et al., 1985).

The *ftz* gene is expressed early in development: *ftz*⁺ transcripts are first detected shortly after the cleavage nuclei have reached the egg surface. At the syncytial blastoderm stage *ftz*⁺ transcripts are accumulated in a regular pattern of seven equally spaced bands each of one segment width (Hafen et al., 1984b). After cellularization the position of the cells that accumulate *ftz*⁺ transcripts appears to coincide with the segment primordia which are deleted in *ftz* mutant embryos (Hafen et al., 1984b; Nüsslein-Volhard et al., 1982; Wakimoto et al., 1984). This supports the idea that *ftz* functions autonomously to establish the fate of the cells at the blastoderm stage, when segmental determination takes place (Wieschaus and Gehring, 1976; Lawrence and Morata, 1977).

How the *ftz* gene is controlled is not known. A number of loci, some of which exert a maternal effect, are known to affect the expression of homeotic genes and alter the spatial distribution of their products (Duncan and Lewis, 1982; Struhl, 1981a; Ingham and Whittle, 1980; Ingham, 1984; Beachy et al., 1985; Jürgens, 1985). In addition, there are certain regulatory interactions among the homeotic genes such that the activity of one gene represses the expression of another (Struhl, 1982; Hafen et al., 1984a). Regulatory loci for the segmentation genes have not yet been identified. The establishment of the regular distribution of *ftz*⁺ transcripts prior to cellularization suggests that the initial activation of the *ftz* gene occurs by interaction of nuclei and determinants of positional information contained in the cortical cytoplasm (Hafen et al., 1984b; Gehring, 1984). Subsequent refinement and/or maintenance of the regular pattern of expression may require activity of other zygotic genes.

As a first step in understanding the control of *ftz* expression, we have mapped the *cis*-acting controlling elements of the *ftz* gene by P-element-mediated germ line transformation. Despite the small size of the transcription unit, the functional unit of the *ftz* gene turned out to be large, including long 5′-flanking sequences. Several controlling elements, including a morphogenetic element necessary for the formation of the "zebra" pattern, were mapped within this region.

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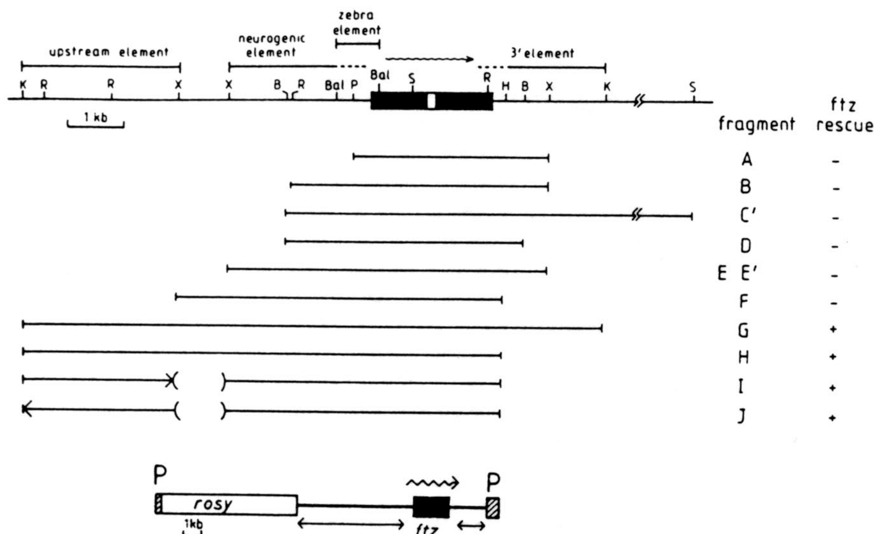


Figure 1. Restriction Map of the *ftz* Gene Used for Transformation

Exons are shown by solid boxes, the intron by an open box. The direction of transcription is indicated by a wavy arrow (from Kuroiwa et al., 1984). Fragments cloned into the P element vector Carnegie 20 are shown below the map. Fragments A through H constitute a simple deletion series. Fragments I and J have an internal deletion (shown by parentheses) in the 5′-flanking region and the upstream region is fused to the gene proximal part in the normal (I) and opposite (J) orientation. P elements are named as P[ry,ftzA], P[ry,ftzB], etc. to indicate the inserted fragments (A, B, etc.). B, Bam HI; H, Hind III; K, Kpn I; P, Pst I; R, Eco RI; S, Sal I; X, Xba I. For Bam HI, Hind III, and Pst I, only relevant restriction sites are shown. The rightmost Sal I site lies 7.1 kb downstream of the right Kpn I site.

The structure of the P[ry,ftzG] element is shown at the bottom of the figure. In all P elements except P[ry,ftzC′] and P[ry,ftzE′], the $ry^+$ gene in the Carnegie 20 vector is present at the 5′ side of the *ftz* gene. P[ry,ftzC′] and P[ry,ftzE′] elements have the *ftz* sequences inserted in the opposite orientation. The extent of 5′ and 3′ deletions are shown.

The ability of the P[ry,ftz] elements to rescue *ftz* lethality is indicated. The limits of the control elements identified are shown above the restriction map.

## Results

### A Long 5′-Flanking Region Is Necessary for the $ftz^+$ Function

In order to identify the functional unit of the *ftz* gene, various chromosomal DNA fragments of the $ftz^+$ gene were inserted into the P element vector Carnegie 20 containing the *rosy*$^+$ ($ry^+$) gene as a marker (Rubin and Spradling, 1983) (Figure 1). All of these fragments contain both the TATA box and the polyadenylation signal, the distance between them being 1.9 kb. These P element constructs were injected into recipient strains whose third chromosomes were marked with the $ry^{506}$ mutation. Transformants were selected by the rescue of the *ry* phenotype and established as balanced stocks.

We have used the rescue of the lethality of *ftz* mutations as a criterion for the $ftz^+$ activity of the P elements. *Ftz* lethality is a recessive character and flies homozygous for the *ftz* mutation on the third chromosome are viable when provided with an extra copy of the ANT-C (Wakimoto et al., 1984). Therefore a single copy of the $ftz^+$ gene is sufficient for normal development to the adult stage. After the establishment of the transformant stocks, appropriate genetic crosses (Figure 2) were carried out, which yield animals having one dose of the P[ry,ftz] element and are heterozygous for two noncomplementing *ftz* mutations in *trans* (P[ry,ftz]/$ftz^{9H34}$/$ftz^{9093}$). As an index of the ability to rescue *ftz* lethality, we define a value, the survival index (SI) which is the ratio of the number of the rescued *ftz* homozygous adults to the expected number of the *ftz* homozygotes carrying the P[ry,ftz] element, calculated from the number of other progeny from the same cross (Figure 2).

Nine constructs (P[ry,ftzA] through P[ry,ftzH], Figure 1), which contain eight different genomic fragments constituting a simple 5′ and 3′ deletion series, were used for the transformation. Between three and eight independent transformant lines were analyzed for each fragment. It turned out that most of the P[ry,ftz] elements do not possess $ftz^+$ activity as assayed by the above criterion. Inclusion of either 5′-flanking sequences up to 3.4 kb from the transcription initiation site (in P[ry,ftzF] element) or 3′-flanking sequences to 8.7 kb downstream of the polyadenylation signal (in P[ry,ftzC′] element) did not result in *ftz* rescue (Table 1).

*Ftz* rescue was achieved only when 6.1 kb of the 5′-flanking sequence was included. All eight transformant lines of the P[ry,ftzG] element yielded survivors with normal segmentation (Table 1). P[ry,ftzH] element, having a *ftz* fragment with 3′-flanking sequence deleted to 400 bp from the polyadenylation signal, also possessed $ftz^+$ function, but there was great variability in the SI among different transformant lines: two had SI values greater than 0.3, one had an SI of 0.15, and four had SIs of 0, i.e., *ftz* was not rescued (Table 1).

There is some evidence that transformant lines that do not yield any adult *ftz* survivors still exhibit partial $ftz^+$ ac-

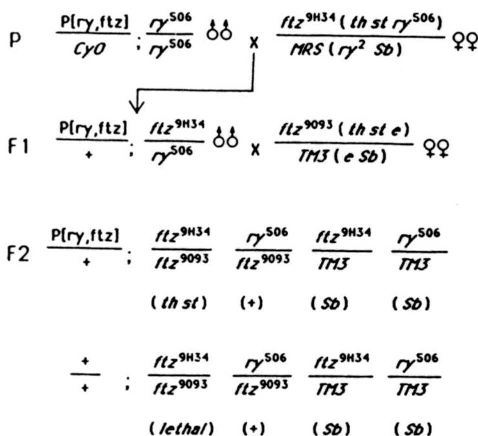


Figure 2. Crosses to Test for the Rescue of *ftz* Lethality

The crossing scheme for a P[ry,ftz] element on the second chromosome is shown. For integrations into the X chromosome, similar crosses were made except that the P[ry,ftz] element was introduced from females (in the P generation) and that the F1 males were hemizygous (P[ry,ftz]/Y). Relevant marker genes are indicated in the parentheses. In the F2 generation, embryos of eight different genotypes are produced. Adult individuals *trans*-heterozygous for two *ftz* alleles can be identified by the presence of *th* and *st* marker genes. The expected number of P[ry,ftz]/$ftz^{9H34}$/$ftz^{9093}$ individuals was calculated by dividing the number of the heterozygous class [wild-type phenotype (+)] by two. Transformants having an insertion on the third chromosome (marked with *th st* $ftz^{9H34}$ and $ry^{506}$) were backcrossed to $ry^{506}$ strain and F1 males were crossed to $ftz^{9093}$/ *TM3* females. The expected number of P[ry,ftz] $ftz^{9H34}$/$ftz^{9093}$ individuals equals the number of the progeny with the wild type phenotype ($ftz^{9093}$/$ry^{506}$).

tivity. We analyzed the cuticle pattern of the embryos that failed to hatch from the cross shown in Figure 2. Four transformant lines of the P[ry,ftzH] element with an SI value of 0 gave rise to embryos having more segments than homozygous *ftz* mutants (Figure 3). Such a phenotype is never observed with embryos *trans*-heterozygous for $ftz^{9H34}$ and $ftz^{9093}$, both of which are strong *ftz* alleles (Jürgens et al., 1984). Our interpretation of this phenotype is that the P[ry,ftzH] element in these lines produce a reduced amount of the *ftz* gene product due to a position effect exerted by the flanking sequences at the different sites of insertion. Indeed, a similar phenotype is observed in hypomorphic *ftz* alleles, such as a temperature sensitive one (Wakimoto and Kaufman, 1984).

We also examined the cuticular phenotype of the transformants carrying P[ry,ftzC′], P[ry,ftzE], and P[ry,ftzF] elements. These P elements also showed some $ftz^{+}$ function, since P[ry,ftz]/$ftz^{9H34}$/$ftz^{9093}$ embryos contain some of the segments missing in the $ftz^{9H34}$/$ftz^{9093}$ embryos (Figure 3). However, the rescue was generally weak and variable. We conclude that the functional unit of the $ftz^{+}$ gene includes more than 3.4 kb of the 5′-flanking region.

The requirement of the unusually long 5′-flanking sequence for the normal $ftz^{+}$ function led us to consider the possibility that this region contains two (or more) elements which are nonoverlapping. To test this possibility, we have constructed two P elements that have an internal deletion in the 5′-flanking region of the $ftz^{+}$ gene and have the far upstream region fused to the gene proximal part in both

Table 1. Transformant Lines of P[ry,ftz] Elements

| P Element | Line[1] | Recipient[2] | Linkage | SI |
|---|---|---|---|---|
| P[ry,ftzA] | 1 | R | X | 0 |
| | 2 | R | II | 0 |
| | 3 | R | II | 0 |
| P[ry,ftzB] | 1* | R | II | 0 |
| | 2* | R | X | 0 |
| | 3* | R | II | 0 |
| P[ry,ftzC′] | 1 | R | II | 0 |
| | 2 | R | II | 0 |
| | 3 | R | II | 0 |
| | 4 | R | II | 0 |
| | 5 | R | X | 0 |
| P[ry,ftzD] | 1 | R | II | 0 |
| | 2 | R | X | 0 |
| | 3 | R | II | 0 |
| | 4 | R | II | 0 |
| P[ry,ftzE] | 1* | R | II | 0 |
| | 2 | R | II | 0 |
| | 3 | R | II | 0 |
| | 4 | R | X | 0 |
| P[ry,ftzE′] | 1* | R | II | 0 |
| P[ry,ftzF] | 1 | F | II | 0 |
| | 2 | F | II | 0 |
| | 3 | F | II | 0 |
| | 4 | F | III | 0 |
| | 5 | F | III | 0 |
| | 6 | F | II | 0 |
| | 7 | F | II | 0 |
| P[ry,ftzG] | 1 | R | II | 0.29 |
| | 2 | R | X | 0.54 |
| | 3 | F | II | 0.58 |
| | 4 | F | III | 0.60 |
| | 5 | F | II | 0.36 |
| | 6 | F | III | 0.53 |
| | 7 | F | X | 0.33 |
| | 8 | F | II | 0.18 |
| P[ry,ftzH] | 1 | R | II | 0.42 |
| | 2 | R | II | 0.31 |
| | 3 | R | II | 0 |
| | 4 | R | II | 0.15 |
| | 5 | R | II | 0 |
| | 6 | R | II | 0 |
| | 7 | R | X | 0 |
| P[ry,ftzI] | 1 | F | III | 0.30 |
| | 2 | F | III | 0 |
| | 3 | F | X | 0.09 |
| | 4 | F | II | 0.36 |
| | 5 | F | II | 0.06 |
| | 6 | F | X | 0.35 |
| | 7 | F | II | 0.16 |
| | 8 | F | III | 0 |
| P[ry,ftzJ] | 1 | O | X | 0.65 |
| | 2 | O | X | 0.70 |
| | 3 | O | II | 0 |
| | 4 | O | X | 0.39 |
| | 5 | O | II | 0.11 |
| | 6 | O | X | 0.66 |
| | 7 | F | III | 0.69 |

[1] Lines with an asterisk were obtained by coinjection with pπ25.1 plasmid.

[2] R, $ry^{506}$; O, $ry^{506}$(ORM); F, *th st* $ftz^{9H34}$ $ry^{506}$ / *MRS*.

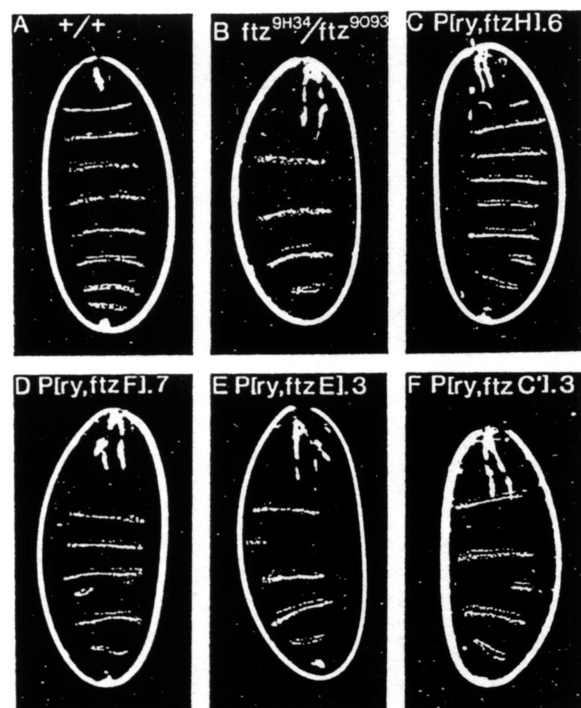


Figure 3. Ventral Cuticular Pattern of Transformant Embryos

(A) and (B) show the normal and the *ftz* mutant pattern, respectively. (C) to (F) show partially rescued phenotype of embryos carrying one dose of the respective P[ry,ftz] element (P[ry,ftz]/$ftz^{9H34}$/$ftz^{9093}$).

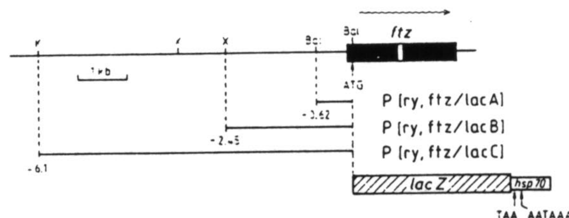


Figure 4. Schematic Diagram of the *ftz/lacZ* Fusion Genes

The indicated region is inserted into the Carnegie 20 vector such that $ry^+$ gene is located at the 5′ side of the fusion gene. The exons are shown by solid boxes, an intron by an open box. The position of the first methionine codon is indicated. Thick lines represent sequences from the $ftz^+$ gene. The fusion point of *ftz* and *lacZ* is at the Bal I site of the *ftz* gene, which cleaves within the second amino acid codon. The translation termination codon and polyadenylation signal are provided by the *hsp70* gene. Bal, Bal I; K, Kpn I; X, Xba I. Not all existing Bal I sites are shown.

orientations (Figure 1, P[ry,ftzI] and P[ry,ftzJ] elements). Both of these elements yielded transformants in which the *ftz* lethality was rescued (Table 1). Therefore the region required for the $ftz^+$ function consists of two parts, the far upstream region which acts in both orientations, and the gene proximal part, separated by a dispensable region.

## The Use of Promoter Fusion Genes to Monitor Gene Activity

As described above, transformation experiments with the $ftz^+$ gene fragments are suited to define the functional unit of the gene, but do not yield information about how the expression is affected in the nonfunctional constructs. In order to analyze the properties of the *ftz* controlling region, we constructed a series of P elements containing fusion genes in which the E. coli *lacZ* gene encoding β-galactosidase is placed under the control of the *ftz* promoter (Figure 4). Transcription from the *ftz* initiation site would produce a fusion transcript containing the *ftz* leader sequence, the β-galactosidase coding sequence, and an *hsp70* trailer sequence with the polyadenylation signal. This transcript should encode an active β-galactosidase, with no contribution from the *ftz* protein sequence. Three fusion genes with varying amounts of the *ftz* 5′-flanking sequences were constructed and several transformed lines were established for each of them.

We first characterized transformants obtained with the P[ry,ftz/lacC] element, since this P element contains all of the 5′-flanking sequence shown to be necessary for $ftz^+$ function in P[ry,ftz] elements. In order to examine the temporal expression pattern of the fusion gene, extracts of the transformant line P[ry,ftz/lacC].1 from various developmental stages were run on native polyacrylamide gels and stained for the β-galactosidase activity. A strongly stained band is observed in the embryonic stages (Figure 5). Since this band comigrates with purified E. coli β-galactosidase and is not present in extracts of the recipient embryos (data not shown), we conclude that it is derived from the *ftz/lacZ* fusion gene encoded in the P element. A faster migrating band is observed starting from the late embryonic stages and having a peak of expression at the pupal stage. This band represents the endogeneous Drosophila β-galactosidase since it is also present in the extract of the recipient strain and is absent in a strain carrying a null allele of the Drosophila *β-Gal-1* locus (data not shown).

The accumulation pattern of β-galactosidase during development of the transformant is consistent with the early zygotic expression of the $ftz^+$ gene. E. coli β-galactosidase is not detected in the adult female abdomen, but is present in early embryos. The peak of accumulation occurs between 4.5 and 10.5 hr after egg laying, during the germ band extension and retraction stages.

## *ftz/lacZ* Fusion Gene Is Expressed in a Segmental Pattern

The spatial distribution of the *ftz/lacZ* fusion gene transcript was analyzed by in situ hybridization. As a probe we used a plasmid pSPGal, containing the E. coli *lacZ* gene sequence. This probe has no homology to Drosophila sequences when analyzed by whole genome Southern hybridization under medium stringency conditions (data not shown). Figure 6 shows the distribution of the fusion gene transcript in a section through a blastoderm of the transformant line P[ry,ftz/lacC].4. The transcripts from the fusion gene accumulate in a regular pattern of seven bands, which is identical to the spatial distribution of the $ftz^+$ transcripts in the wild-type embryos (Hafen et al., 1984b). Therefore sequences from the $ftz^+$ gene contained in P[ry,ftz/lacC] element are sufficient to give rise to a segmental pattern of fusion gene expression.

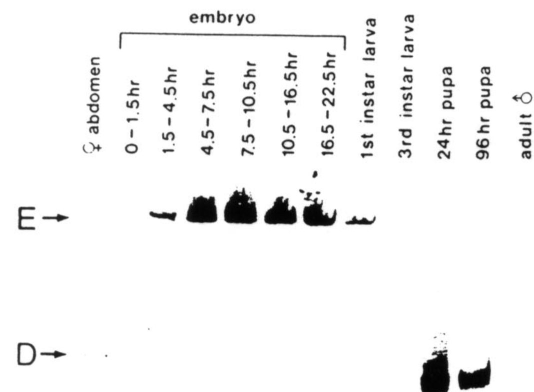


Figure 5. Developmental Profile of β-galactosidase Activity in the P[ry,ftz/lacC]. 1 Transformant Line

Extracts from various developmental stages were run on a native polyacrylamide gel and stained for β-galactosidase activity. Each lane contains extract from 5 mg (wet weight) of material. The β-galactosidase band derived from the *ftz/lacZ* fusion gene is designated E. The weak band at 0–1.5 hr of embryogenesis could be an artifact, since we find that this sample is contaminated with ~10% of gastrula stage embryos. The band D shows the β-galactosidase of Drosophila. This band is not present in a strain carrying a null allele of Drosophila *β-Gal-1* locus (data not shown).

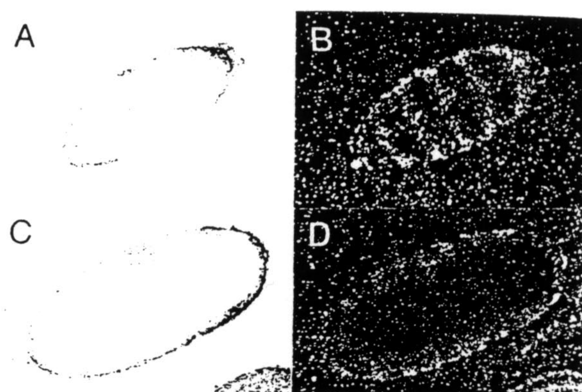


Figure 6. Localization of Transcripts from the *ftz/lacZ* Fusion Gene by In Situ Hybridization

Sections of blastoderm embryos of P[ry,ftz/lacC].4 transformant line. (A), (B) A superficial section through the blastoderm epithelium. (C), (D) A nearly horizontal section. The anterior end of the embryo points to the left. (A), (C) bright field; (B), (D) dark field optics. Tissue sections were hybridized to a nick translated pSPGal plasmid containing E. coli *lacZ* sequences. No specific hybridization was observed in recipient embryos (data not shown).

The expression of the *ftz/lacZ* fusion gene can also be visualized by localizing the β-galactosidase protein by activity staining of the enzyme. Figure 7 shows the distribution of β-galactosidase in whole mount embryos of a transformant line P[ry,ftz/lacC].4. β-galactosidase is first detected at the cephalic furrow stage where it is present in seven belts of cells surrounding the embryo (Figure 7A). The staining becomes more concentrated in the ventral region as the ventral furrow formation takes place. During the germ band extension stage the stained cells form seven sharp bands along the germ band (Figure 7B, 7C). The most anterior band is seen at the posterior side of the cephalic furrow. The seventh band, at the caudal end of the germ band, is wider than the other six bands and is occasionally seen as two bands, the more posterior one being approximately half the width of the other six bands (see also Figure 9C). This may indicate that the fusion gene is also expressed in part of the tenth abdominal segment.

Electrophoretic analysis of the embryo extracts has revealed that β-galactosidase in transformants persists until late embryogenesis (Figure 5). The high resolution and sensitivity of the β-galactosidase staining method allowed us to follow the expression of the *ftz* gene after completion of the germ band extension. Figure 7D shows an embryo shortly before the germ band starts to retract. In addition to seven bands of cells, large stained cells are observed regularly spaced between each band. A ventral view of the same embryo reveals that each repeat unit of stained bands contains two pairs of stained cells close to the midline (Figure 7E). Based on the distribution of these cells and the later staining of the ventral ganglia (see below), we conclude that these cells are neuroblasts that migrate inward from the outer ectoderm (Poulson, 1950; Turner and Mahowald, 1977; Hartenstein and Campos-Ortega, 1984). Another two pairs of labeled cells are located more laterally between the midline pairs (Figure 7E).

A 9 hr old embryo that has completed germ band retraction and with individual segments already visible is shown in Figure 7F. The epidermal cells containing β-galactosidase form bands approximately one segment wide, which do not correspond to segments but are out of phase: each band spans every other segmental boundary and includes the posterior part of one segment and the anterior part of the next one. This confirms our earlier interpretation based on the comparison of the position of the cells accumulating *ftz*⁺ transcripts in the blastoderm and the fate map (Hafen et al., 1984b). The region of fusion gene expression coincides exactly with the region deleted in *ftz* mutants (Nüsslein-Volhard et al., 1982; Wakimoto et al., 1984). The metameric units of *ftz*⁺ expression appear to coincide with parasegments defined by Martinez-Arias and Lawrence (1985).

In addition to cells of the epidermis, cells of the embryonic ventral nervous system (VNS) are strongly stained. Figure 8A shows the VNS dissected from a 12 hr embryo. In contrast to the epidermal cells, which express *ftz/lacZ* fusion gene in a "pair rule" fashion, cells containing β-galactosidase are found in ganglia of all segments. These cells are arranged in a regular pattern that is invariant from embryo to embryo.

## A Short 5′-Flanking Sequence Is Sufficient for Expression of the "Zebra" Pattern

We then analyzed the effects of deletions of the 5′-flanking

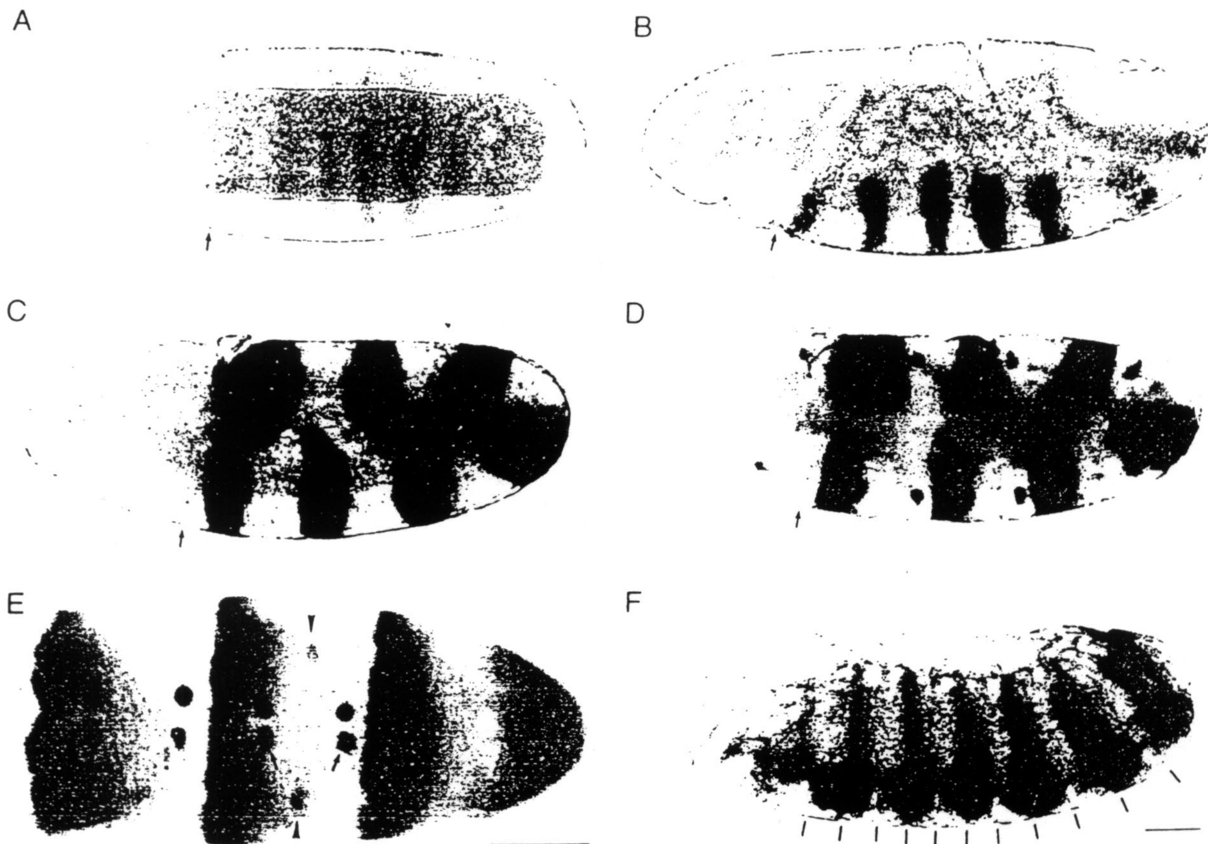


Figure 7. Localization of β-galactosidase in P[ry,ftz/lacC].4 Whole Mount Embryos

Embryos were fixed and stained for β-galactosidase activity. Anterior is to the left. (A) A cephalic furrow stage (3.5 hr). (B, C) Germ band extension stage (4–5.5 hr). (D) An embryo with fully extended germ band (7 hr). Note the presence of large stained cells between the stripes. (E) Ventral view of the same embryo. Each repeat unit contains two pairs of stained cells close to the midline (arrow). Another two pairs of cells located more laterally (arrowhead) also contain β-galactosidase. The second pair of the lateral cells is located in the stained bands and are not visible in this photograph. (F) An embryo that shows cuticular segmentation (9 hr). Note that the stained bands do not correspond to segments but are out of phase, overlapping the segmental boundaries (vertical lines). Arrows in (A) to (D) show the position of the cephalic furrow. Bar represents 50 μ.

sequences on expression of the *ftz/lacZ* fusion gene. In order to examine whether the fusion genes are expressed, the β-galactosidase level in transformant embryos was quantified. All transformant lines showed levels of β-galactosidase significantly higher than the recipient strains (Table 2). In most cases, β-galactosidase specific activity in transformant lines of two shorter fusions (P[ry,ftz/lacB] and P[ry,ftz/lacA] elements, see Figure 4) was about 50% of that in P[ry,ftz/lacC] transformant lines. One notable exception was P[ry,ftz/lacB].3 line, which had the highest specific activity of all lines. Southern blotting experiments showed that all lines contain a single copy integration of the respective P elements (data not shown).

The spatial distribution of β-galactosidase in flies transformed with the shorter fusion genes revealed two interesting features. First, the "zebra" pattern of β-galactosidase expression is maintained even when the 5′-flanking sequence is deleted to 0.62 kb from the transcription initiation site (Figure 9). Second, β-galactosidase is expressed not only in the seven bands of cells along the germ band, but also in one or in some cases two additional bands of cells anterior to the cephalic furrow (Figure 9). Such expression is never observed in embryos of any of the P[ry,ftz/lacC] transformant lines.

Deletion of the 5′-flanking sequence also affects the expression in the VNS. In P[ry,ftz/lacA] transformants, neuroblast staining (as in Figure 7D, 7E) was not observed (data not shown). Consequently, the cells of the VNS did not contain β-galactosidase, although epidermal staining was evident (Figure 8C). Expression of the P[ry,ftz/lacB] element was mainly in the VNS. Staining of the epidermal cells was considerably weaker than in P[ry,ftz/lacC] transformants (Figure 8B).

## Discussion

Using P-element-mediated germ line transformation we have shown that the functional *ftz*⁺ gene includes a large controlling region flanking the 5′ end of the transcription unit. *Ftz* mutations can only be complemented when 6.1 kb of 5′-flanking sequences are included in the transforming DNA sequences, whereas 3.4 kb are insufficient. Sev-

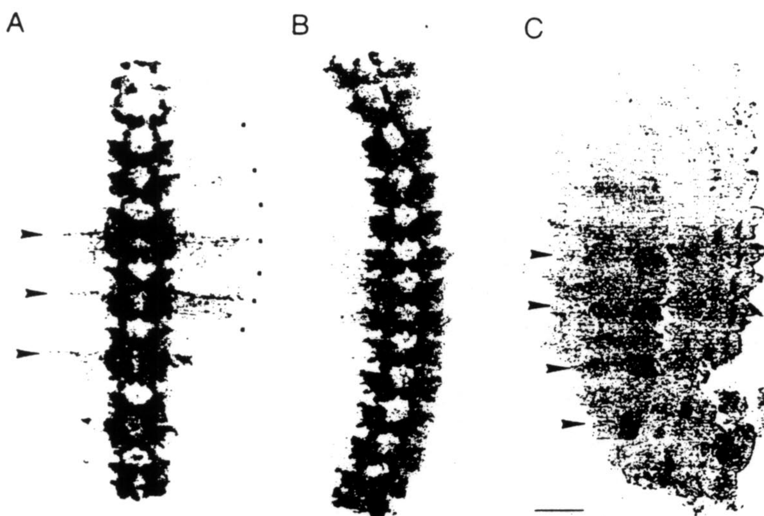


Figure 8. Localization of β-galactosidase in the Ventral Nervous System of the Transformants

The ventral nervous system was dissected from 10–12 hr embryos stained for β-galactosidase activity. Anterior is on the top. (A) A regular pattern of stained ganglion cells is observed in P[ry,ftz/lacC].4 transformants. Note that cells of all segmental ganglia contain β-galactosidase, whereas epidermal staining (arrowheads) obeys the "pair rule." Segmental boundaries in the cuticle are indicated by dots. (B) The same pattern of stained ganglion cells is observed in the P[ry,ftz/lacB].2 transformants but labeling of the epidermis is weaker. (C) P[ry,ftz/lacA].4 transformants do not show appreciable staining of the ventral nervous system, while epidermal staining is evident. Bar represents 50 μ.

eral *cis*-acting controlling elements have been mapped within the *ftz* functional unit.

### The Morphogenetic Control Element Generating the "Zebra" Pattern Is Located at the 5′ Side of the *ftz* Gene

The most striking property of *ftz* gene expression is that its transcripts accumulate in seven narrow bands of cells at the blastoderm stage (Hafen et al., 1984b). The P[ry,ftz/lacA] element, which contains only 0.74 kb from the *ftz*+ gene, directs accumulation of β-galactosidase in a segmental fashion, although there is some deviation from the normal pattern (discussed below). We conclude that the basic information necessary for generating the "zebra" pattern, the "zebra element", is encoded in this 0.74 kb fragment including the untranslated leader sequence and 0.62 kb of 5′-flanking sequences (Figure 1).

The regular pattern of accumulation of the *ftz*+ transcripts is established shortly after the thirteenth nuclear division, before the cell membranes are formed. At the eleventh nuclear division, the *ftz*+ gene is expressed uniformly in nuclei between 15% and 65% egg length (Hafen et al., 1984b, 0% egg length is the posterior end of the embryo). The mechanism by which this regular spatial distribution is achieved is not known. One obvious model is that the *ftz*+ gene is specifically transcribed in those cells that accumulate the transcripts. An alternative possibility is that selective degradation and/or transport of the transcripts are involved in their segmental distribution. Since our *ftz/lacZ* fusion gene also includes the untranslated leader sequences of the *ftz*+ gene, we cannot definitely resolve this problem. However, the fact that the spatial pattern of expression is altered by deletion of the 5′-flanking sequence strongly suggests that the primary regulation is at the transcriptional level. The possibility that the *ftz*+ leader sequence also plays a role in the stability of the mRNA cannot be ruled out.

Table 2. Transformant Lines of P[ry,ftz/lac] Elements

| P Element[1] | Line[2] | Linkage | β-galactosidase Specific Activity[3] (U/mg protein) |
|---|---|---|---|
| P[ry,ftz/lacA] | 1 | X | 12.6 ± 3.0 |
| | 2 | II | 18.1 ± 1.6 |
| | 3* | II | 8.1 ± 0.4 |
| | 4* | III | 20.2 ± 1.1 |
| | | average | 14.8 |
| P[ry,ftz/lacB] | 1* | II | 14.1 ± 3.1 |
| | 2* | II | 15.8 ± 1.3 |
| | 3* | II | 48.2 ± 9.8 |
| | 4 | X | 8.7 ± 2.3 |
| | 5* | II | 12.7 ± 2.3 |
| | | average | 19.9 |
| P[ry,ftz/lacC] | 1 | III | 23.7 ± 2.6 |
| | 2 | X | 28.0 ± 0.2 |
| | 3 | X | 37.9 ± 7.8 |
| | 4 | II | 32.8 ± 4.2 |
| | | average | 30.6 |

[1] Recipient strains for the transformation were as follows: P[ry,ftz/lacA]; *βGal-1*$^{n1}$; *ry*$^{506}$, P[ry,ftz/lacB]; *ry*$^{506}$, P[ry,ftz/lacC]; *ry*$^{506}$ (ORM).

[2] Lines with an asterisk were either homozygous lethal or poorly fertile. Eggs were collected from heterozygous individuals and β-galactosidase specific activity was multiplied by two, compensating the gene dosage effect.

[3] Each value is obtained from measurement of three separate egg collections. β-galactosidase specific activities of all recipient strains were below 1.0.

### Element Responsible for Expression in the VNS

We have found that in P[ry,ftz/lacC] transformants the cells of the VNS stain strongly for β-galactosidase. In contrast to epidermal expression, which obeys the "pair rule", VNS expression takes place in certain ganglion cells of all segments. Prior to germ band retraction, a subset of the neuroblast population contains β-galactosidase. Since each neuroblast divides repeatedly to produce a number

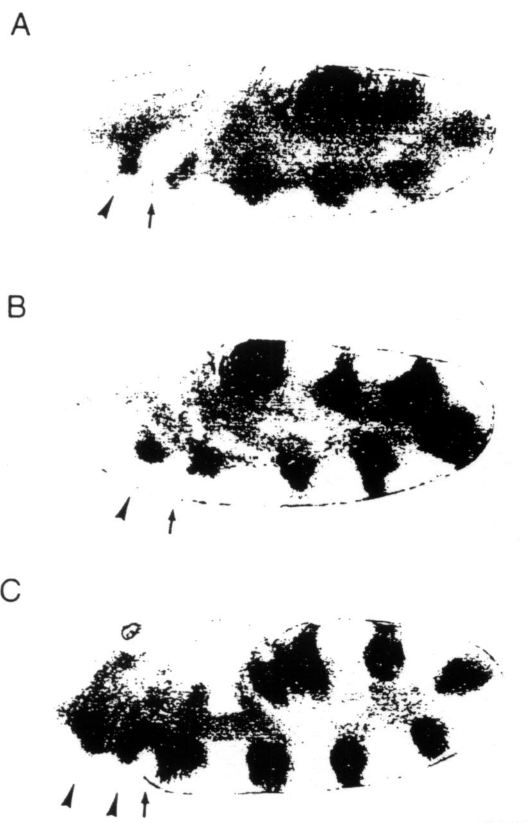


Figure 9. Expression of the Shorter *ftz/lacZ* Fusion Genes

β-galactosidase staining of whole mount embryos of the germ band extension stage (4.5–5.5 hr). Arrows show the position of the cephalic furrow. Extra bands anterior to the cephalic furrow are indicated by arrowheads. (A) P[ry,ftz/lacA].4, (B) P[ry,ftz/lacB].2, (C) P[ry,ftz/lacB].3. Bar represents 50 μ.

of ganglion cells (Poulson, 1950; Seecof et al., 1973), the strong staining of the ganglion cells suggests that the presence of β-galactosidase in the VNS is due to a de novo expression of the fusion gene in the neuroblasts and the ganglion cells. A small amount of *ftz*⁺ transcripts in late embryos detected by Northern blot analysis (Kuroiwa et al., 1984; Weiner et al., 1984) may represent expression of the *ftz*⁺ gene in the VNS.

Deletion of the 5′-flanking sequence to the Bal I site 0.62 kb upstream of the transcription initiation site (in P[ry,ftz/lacA]) abolishes expression in the neuroblasts and the VNS. We therefore conclude that the deleted region contains an element necessary for the late *ftz* expression in the VNS (the "neurogenic element", Figure 1). Whether this element itself confers VNS-specific expression or acts as a tissue-specific enhancer cannot be concluded from our experiments.

### The Far Upstream Element Has Properties of an Enhancer

The third element we have identified lies in the far upstream region, defined by the 2.7 kb Kpn I–Xba I fragment (Figure 1, the "upstream element"). This region is absolutely necessary for the *ftz*⁺ function: deletion in P[ry,ftz] constructs results in the inability to rescue *ftz* lethality. This region has several interesting features which may give an insight to the property of the encoded element. First, *ftz/lacZ* fusion genes lacking this region produce only about half the amount of β-galactosidase of the P[ry,ftz/lacC] element. Second, this element acts at variable distances from the gene, since the 3′ adjacent 0.95 kb Xba I fragment can be deleted without affecting *ftz*⁺ function. Third, this element can function in both orientations. These properties are common to enhancer elements (reviewed by Serfling et al., 1985). It remains to be tested whether this element can function when inserted at the 3′ side of the gene.

### A Possible Role of the 3′-Flanking Region in Gene Expression

There is some indication that a putative element at the 3′ side of the gene plays a general role in gene expression (the 3′ element, Figure 1). When this region is deleted from the P elements, the encoded *ftz*⁺ gene is subject to severe position effects, a fraction of the transformant lines exhibiting only partial *ftz*⁺ function.

Transformation studies with a number of genes have revealed that insertions into certain chromosomal locations influence the expression quantitatively without altering the tissue specificity (Spradling and Rubin, 1983; Goldberg et al., 1983; Scholnick et al., 1983; Bourouis and Richards, 1985). Although we have not attempted to quantify the amount of *ftz*⁺ RNA produced from P[ry,ftz] elements, the partially rescued phenotype of the lines with the position effect suggests that the P elements in these lines make normal *ftz*⁺ product in reduced quantity. It remains to be seen whether this 3′-flanking region encodes an element with a specific property, or only serves to physically separate the gene from the surrounding sequences.

### Partial Deletion of the *ftz* Control Region Results in Derepression of Expression in the Anterior Head Segments

We have found that deletion of the 5′-flanking region of the *ftz* gene results in a change in the spatial pattern of expression. In transformant embryos of both P[ry,ftz/lacB] and P[ry,ftz/lacA] elements, cells containing β-galactosidase were found in the region anterior to the cephalic furrow, where no signal is detected in the P[ry,ftz/lacC] transformants. *Ftz*⁺ transcripts are not found in this region by in situ hybridization either (Hafen et al., 1984b).

It is rather surprising that the expression of the fusion gene in the anterior head region is also confined to narrow bands, even though *ftz*⁺ gene is never expressed there. This suggests that the mechanism necessary for expression in the position-dependent segmental pattern extends to the anterior part of the embryo. Indeed, the *engrailed* gene, which is required to maintain the anterior–posterior compartment border and is expressed in the posterior compartment of each segment, is also expressed in at least two bands of cells in the region anterior to the cephalic furrow (Fjose et al., 1985; Kornberg et al., 1985). Al-

though the head region of Drosophila is not overtly segmented, the segmental Anlagen of the blastoderm has been proposed to contain six head segments (Struhl, 1981b), of which the most posterior two accumulate *ftz*⁺ transcripts in wild-type embryos (Hafen et al., 1984b). If we extrapolate the *ftz*⁺ expression pattern to the anterior head region, two additional regions of expression can be expected. Extra bands of cells expressing the fusion gene in P[ry,ftz/lacA] and P[ry,ftz/lacB] transformants may correspond to the cells in the anterior head region having the same positional identity as the cells expressing *ftz*⁺ transcripts in the posterior region of the embryo.

This further suggests that there is a mechanism that represses *ftz*⁺ expression in the anterior head region. Deletion of the far upstream region of the *ftz*⁺ gene appears to have caused the derepression of the fusion gene expression in that region.

The genetic mechanism of the repression and the nature of the derepression has still to be found. One possibility is that there is a gene (or genes) that represses *ftz*⁺ expression in the anterior head segments, and that the region deleted in the shorter fusions contains the binding sites for the repressor gene products. Alternatively, deletion of the upstream region may have caused a change in the chromatin structure, which resulted in a less strict control of expression, relieving the repression in the head segments.

A number of homeotic genes in Drosophila are clustered in two regions of the genome, the *Bithorax* complex and the ANT-C. Similar clustering of the homeo-box-containing genes has also been found in mouse and man (Joyner et al., 1985; Rabin et al., 1985). Our results showing that the *ftz*⁺ gene can exert complete function at ectopic sites prove that its location in the ANT-C is not a prerequisite for its function, although it may have an evolutionary significance. Identification of the functional unit of the *ftz* gene now allows a systematic study of the effect of mutations in the homeo box on embryonic development.

## Experimental Procedures

### Drosophila Strains

Two *ftz* alleles, $ftz^{9H34}$ and $ftz^{9093}$ (Jürgens et al., 1984), were provided by C. Nüsslein-Volhard. These mutations were induced on the multiply marked chromosome *rucuca* (Lindsley and Grell, 1968). A chromosome carrying the *th*, *st*, $ftz^{9H34}$, and $ry^{506}$ alleles was constructed by recombination with the chromosome having $ry^{506}$ allele (provided by A. Spradling). The following balancer stocks were used to maintain transformant lines: (1) *FM6*; $ry^{506}$, (2) *CyO*; $ry^{506}$, and (3) *MRS*, $ry^2$ *Sb*. The *CyO*; $ry^{506}$ strain was obtained from R. Klemenz. For a description of marker genes and balancer chromosomes, see Lindsley and Grell (1968). Flies were reared on standard cornmeal-yeast-agar medium at 25°C.

### P-Element-Mediated Transformation

Germ line transformation was done essentially as described by Rubin and Spradling (1982). In most cases the helper P element was pπ25.7WC (Karess and Rubin, 1984). Some initial transformants were obtained with the pπ25.1 plasmid. Embryos were injected with 100 μg/ml of the helper plasmid and 300–400 μg/ml of the Carnegie 20 constructs. In most of the experiments the recipient was the $ry^{506}$ strain. The *th st* $ftz^{9H34}$ $ry^{506}$/*MRS* strain was used for some of P[ry,ftz] elements and the $\beta$-*Gal-1*$^{n1}$; $ry^{506}$ strain (obtained from J. Lis) was used for the P[ry,ftz/lacA] element. We and others (Bourouis and Richards, 1985) have found that the second chromosomes of the $ry^{506}$ stock are heterogeneous for mutations that cause lethality and reduced female fertility. Consequently, when the P transposon was integrated into the second chromosome, homczygous transformant lines could not be established. We therefore exchanged the second chromosomes of the $ry^{506}$ stock with those of the Oregon R München wild-type strain. This stock, designated $ry^{506}$ (ORM) was used as the recipient for the transformation with the P[ry,ftzJ] and P[ry,ftz/lacC] elements.

### Plasmid Construction

Twelve P[ry,ftz] elements (Figure 1) were constructed by subcloning *ftz* genomic fragments into the polylinker region of the Carnegie 20 vector (Rubin and Spradling, 1983) by standard techniques (Maniatis et al., 1982). The source of the DNA fragments was lambda phage clone 523 (Garber et al., 1983), except for fragment D, which was taken from lambda phage clone 605 isolated from the homozygous *Ns* genomic library (AK unpublished). The right Sal I fragment of P[ry,ftzC'] is also derived from clone 605. The P[ry,ftzI] element has an internal deletion of the 0.95 kb Xba I fragment: this was made by digestion of the P[ry,ftzH] with Xba I and religation. The P[ry,ftzJ] element also has the same deletion and the upstream 2.7 kb Kpn I–Xba I fragment is inserted in the opposite orientation. This was achieved by first cloning the upstream fragment into the polylinker of pUC18, so that an Xba I site is provided 5′ to the Kpn I site. The junction point of Kpn I and Xba I sites in P[ry,ftzJ] element contains Bam HI, Sph I, and Sal I sites which originate from the polylinker.

Three P elements containing *ftz/lacZ* fusion genes (P[ry,ftz/lac] elements, Figure 4) were constructed using restriction sites present in the parental plasmids. The source of the DNA fragments was as follows: *ftz*; lambda clone 523 (Garber et al., 1983), *lacZ*; pUR288 (Rüther and Müller-Hill, 1983) and pUK230 (Koenen et al., 1982), *hsp70*; 56H8 (Schedl et al., 1978). The central part is the E. coli *lacZ* gene, which lacks the sequences encoding the first five amino acids and the termination codon, but otherwise should give a functional $\beta$-galactosidase protein when translated. The sequences from the *ftz* gene contribute to the 5′ portion. The *ftz* sequence in clone 523 differs from that described by Laughon and Scott (1984) in that the sequence of the second amino acid codon is GCC rather than GCT. This change creates a Bal I site (TGGCCA) that can be cleaved within the second amino acid codon. The 5′-flanking sequence and the leader sequence are fused at this Bal I site to the *lacZ* gene via polylinker sequences. The 3′ portion is derived from the *hsp70* gene, which provides the termination codon and the polyadenylation signal to the fusion gene. These fusion genes are inserted into the polylinker of the Carnegie 20 vector such that the *rosy*⁺ gene is located at the 5′ side of the fusion genes. A detailed protocol of the plasmid construction is available on request.

### *ftz* Rescue Assay

The ability of P[ry,ftz] elements to rescue the lethality of the *ftz* mutation was assayed by scoring the progeny of the cross shown in Figure 2. The expected number of p[ry,ftz]/$ftz^{9H34}$/$ftz^{9093}$ individuals was calculated by dividing the number of the mutant heterozygous class (P[ry,ftz]/$ftz^{9093}$/+ and $ftz^{9093}$/+) by two. The survival index (SI) is defined as the ratio of the number of the P[ry,ftz]/$ftz^{9H34}$/$ftz^{9093}$ individuals surviving as adults to the expected number of that class.

Cuticle preparations of the embryos were made according to the protocol of Van der Meer (1977). Embryos were collected for 12 to 24 hr at 25°C on apple juice–agar plates (Nüsslein-Volhard, 1977) and were allowed to develop further for 24 hr before mounting.

### In Situ Hybridization

In situ hybridization for detecting specific transcripts on tissue sections was done essentially as described by Hafen et al. (1983), except that the embryos were embedded without dechorionation and prefixation. Plasmid pSPGal, which contains a 1.9 kb fragment of the coding region of the *lacZ* gene in the pSP64 vector (Melton et al., 1984), was used as a probe. The specific activity of the probe was $7.2 \times 10^7$ cpm/μg.

### Detection and Measurement of $\beta$-Galactosidase Activity

For quantitative measurement of $\beta$-galactosidase activity in the transformant embryos, eggs were collected for 6 hr and were aged for 3 hr at 25°C. Dechorionated embryos were homogenized in Z buffer (0.1 M

phosphate buffer, pH 7, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) (20 μl/mg embryos) and centrifuged for 15 min at 10,000 g and 4°C to remove debris. Measurement of β-galactosidase activity was done according to Miller (1972) using 1.25 mg of embryos per assay. Protein concentration was measured with the Protein Assay (Biorad), using BSA as a standard.

The electrophoresis and staining of β-galactosidase in native polyacrylamide gels were performed essentially as described by Knipple and McIntyre (1984), except that the citric-phosphate buffer used was pH7 instead of pH6.

To stain embryos for the β-galactosidase activity, dechorionated embryos were fixed with heptane saturated with the fixative (25% glutaraldehyde, 50 mM cacodylate buffer, pH 7.3) for 10 min (Zalokar and Erk, 1977). Fixed embryos were transferred onto a double stick tape, covered with a drop of Drosophila Ringer solution, and the vitelline membrane was removed mechanically. The embryos were rinsed with citric-phosphate buffer (pH 8) and subsequently stained overnight for β-galactosidase activity with a solution containing 0.2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM $K_4[Fe(CN)_6]$, 5 mM $K_3[Fe(CN)_6]$ in citric-phosphate buffer (pH 8). All procedures were carried out at room temperature.

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