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Repression of the *Drosophila fushi tarazu (ftz)* segmentation gene

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The striped expression of the *Drosophila* segmentation gene *fushi tarazu* in alternate parasegments of the early embryo is controlled by the 740 bp zebra element. Among multiple protein factors that bind to the zebra element, FTZ-F2 behaves as a transcriptional repressor of *ftz*. Point mutations in the zebra element which disrupt FTZ-F2 binding to DNA cause ectopic expression of zebra-*lacZ* activity in transformed embryos. The mutant constructs are expressed from the zygotic genome in preblastoderm embryos as early as the third nuclear division cycle. This unprecedented early transcription suggests that *ftz* requires active repression during initial nuclear division cycles, a novel type of embryonic gene regulation. A putative FTZ-F2 cDNA clone isolated by recognition site screening of an expression library was found to be identical in sequence with the zinc finger protein *tramtrack* (Harrison and Travers, 1990).

Key words: ftz/preblastoderm embryo/repressor/segmentation/zebra element

Introduction

The process of segmentation in the *Drosophila* embryo is regulated by a network of maternal and zygotic genes (for reviews see: Akam, 1987; Nüsslein-Volhard *et al.*, 1987; Scott and Carroll, 1987; Ingham, 1988). The *fushi tarazu* (*ftz*) gene is a member of the 'pair-rule' class of segmentation genes that are expressed with double segment periodicity in the early embryo (Nüsslein-Volhard and Wieschaus, 1980). In the absence of a functional *ftz* product, the even numbered parasegments fail to develop, and the embryo dies late in embryogenesis (Wakimoto and Kaufman, 1981).

A low level of *ftz* expression is first observed at the syncytial blastoderm stage after the ninth nuclear division, when *ftz* transcripts are detected over nuclei along the entire length of the egg (Hafen *et al.*, 1984; Weir and Kornberg, 1985). During subsequent nuclear divisions, expression of *ftz* RNA becomes restricted to a region between 15% and 65% of egg length (0% is at the posterior pole). By early nuclear division cycle 14, *ftz* RNA and protein expression is further restricted transiently to several broad bands which subdivide later in cycle 14 (2–3 h of development) to seven evenly-spaced 'stripes' that encircle the cellularized blastoderm (Carroll and Scott, 1985; Krause *et al.*, 1988; Karr and Kornberg, 1989). These seven stripes of *ftz* expression, each 3–4 cells wide, are located in the even parasegmental primordia (Lawrence *et al.*, 1987) and are approximately coincident with the primordia of the body elements that are missing in *ftz*$^-$ embryos.

Expression of *ftz* RNA and protein persists during the early stages of gastrulation, but by the time the germ band is fully extended (4.5 h of development) *ftz* RNA and protein are no longer detectable throughout the embryo. Subsequently, between 5 and 12 h of development, *ftz* protein is newly expressed in a segmentally repeated subset of neuronal precursor cells of the ventral nervous system (Carroll and Scott, 1985; Doe *et al.*, 1988), and, between 12 and 15 h of embryogenesis, in a section of the hindgut (Krause *et al.*, 1988).

The temporal and spatial pattern of *ftz* expression is controlled primarily at the level of transcription. When the *ftz* transcription unit is replaced by the *lacZ* gene in promoter fusion experiments, the pattern of β-galactosidase activity observed in germ-line transformed embryos remains similar to the natural pattern of *ftz* expression (Hiromi *et al.*, 1985). The zebra element, a 740 bp region immediately upstream of the translational start, confers a weak, but nonetheless striped pattern of β-galactosidase activity in the embryonic mesoderm. Two other, more broadly defined elements (the neurogenic and the far upstream enhancer elements) comprise the *cis*-regulatory sequences required for appropriate levels of *ftz* expression in the blastoderm and nervous system (Hiromi *et al.*, 1985).

The lack of *ftz* expression in specific cells of the developing embryo is also important for embryonic development, since indiscriminate expression of *ftz* throughout the embryo results in a mutant phenotype (Struhl, 1985; Ish-Horowicz *et al.*, 1989). In embryos carrying *ftz–lacZ* constructs lacking sequences upstream of the neurogenic element, β-galactosidase is expressed ectopically in the anterior head region, suggesting that those sequences mediate repression of *ftz* in the anterior part of the embryo (Hiromi *et al.*, 1985). Subregions of the zebra element which mediate negative regulation have also been defined (Dearolf *et al.*, 1989a). Microinjection studies with the protein synthesis inhibitor cycloheximide have revealed two systems by which *ftz* transcription is repressed in early embryos (Edgar *et al.*, 1986a). One system, called polar repression, eliminates *ftz* expression from the anterior 35% and posterior 15% of the embryo, and a second system, called periodic repression, eliminates *ftz* expression in the interstripes.

We and others have conducted a search for specific *trans*-acting factors that interact directly with the *ftz* transcriptional

control sequences. Two specific *trans*-acting regulators of *ftz* transcription have recently been elucidated. FTZ-F1, a nuclear factor whose synthesis or activity is modulated during embryogenesis, binds to reiterated sites within the zebra element and the *ftz* coding sequence, and is implicated in the activation of *ftz* transcription, especially in the anterior three *ftz* stripes and stripe 6 (Ueda *et al.*, 1990). The product of the homeobox gene *caudal* (*cad*) binds to reiterated sequences within the zebra element and is implicated in activating *ftz* transcription in the posterior region of the embryo (Dearolf *et al.*, 1989b).

In analyzing multiple activities: FTZ-F2, FTZ-F3, and FTZ-F1 that interact with a 69 bp sub-region of the zebra element, we have found that mutations which disrupt binding by the FTZ-F2 proteins cause derepression of *lacZ* in embryos transformed with the mutated zebra-*lacZ* constructs.

Results

FTZ-F2 activity in developmentally staged extracts

*Exo*III protection experiments performed *in vitro* revealed an activity in embryo extracts that conferred resistance to convergent *Exo*III digestion at positions −131 and −191 upstream of the *ftz* transcription start site. When a ^{32}P-labeled, '69 bp' *Ban*I−*Hpa*II restriction fragment (−131 to −200) was incubated with nuclear extracts prepared from embryos staged at intervals of several hours, beginning at the syncytial stage (1.5 h of development) and analyzed by native gel elecrophoresis, at least four protein−DNA complexes of different gel mobilities were observed (Figure 1A). We refer to these electrophoretically distinct activities as FTZ-F2(A-D), the most prominent being FTZ-F2B.

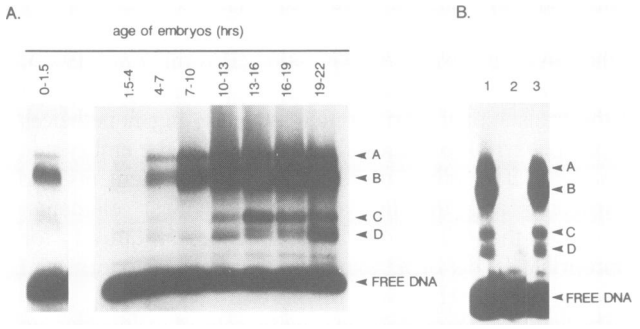

Fig. 1. Gel mobility shift assay showing factors that interact with the 69 bp subregion of the zebra element. **A.** Autoradiogram of a 1% agarose gel showing staged embryo proteins binding to a ^{32}P-labeled, 69 bp *Ban*I−*Hpa*II fragment (position −131 to −200) of the *ftz* gene. Extracts from embryos 1.5 h and older are from isolated nuclei; the amount of nuclear extract used in the assay was normalized to the DNA content of the nuclear pellet obtained from each extraction. 0−1.5 h embryos did not yield sufficient nuclei, hence whole embryos extracts were assayed for this stage. The amounts of binding activity are directly comparable for only stages subsequent to 1.5 h. The four protein−DNA complexes are denoted A−D. Not indicated are the cryptic complexes of FTZ-F1 and FTZ-F3 with the 69 bp fragment (see Figures 4 and 5). FTZ-F1 has a 10-fold lower affinity for the binding site in the 69 bp fragment (Ueda *et al.*, 1990) relative to the three other sites on the *ftz* gene, and is not obvious in 1.5−4 h embryo extracts. **B.** Specificity of DNA-binding. 16−19 h embryo extract was incubated with labeled 69 bp fragment in a gel mobility shift assay. In addition to the standard components used in the assay, each incubation contained 1 μg carrier *E.coli* DNA, and lane 1: no competitor DNA; lane 2: a 50-fold molar excess of unlabeled 69 bp fragment, and lane 3: a 50-fold molar excess of fragmented *E.coli* DNA.

FTZ-F2D appears at times as a double band on the gel. Competition experiments indicate that the binding of FTZ-F2 activities to labeled 69 bp DNA is sequence-specific (Figure 1B). The different gel mobilities of the FTZ-F2 factors may be due to covalent modification, proteolytic degradation, or to the association of one or more factors. Not evident in this analysis is a minor activity, FTZ-F3, which migrates to the same position as FTZ-F2A, and is revealed only after elimination of FTZ-F2A binding by mutation of the 69 bp fragment (see below).

The levels of FTZ-F2 DNA binding activities change as embryogenesis proceeds. Some FTZ-F2 activity is present in preblastoderm stage embryos (0−1.5 h) but the scarcity of nuclei at this stage necessitated the assay of whole embryo rather than nuclear extracts, and the amount of DNA binding activity is not directly comparable with the normalized activities in nuclear extracts after 1.5 h of development. In 1.5−4 h embryo nuclear extracts, when *ftz* expression in stripes is high, FTZ-F2 activities are extremely low. FTZ-F2A and FTZ-F2B activities rise progressively after 4 h of development, and reach a high level (a 30-fold increase) in

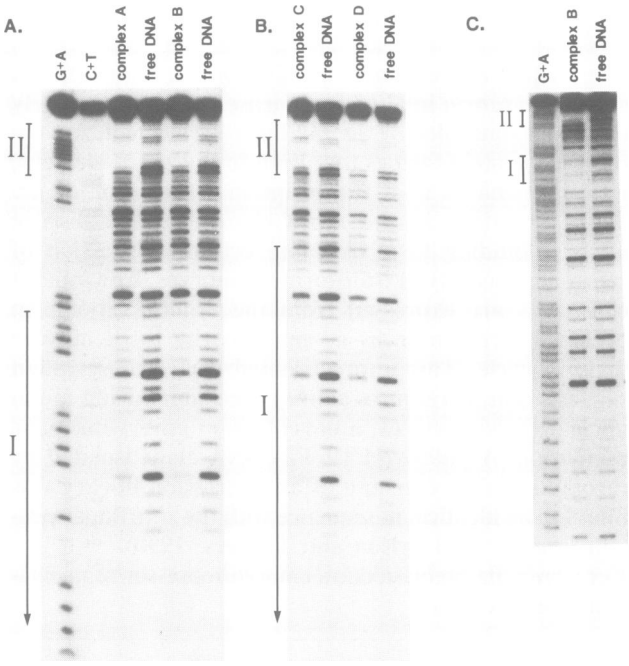

Fig. 2. DNase I protection analysis of the FTZ-F2 interaction with DNA. **A, B.** Autoradiograms of a 12% sequencing gel showing sequences on the upper strand of the 69 bp *Ban*I−*Hpa*II fragment that are protected from DNase I cleavage. Extract from 16−19 h embryos was incubated with ^{32}P-labeled fragment, the DNase I reaction was performed on the mixture, and the bound and free DNA were separated by electrophoresis. The DNA from complexes A−D, and from unbound (free) DNA were recovered and displayed on a sequencing gel. The extent of the sequences protected at two sites (I and II) is indicated by the vertical lines. The G+A and C+T lanes show the pattern of chemical cleavage at those residues. A weak DNase I protection was also detected on the lower strand. **C.** Autoradiogram of an 8% sequencing gel showing sequences on the upper strand of a 136 bp *Bss*HII−*Hpa*II fragment (positions −131 to −267) of the *ftz* gene that are protected from DNase I cleavage by FTZ−F2B. A similar protection pattern was observed for the other complexes (data not shown). The DNase I protection and the methylation interference experiments (Figure 3) reveal major protein−DNA interactions; interactions involving a minor fraction of the DNA would be obscured.

the latter half of embryogenesis. FTZ-F2C and FTZ-F2D activities are low in roughly the first half of embryogenesis, and increase slightly in the latter half. The temporal pattern of FTZ-F2 activity is generally consistent with the temporal pattern of *tramtrack* RNA expression as determined by in situ hybridization (Harrison and Travers, 1990).

Two related DNA sequences that interact with FTZ-F2 activities

The sites of FTZ-F2 binding within the 69 bp fragment were determined by DNase I protection analysis. FTZ-F2A and FTZ-F2B each protect two sites (sites I and II) at either end of the 69 bp fragment, leaving the intervening DNA free (Figure 2A). FTZ-F2C and FTZ-F2D each protect the same two sites; however, the inner boundary of protection at site I is extended further by several nucleotides (Figure 2B). Since the 5′ border of sequences protected at site I was close to the edge of the 69 bp fragment, a similar protection experiment was carried out with a longer, 136 bp fragment (Figure 2C).

The G residues on the 69 bp fragment that contact FTZ-F2(A−D) were identified using a methylation interference assay (Figure 3A). Methylation of either of two Gs on the upper strand at site I or site II was found to interfere with the binding to protein (Figure 3B). All four FTZ-F2 activities exhibit a similar pattern of methylation interference, although for FTZ-F2C and FTZ-F2D, the interference is apparently minor. No significant methylation interference was detectable on the lower strand (data not shown).

The two sites protected from DNase I digestion share a TGC__AGGAC__T sequence that is identical at 9/11 positions; when methylated, the two central Gs in the repeated sequence interfere with FTZ-F2 binding (Figure 3B). Intriguingly, FTZ-F2 binding site II overlaps one of the four binding sites for the transcriptional activator FTZ-F1 (FTZ-F1 binding site IV) (Figure 3B; Ueda *et al.*, 1990). Although the binding site for FTZ-F1 within the 69 bp fragment is 10-fold weaker than the binding sites located elsewhere on the *ftz* gene (Ueda *et al.*, 1990), an interaction of FTZ-F1 with the 69 bp fragment can also be observed (see below).

FTZ-F2 binding to site I and site II individually

To test the ability of the FTZ-F2 activities to interact with each binding site individually, single binding sites containing one TGC__AGGAC__T sequence were synthesized and used as templates for the gel shift assay. Only FTZ-F2C and FTZ-F2D are able to bind to site I or site II individually (Figure 4A, 4B). FTZ-F2A and FTZ-F2B do not bind to the individual sites, suggesting that these activities require a simultaneous interaction with both site I and site II in order to form a stable protein−DNA complex.

The FTZ-F2 group is completely separate and distinct from FTZ-F1. In addition to a different activity profile during embryonic development, FTZ-F2 and FTZ-F1 are separable by chromatography, and FTZ-F2 binding to DNA is not inhibited by the presence of antibodies raised against FTZ-F1 that eliminate FTZ-F1 binding to DNA (data not shown). As mentioned above, FTZ-F2 binding site II contains a sequence weakly recognized by the activator FTZ-F1 (Ueda

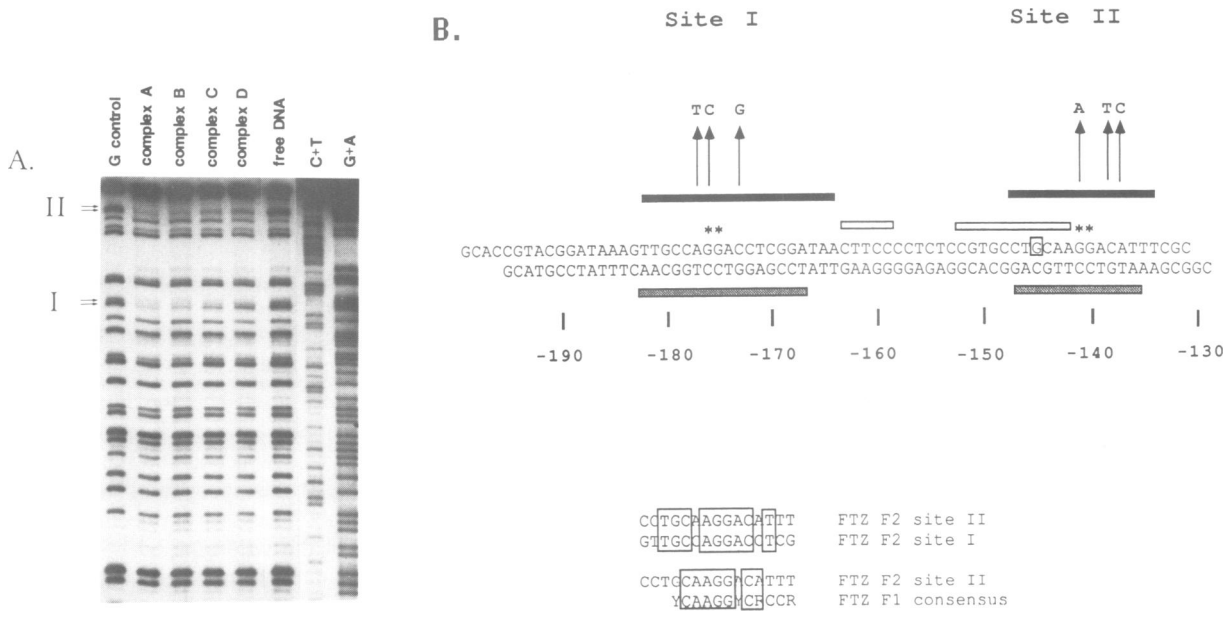

Fig. 3. Methylation interference analysis of FTZ-F2 binding to DNA. **A.** Autoradiogram of an 8% sequencing gel showing chemical cleavage at sites of G methylation on the upper strand of the 136 bp *Bss*HII−*Hpa*II upstream fragment (positions −131 to −267) of the *ftz* gene. Arrows indicate guanine residues which when methylated interfere with protein-binding (16−19 h embryo extract) to DNA. The degree of interference for FTZ-F2C or FTZ-F2D appears to be less than the interference for FTZ-F2A or FTZ-F2B. This is caused by the ability of FTZ-F2C and FTZ-F2D to bind either site I or to site II on the 69 bp fragment; hence, methylation at one site does not interfere with binding to the other site. **B.** Summary showing the sequences of the 69 bp region of the zebra element which interact with nuclear factors. Sequences protected from DNase I cleavage by FTZ-F2A,B binding are indicated by a solid bar for the upper strand, and shaded bar for the lower strand; the protection over the lower strand is weak, but reproducible; (data not shown). Asterisks denote G residues which when methylated interfere with FTZ-F2 binding to the 69 bp fragment. Open bars denote sequences protected from DNase I digestion by the binding of FTZ-F3, and the boxed nucleotide shows the single G which when methylated interferes with the FTZ-F3 binding to DNA (data not shown). The three-base substitution mutations at site I and site II are indicated by the arrows. The lower panel shows the boxed TGC__AGGAC__T consensus from site I and site II. The site II sequence is aligned with the consensus binding sequence for FTZ-F1 (Ueda *et al.*, 1990), and identical sequences are shown boxed. Y and R denote pyrimidines and purines, respectively.

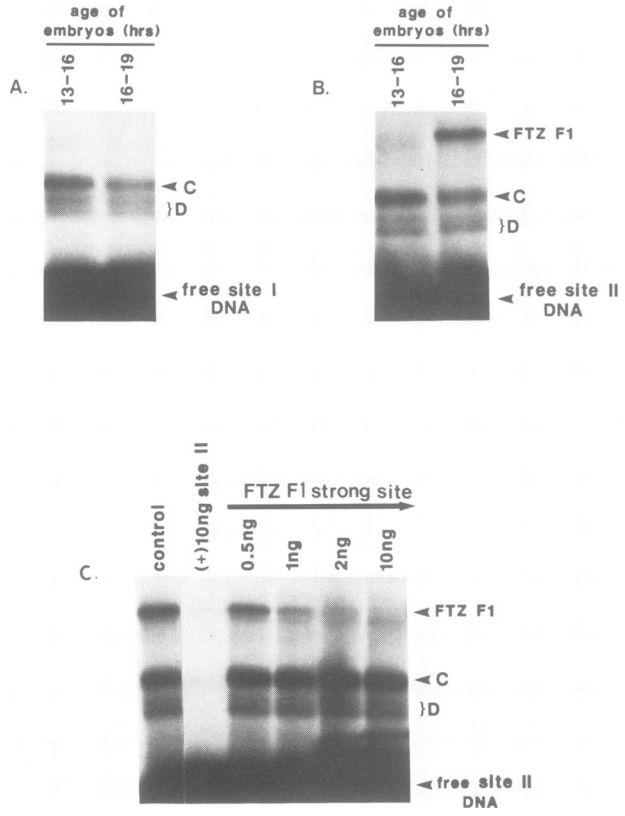

Fig. 4. FTZ-F1 and FTZ-F2 binding to fragments containing only site I or site II. **A,B.** Autoradiograms showing gel mobility shift analysis of stage embryo proteins binding to ^{32}P-labeled subfragments containing site I or site II. Double-stranded binding sites were prepared by annealing synthetic oligonucleotides (positions −194 to −156 for site I, and −164 to −120 for site II; a 4 base, 5′ extension was added to upper and lower strands). FTZ-F2C and FTZ-F2D were identified on the basis of their developmental profiles in staged embryo extracts, and from the agarose gel mobilities of the protein−DNA complexes, which do not change significantly with the changes in DNA fragment length (data not shown). **C.** Gel mobility shift assay of proteins in a 16−19 h embryo extract binding to ^{32}P-labeled site II DNA, in the presence of 10 ng of unlabeled site II DNA, or increasing amounts of a strong binding sequence for FTZ-F1 (positions −124 to −156; Ueda *et al.*, 1990).

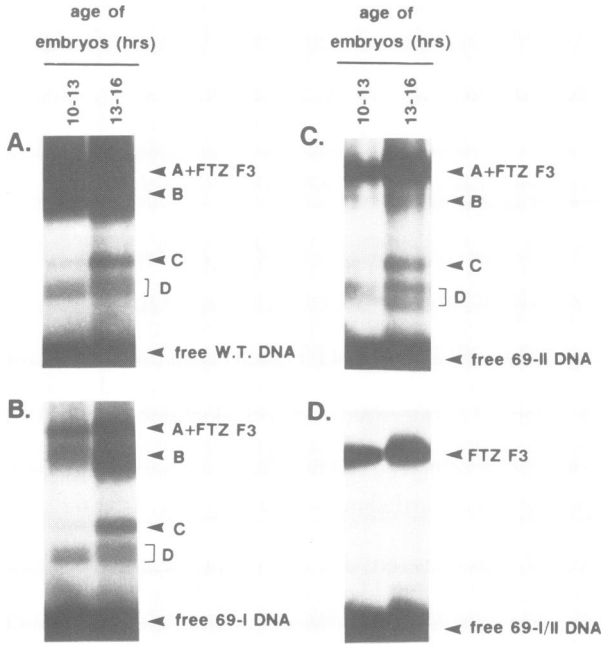

Fig. 5. FTZ-F2 and FTZ-F3 binding to the wild-type and mutated 69 bp fragment. Autoradiograms showing gel mobility shift analyses of staged embryo proteins binding to: **A.** the wild-type, 69 bp *Ban*I−*Hpa*II fragment (W.T.); **B.** the 69 bp fragment mutated at site I (69-I); **C.** the fragment mutated at site II (69-II); and **D.** the fragment mutated at sites I and II (69-I/II). The electrophoretic positions of unbound (free) DNA, and of bound FTZ-F3 and FTZ-F2(A−D) are indicated. The specific activities of the labeled DNAs in this figure are equivalent; hence the amounts of each of the complexes formed can be compared directly. The substitution mutations constructed within sites I and II are shown in Figure 3B.

et al., 1990). A distinct interaction of binding site II with FTZ-F1 is observed when an extract from 16−19 h embryos containing both FTZ-F1 and FTZ-F2 activities was analyzed (Figure 4B). No interaction of binding site II with FTZ-F1 is observed using a 13−16 h embryo extract that has very low FTZ-F1 activity (Ueda *et al.*, 1990). We confirmed the identity of the FTZ-F1−binding site II complex by showing inhibition of complex formation with an excess of unlabeled, strong FTZ-F1 binding sequence (Figure 4C). FTZ-F1 has the same gel mobility as FTZ-F2B (data not shown); hence, the minor binding of FTZ-F1 to the whole 69 bp fragment is masked by the binding of FTZ-F2B in extracts containing both activities.

Mutation of the TGC__AGGAC__T sequences disrupts FTZ-F2 binding and reveal a new activity, FTZ-F3

To further elucidate the interactions of the FTZ-F2 activities with the 69 bp region, three-base substitution mutations were introduced in the TGC__AGGAC__T sequences at binding site I (mutant 69-I), at site II (mutant 69-II), and at sites I and II combined (mutant 69-I/II) (Figure 3B). When compared with the binding to the wild-type 69 bp fragment (Figure 5A) mutation of site I resulted in a 4-fold decrease in binding to FTZ-F2B, and no change in the binding to FTZ-F2C and FTZ-F2D (Figure 5B). Mutation of site II resulted in a substantial decrease (17-fold) in the binding to FTZ-F2B, and decreased to a small extent the binding to FTZ-F2C and FTZ-F2D (Figure 5C). Mutation of both site I and site II completely abolished the binding to FTZ-F2(B,C,D) (Figure 5D). Since the mutations introduced into the TGC__AGGAC__T sequence at site I are not identical to the mutations at site II, the severity of the effect of the site II mutations does not necessarily reflect the dominance of site II over site I in the wild-type 69 bp sequence.

All of the above mutations do not affect the binding of a different activity, termed FTZ-F3, which migrates with the same gel mobility as FTZ-F2A (Figure 5). DNase I protection analysis of FTZ-F3 binding showed that its sequence specificity is distinct from the specificity of the FTZ-F2 group. The sequences protected by FTZ-F3 are located between FTZ-F2 binding sites I and II, and extended into part of site II (data not shown; sequences protected are noted in Figure 3B). In order to distinguish the binding of FTZ-F3 from the binding of FTZ-F2A on the wild-type 69 bp fragment, we separated FTZ-F3 from the FTZ-F2 activities by DNA-affinity chromatography using a synthetic fragment based on the FTZ-F3 binding site as ligand. The

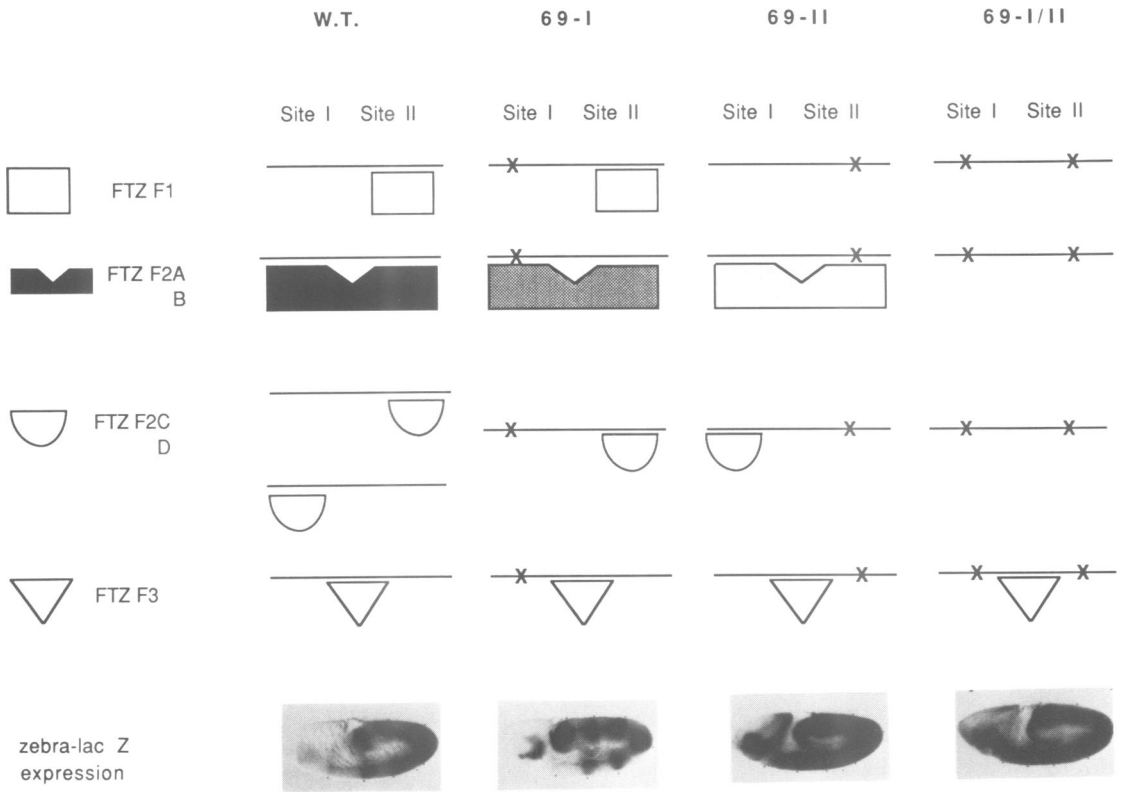

Fig. 6. Diagrammatic representation of factors binding to the wild-type and mutated 69 bp fragment. The X represents the base substitution mutations that were introduced in the TGC__AGGAC__T sequences at sites I and II. In the presence of excess 69 bp binding sequences in the gel shift assay, mutation of either site I or site II does not significantly decrease binding to FTZ-F2C and FTZ-F2D, because these activities are capable of binding to the remaining unmutated site. However, a decrease in FTZ-F2A and FTZ-F2B binding is caused by mutation of either site I or site II, represented by decreased shading of the cartoon, because both sites are required for stable complex formation. Only when site I and site II are both mutated is the interaction with all four FTZ-F2 activities abolished. The effects of the mutations on zebra-*lacZ* expression in transformed embryos is shown in the bottom.

bulk of the FTZ-F2 activities flowed through the FTZ-F3-specific affinity column, with some activity in the wash and low salt fractions, while the FTZ-F3 activity was primarily eluted in the high salt fractions (data not shown). With the depletion of FTZ-F3 from the extract, it was possible to determine that the low level of binding by FTZ-F2A to the 69 bp fragment is also abolished by the dual-site mutation (data not shown). A summary of factor interactions on the wild-type and mutated zebra element is given in Figure 6.

A putative FTZ-F2 cDNA clone is identical to tramtrack

We screened a 0−16 h embryo cDNA expression library in lambda gt11 with a labeled trimer of the 69 bp region essentially according to the procedure described by Vinson *et al.* (1988). Gel shift analysis of extracts prepared from a lysogen of a positive phage (2−18) showed specific binding of the recombinant protein to wild-type, but not to mutated FTZ-F2 binding sequences (Figure 7). In addition, methylation interference analysis showed that the recombinant protein makes close contact with the same G residues on the 69 bp fragment as does native FTZ-F2 protein. The results suggest that phage 2−18 carries the DNA-binding domain of a FTZ-F2 protein. By using the cDNA insert of phage 2−18 to rescreen the library, overlapping cDNA clones encompassing the entire FTZ-F2 open reading frame were isolated. The clones were sequenced (data not shown), and found to be identical in DNA sequence to the *tramtrack* (*ttk*) gene, which was previously cloned by a cDNA library screen using a sequence from the far upstream enhancer of the *ftz* gene (Harrison and Travers, 1990). The *ttk* gene encodes a zinc finger protein, which, for the purposes of discussion, we shall assume is related to the FTZ-F2 activities; a conclusive demonstration awaits immunological or protein sequence analysis.

In vivo effects of mutations in FTZ-F2 binding sites

To test the effects of mutating the TGC__AGGAC__T sequences at site I and site II *in vivo*, mutant zebra elements were joined to the *lacZ* gene and introduced into flies by P element-mediated germ line transformation. Multiple independent transformant lines carrying the wild-type and mutant zebra-*lacZ* constructs were established as homozygous stocks. The patterns of β-galactosidase gene expression in embryos collected from each transformant line were examined by indirect immunohistochemical staining. As reported previously (Hiromi *et al.*, 1985), the expression of zebra-*lacZ* constructs is undetectable at the cellular blastoderm stage, presumably because these constructs lack the upstream enhancer element, but is detectable shortly thereafter, during extension of the germ band, when the β-galactosidase protein has accumulated to sufficient levels. The staining pattern of embryos carrying the wild-type zebra-*lacZ* construct shows seven stripes primarily in the mesoderm. The anterior three stripes, especially stripe one,

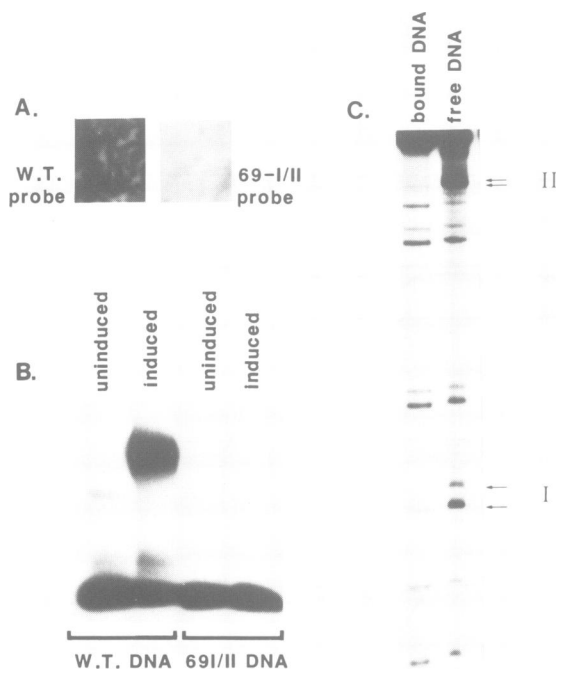

Fig. 7. The FTZ-F2 cDNA expression screen. **A.** Autoradiogram of nitocellulose filter sections carrying phage 2-18 screened for interaction with wild-type (W.T.) or mutated (69-I/II) 69 bp fragment, labeled to comparable specific activities. **B.** Autoradiogram showing a gel mobility shift assay using extracts prepared from uninduced and induced strains lysogenized with phage 2-18, and wild type or mutated 69 bp DNAs. **C.** Autoradiogram of a methylation interference assay showing G residues which when methylated, interfere with the binding of the recombinant fusion protein to the wild-type 69 bp fragment. The fusion protein makes close contact with the same four G residues (arrows) as does native FTZ-F2.

are relatively weak (Figure 8A). The pattern of *lacZ* expression in embryos carrying a mutation of the TGC__AGGAC__T sequence at FTZ-F2 binding site I (causing a 4-fold decrease in FTZ-F2B binding *in vitro*) is indistinguishable from the expression of the wild-type construct in all lines examined (Figure 8B). Mutation of the TGC__AGGAC__T sequence at site II (causing a 17-fold decrease in FTZ-F2B binding *in vitro*), and mutations at both TGC__AGGAC__T sequences (causing complete abolition of FTZ-F2 binding *in vitro*) result in aberrant *lacZ* expression, or a loss of repression, in the regions corresponding to the interstripes; faint striping can still be detected above the overall expression in the germ band extension stage embryo (Figure 8C and D). In addition, aberrant *lacZ* expression is consistently observed in the posterior midgut invagination, and in the ventral region anterior to the cephalic furrow.

In order to learn when *lacZ* derepression first becomes evident, we examined the staining pattern of embryos at earlier developmental stages. Prior to embryonic stage 9 (Campos-Ortega and Hartenstein, 1985), *lacZ* expression is undetectable in embryos carrying the wild-type zebra-*lacZ* construct. Remarkably, with the dual-site mutated construct, we could detect *lacZ* expression in very early embryos (stage 2: nuclear division cycles 3−8), well before the onset of expression of the endogenous *ftz* gene (Figure 8E−H). The precocious expression is observed as halos of stained β-galactosidase protein surrounding cleavage stage nuclei in the egg interior as early as the third nuclear division cycle (eight nuclei total) (Figure 8E). The widespread presence of β-galactosidase is also observed at the cellular blastoderm stage (Figure 8J). Embryos carring the wild-type construct do not show precocious *lacZ* expression (Figure 8I).

The very early expression of β-galactosidase could be due to transcription from either the maternal or the zygotic genome. We have determined that it is the zygotic genome that is transcribed by introducing the zebra-*lacZ* construct from the sperm only. Embryos resulting from a cross between males from each of two independent lines carrying the mutated zebra-*lacZ* construct and females that lack any construct expressed *lacZ* activity in a pattern identical to the embryos shown in Figure 8E−H. It should be noted that some of the *lacZ* RNA or protein could also be introduced by the sperm.

Discussion

We have described interactions of multiple DNA-binding factors with a 69 bp sub-region of the *ftz* zebra element. Two copies of a negative regulatory sequence TGC__AGGAC__T have been identified; substitution mutations in the TGC__AGGAC__T sequences cause a derepression of zebra-*lacZ* activity in transformed embryos at an extremely early stage of development. Among the protein factors FTZ-F1, FTZ-F2(A-D), and FTZ-F3 that interact with the 69 bp region, the best candidates for repressor function are the FTZ-F2 proteins, particularly FTZ-F2A and FTZ-F2B, since the mutant phenotypes are tightly correlated with substantial loss of FTZ-F2A and FTZ-F2B binding. It is possible that a role in repression is also served by FTZ-F1; however, the FTZ-F1−DNA interaction at this site is minor compared with the FTZ-F2−DNA interactions, and a neighbouring FTZ-F1 binding site on the zebra element behaves as a positive, rather than a negative regulatory element (Ueda *et al.*, 1990). Because FTZ-F3 binding *in vitro* is unaffected by the mutations that cause derepression, FTZ-F3 seems unlikely to be directly responsible for the repression of *ftz*. It is also unlikely that the mutant phenotype is caused by the adventitious binding of an undetectable factor that specifically recognizes the substitution mutations only. An alternate mutation (comprising a four-base deletion and a three-base substitution in the TGC__AGGAC__T sequences) resulted in a phenotype identical to the substitutions at both TGC__AGGAC__T sequences (S.Sonoda and M.P.Scott, unpublished observations).

The mechanism by which transcription of the *ftz* gene is repressed by FTZ-F2 is unknown. Binding of FTZ-F2 could exclude by steric hindrance transcriptional activators such as FTZ-F1, since both FTZ-F1 and FTZ-F2 contact the same G residues at overlapping binding sites. FTZ-F2 binding may also cause repression by directly or indirectly disrupting the formation of a productive transcription complex.

Relationship between FTZ-F2 and tramtrack

A comparison of the sequence of the putative FTZ-F2 cDNA showed identity with the sequence of the *tramtrack* (*ttk*) gene, which was cloned by recognition site screening of a cDNA library using a sequence from the far upstream enhancer of the *ftz* gene that binds specifically to a nuclear factor

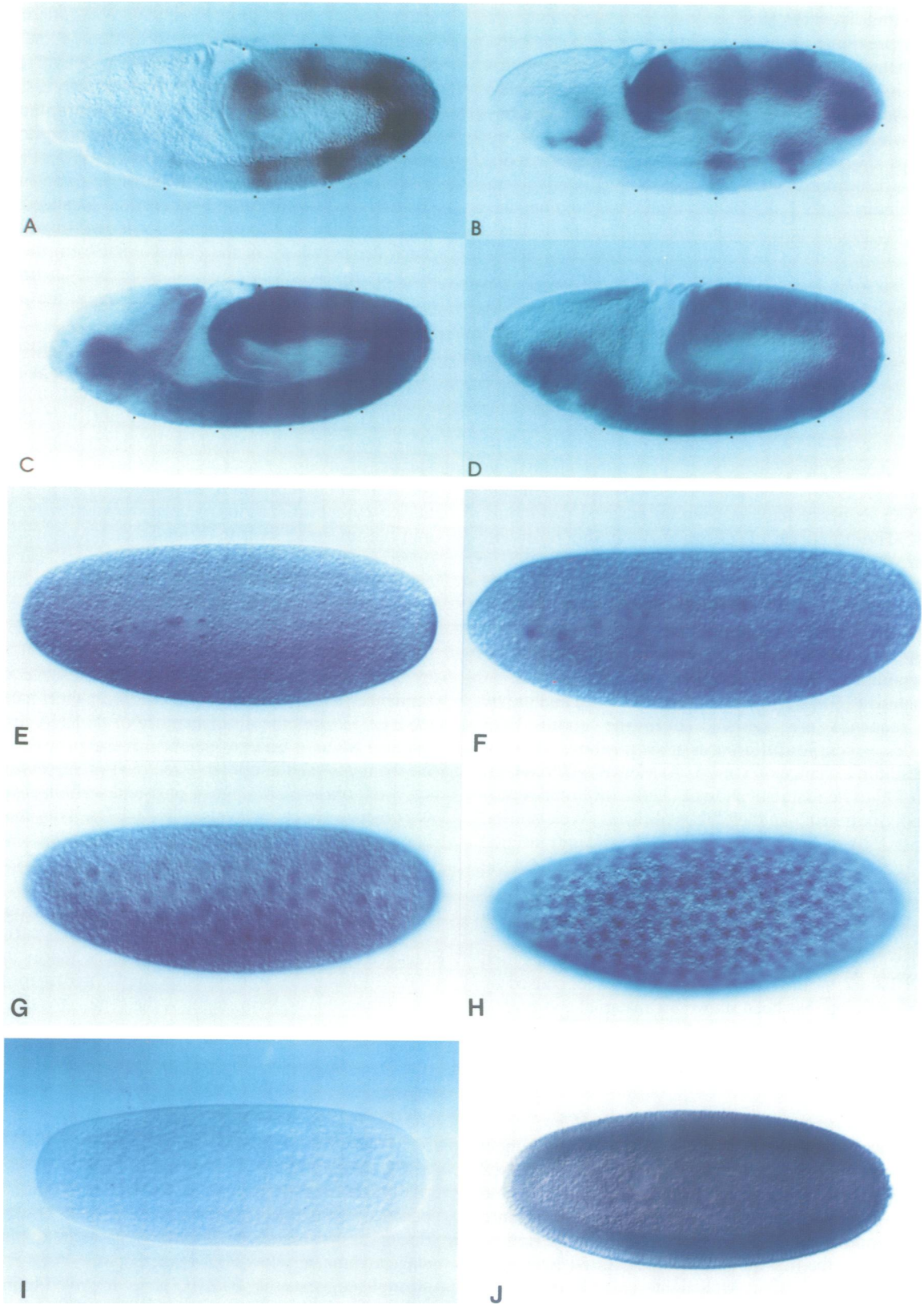

Fig. 8. *LacZ* expression detected by immunohistochemical staining of embryos transformed by wild-type and mutant zebra element-*lacZ* constructs. **A-D.** *LacZ* expression in germ band extension stage embryos carrying **A.** the wild-type zebra-*lacZ* construct; **B.** the zebra element mutated at FTZ-F2 binding site I; **C.** the mutation at site II; and **D.** the dual-site mutation. The approximate locations of staining in stripes are denoted by dots. The staining patterns shown are typical of at least four independent transformant lines for each construct. There is ectopic head staining specific to the line shown in 8B. **E–H.** *LacZ* expression in presyncytial blastoderm stage embryos carrying the zebra-*lacZ* construct mutated at FTZ-F2 binding sites I and II. The antero-posterior axis is from left to right. β-galactosidase expression is detectable as darkly-stained islands of cytoplasm surrounding cleavage stage nuclei. **I.** Lack of β-galactosidase expression in a presyncytial stage embryo carrying the wild-type zebra-*lacZ* construct. **J.** β-galactosidase expression of the dual site-mutated zebra-*lacZ* construct at the cellular blastoderm stage.

(Harrison and Travers, 1988, 1990). No *in vivo* function of *ttk* has previously been reported, although it has been speculated that *ttk* may negatively regulate *ftz*. The recognition site in the enhancer used to clone *ttk* has partial homology to the 69 bp region of the zebra element, and this similarity most likely accounts for the isolations of *ttk* by both groups. Two locations of *ttk* protein binding to the zebra element, as determined by DNase I footprinting, overlap with FTZ-F2 binding sites I and II, and a third *ttk* binding site, closer to the transcriptional start, overlaps a site of weak FTZ-F2 interaction [Harrison and Travers (1990); J.L.Brown and C. Wu, unpublished results]. However, the core consensus sequence TTATCCG noted by Harrison and Travers (1990) for *ttk*-binding—the TTATCCG sequence which lies immediately adjacent to a TGC__AGGAC__T sequence in the 69 bp region—does not seem to reflect the nucleotides crucial for FTZ-F2/*ttk* recognition. The methylation interference analysis we performed did not reveal close contacts to the TTATCCG sequence. An absolute match to the TTATCCG sequence does not bind *ttk* protein (Harrison and Travers, 1990). The functional importance of the TTATCCG sequence has not been tested by a mutational analysis. The footprint of *ttk* protein on the upstream enhancer (Harrison and Travers, 1990) does include a sequence TTAATGGACGT next to the TTATCCG sequence which is similar to the TGC__AGGAC__T consensus that we have identified. Therefore, we believe that the most accurate representation of *ttk* binding specificity should contain the invariant residues TnnnnGGACnT.

The putative FTZ-F2 protein coding sequence and the *ttk* coding sequence are identical over the entire 1923 nucleotides, except for differences at six positions, three of which result in a change of Thr-253 to Met, His-279 to Gln, and Thr-499 to Ile (data not shown). These differences may reflect polymorphisms in the cDNA libraries. We also find that the cDNA sequence diverges in the 5′ untranslated region, 166 nt upstream of the translational start. Since the *ttk* cDNA clone was isolated from a 0−4 h embryo cDNA library, while the FTZ-F2 cDNA clones were isolated from a 0−16 h embryo cDNA library, it is possible that this divergence may reflect differences in the maternal and zygotic FTZ-F2/*ttk* mRNAs. Such a difference, caused by alternative splicing, has been shown for maternal and zygotic mRNAs transcribed from the *hunchback* segmentation gene, which also encodes a zinc finger protein (Schröder *et al.*, 1988).

Repression during early nuclear division cycles

We have discovered that mutations in the zebra element which disrupt FTZ-F2 binding to DNA cause zebra-*lacZ* transcription from the zygotic genome in embryos prior to formation of the syncytial blastoderm, as early as the third nuclear division cycle. The zygotic genome is transcriptionally silent during the first nine, rapid nuclear division cycles (Lamb and Laird, 1976; McKnight and Miller, 1976; Anderson and Lengyel, 1979; Edgar and Schubiger, 1986), although in one instance expression of the *engrailed* protein was detected in pre-cycle 9 embryos (Karr *et al.*, 1989b). It is usually assumed that transcriptional inactivity at this early developmental stage is simply due to a general absence of activators or to inactivity of the general transcriptional apparatus. The unprecedented transcription we have observed suggests, however, that the general transcription components are present and potentially active, and that some activators are present throughout the preblastoderm embryo. The inactivity of *ftz* prior to formation of the syncytial blastoderm is thus likely to be due to active repression, probably directed by FTZ-F2. Consistent repression during early nuclear division cycles may be an absolute requirement for genes like *ftz*, which are subject to positive autoregulation (Hiromi and Gehring, 1987; Pick *et al.*, 1990). Even a transient activation of *ftz* might initiate the positive feedback loop and subvert the localized cues that regulate proper striped expression when the migrating nuclei reach the periphery of the embryo and form the syncytial blastoderm. We speculate that other early-acting zygotic genes may also be subject to repression at the presynctial stage.

The timely relief of early repression is critical for the initiation and evolution of the *ftz* pattern in the syncytial and cellular blastoderm. Given a finite amount of maternally provided repressor like FTZ-F2, an increase in the DNA:repressor ratio caused by the exponentially increasing number of dividing nuclei could provide a simple mechanism for terminating repression. Regulation by an increase in the nucleo-cytoplasmic ratio has been shown previously for the mid-blastula transition in *Xenopus* embryos, whereby the progressive titration of maternal substances lead to a new cell program, or to activation of an injected yeast tRNA gene (Newport and Kirshner, 1982a and 1982b). The nucleo-cytoplasmic ratio has been shown to affect the nuclear division cycle in *Drosophila* (Edgar *et al.*, 1986b). As noted by Harrison and Travers (1990), the presence of PEST sequences characteristic of proteins with short half-lives in *ttk*/FTZ-F2, and the disappearance of *ttk* RNA just around the time when *ftz* transcription begins also provide additional mechanisms for the effective removal of repression by the syncytial blastoderm stage. Consistent with this hypothesis, we observe very low levels of FTZ-F2 activity in 1.5−4 h embryo nuclear extracts.

Repression at later stages

The detection of significant levels of β-galactosidase protein at the cellular blastoderm stage in embryos carrying the mutated zebra-*lacZ* construct does not necessarily imply that FTZ-F2 has a role in repression (in the interstripes) during the cellular blastoderm stage, as the β-galactosidase protein is likely to have persisted from the derepression at earlier stages. There is a slight suggestion, from the somewhat decreased level of the accumulated β-galactosidase in the polar regions (Figure 8J), that FTZ-F2 is also not involved with polar repression during nuclear division cycles 11-13. Repeated attempts to visualize zebra-*lacZ* transcription by *in situ* hybridization, which would circumvent the persistence of β-galactosidase protein, were unsuccessful, possibly because these mRNAs are at low levels. However, the disappearance of *ttk* RNA during the cellular blastoderm stage and the very low FTZ-F2 activity in 1.5−4 h embryos strongly suggests that FTZ-F2 is *not* involved in interstripe repression. There is compelling genetic evidence that interstripe repression is mediated by *hairy* protein and perhaps other factors (Carroll and Scott, 1986; Howard and Ingham, 1986; Frasch and Levine, 1987; Hiromi and Gehring, 1987; Ish-Horowicz and Pinchin, 1987; Carroll *et al.*, 1988; Hooper *et al.*, 1989). *Hairy* protein is expressed in regions that are roughly reciprocal to the *ftz* stripes, and the localized *hairy* protein may be the major component of the interstripe repression system. The striped expression of *hairy* itself

appears to be generated from overlapping concentration gradients of the gap segmentation gene products (Pankratz *et al.*, 1990; Carroll, 1990).

The ectopic β-galactosidase activity detected at the germ band extension stage in transformed embryos carrying a mutated zebra element suggests a second, late repressive role for FTZ-F2, and this role is consistent with the increasing levels of FTZ-F2 DNA-binding activity (this paper), and with the distribution of *ttk* RNA (Harrison and Travers, 1990) after 4 h of embryogenesis. The post-blastoderm phenotype we have observed is also consistent with the phenotype obtained using a deletion analysis of the zebra element that suggested the presence of negative regulatory sequences between −112 and −222 upstream of the *ftz* transcriptional start (Dearolf *et al.*, 1989a). The deletion analysis did not uncover the early derepression, possibly because the analysis was not extended to preblastoderm stages, or because *cis*-elements important for early, but not late expression were removed by the deletions. It will be interesting to analyze further late repression in subsequent developmental stages of the embryo, larva and adult.

Conclusions and prospects

The identification by biochemical screens of new regulators of *ftz* underscores the possibility that important regulatory components may not be uncovered by genetic screens. In our studies with FTZ-F1 and FTZ-F2, a transcriptional activator and repressor of *ftz* respectively, it is likely that contribution of wild-type transcription factor (RNA or protein) to the egg by the nurse cells of the mother is sufficient to mask the zygotic phenotype or lethality. The possibility that these transcription factors may also be required for the proper expression of other genes not involved in the segmentation pathway further complicates their identification purely on the basis of a segmentation phenotype. Given that the maternal contribution to the *Drosophila* egg represents a large fraction of the genome (Perrimon and Mahowald, 1988), screens based on biochemical criteria should be employed as a complement to the genetic dissection of complex developmental systems.

Materials and methods

Preparation of embryo extracts

Nuclear extracts from staged *Drosophila* embryos were prepared as described in Ueda *et al.* (1990). For the 0−1.5 h whole embryo extract, 5 g of embryos were dechorionated and homogenized in a Dounce homogenizer in 15 ml homogenization buffer (0.35 M sucrose solution I (Ueda *et al.*, 1990)). All procedures were carried out at 4°C. The homogenate was filtered through 2 layers of nylon cloth (63 micron mesh), the mesh was washed with 15 ml homogenization buffer and the combined filtrate was centrifuged 10 min at 400 *g* to remove debris and unbroken cells. The supernatant was centrifuged 10 min at 4500 *g*, transferred to a fresh tube, centrifuged 50 min at 100 000 *g*, frozen in liquid nitrogen and stored at −70°C.

Gel mobility shift assay

1−3.6 μl of nuclear extract was incubated in a total volume of 10 μl with 20 fmoles labeled binding site, 10 μg tRNA, 4 μg poly (dI−dC)·poly (dI−dC), 75 mM NaCl, 15 mM Tris−HCl, pH 7.4, 2 mM Na phosphate, pH 7.0, and 5% glycerol. Samples were incubated for 15 min at 25°C, supplemented with 1 μl of 5% BSA, and 2 μl of gel-loading buffer (2.5% Ficoll 400, 0.5 × TBE, and tracking dyes), and electrophoresed on a 1% agarose, 0.5 × TBE gel.

DNase I footprinting of gel shift complexes

DNase I protection assays on specific complexes were obtained as described in Ueda *et al.* (1990).

Methylation interference assay

The 136 bp *Bss*HII−*Hpa*II fragment, 5′ end-labeled at the *Bss*HII site was partially methylated with DMS (Maxam and Gilbert, 1980). DNA was purified, incubated with 10 μl of 16−19 h extract, in a 50 μl volume under gel shift assay conditions, and electrophoresed on a 1% agarose, 0.5 × TBE gel. After electrophoresis, free DNA and protein−DNA complexes were transferred onto NA45 DEAE membrane (Schleicher and Schuell), located by autoradiography, and recovered as described in Ueda *et al.* (1990). DNA was cleaved at sites of G modification by piperidine treatment (Maxam and Gilbert, 1980), and equal amounts of radioactivity from bound and free DNA were electrophoresed on an 8% sequencing gel.

Affinity ligands

The affinity ligand specific for FTZ-F2 was *Bam*HI-linearized pJLB48, a plasmid which contains 48 directly repeated copies of the 69 bp *Ban*I−*Hpa*II fragment (positions −131 to −200) of the *ftz* gene. pJLB48 was constructed following the approach of Rosenfeld and Kelly (1986). The *Ban*I−*Hpa*II fragment was blunt-ended with Klenow polymerase and subcloned into the *Sma*I site of pUC19. *Bgl*II linkers were inserted into the *Kpn*I site (blunted with T4 DNA polymerase) to generate pJLB2, from which the 69 bp fragment was isolated on an 86 bp *Bgl*II−*Bam*HI fragment. This 86 bp fragment was the basic unit for multiple site construction. Directly repeated copies of this fragment were selected by ligation with T4 ligase, in the presence of *Bam*HI and *Bgl*II. The mixture of fragments was cloned into the *Bam*HI−*Bgl*II vector fragment of pJLB2, to yield pJLB6, containing 6 directly repeated copies of the unit fragment on a 516 bp *Bgl*II−*Bam*HI segment. This segment was isolated and cloned into pJLB6 linearized at the *Bam*HI site to generate pJLB12. The plasmids pJLB24 (24 copies of the unit), and pJLB48 (48 copies) were generated following the same principle. 1.0 mg of pJLB48 DNA was cleaved with *Bam*HI and ligated to CnBr-activated Sepharose according to Wu *et al.* (1988).

The affinity ligand specific for FTZ-F3 was made by annealing two complementary synthetic oligonucleotides based on the FTZ-F3 binding site (positions −140 to −172 of the *ftz* gene; nucleotide −141 was changed to a G; see Figure 3b). A 4-base, 5′ extension was added to both upper and lower DNA strands. The ligand was coupled to Sepharose according to Wu *et al.* (1988).

Mutant constructions

Mutants pM69-I and pM69-II were constructed by site-directed mutagenesis of p19BS (Ueda *et al.*, 1990). As a result, *Xho*I and *Bgl*II restriction sites were introduced into FTZ-F2 binding sites I and II, respectively. pM69-I/II was generated by further mutagenesis of pM69-II. The mutant constructs were sequenced over the site of the mutations.

Construction and transformation of zebra element-lacZ fusion genes

The P-element transformation vector, pCaSpeRβgal(Xho) was used to construct the wild-type and the mutant zebra element-*lacZ* fusion genes (Ueda *et al.*, 1990). Mutated zebra elements were cleaved from pM69-I, pM69-II, and pM69-I/II as 0.7 kb *Eco*RI−*Sal*I fragments and ligated to pCaSpeRβgal(Xho) cut with *Eco*RI and *Xho*I. Germ-line transformations were carried out as described (Ueda *et al.*, 1990). Linkage group assignments for transformant lines were determined by crossing individual males with *white*$^+$ activity to both *w;TM3/TM6B* and *w;Gla/CyO* or *w;Sco/Cyo* females; progeny were then backcrossed to establish the linkage of the insert.

Immunohistochemical staining of embryos

LacZ expression in transformant embryos was monitored by indirect immunohistochemical staining according to Carroll and Scott (1986) with the following revisions. After blocking with PBS-BSA, embryos were incubated with 200 μl of a 1:10 000 dilution of affinity-purified rabbit anti-β-galactosidase antibody that was previously incubated with 0−24 h *Drosophila* embryos for 4 h at room temperature. Embryos were blocked further with normal goat serum for 30 min at room temperature. Embryos were incubated for 2−4 h at room temperature with 13.5 μg/ml of biotinylated goat anti-rabbit IgG. After copious rinsing, embryos were incubated with 200 μl of the Vectastain Elite kit ABC reagent for 1 h at room temperature. The embryos were rinsed again extensively prior to incubation with 0.5 mg/ml diaminobenzidine, 0.4 mg/ml $NiCl_2H_2O$ and 0.001% for H_2O_2 for 5 min at room temperature.

Expression library screening

A 0−16 h embryo cDNA library in lambda gt11 (gift of Bernd Hoveman) was screened with a trimer of the 69 bp fragment. The trimer was constructed as described in the affinity ligand section for the pJLB6 insert, and was labeled by nick-translation according to standard procedures. The library screen was performed as described by Vinson *et al.* (1988), with these modifications: 10 mM Tris−HCl pH 7.5, 50 mM NaCl and 1 mM DTT

was substituted as the binding buffer and tRNA (final concentration 0.35 mg/ml) was included as carrier in the binding reaction. Out of 1.2×10^6 plaques, nine showed positive interaction with the probe. After plaque purification, three of the nine original positives bound to wild-type 69 bp DNA, but not to the 69-I/II mutant DNA, thus identifying candidate cDNA clones for FTZ-F2; the remaining positives bound to both DNAs, or showed no binding at all. DNA blot, restriction enzyme and partial sequence analysis of the cDNA inserts of the three putative FTZ-F2 cDNA clones suggested that two (2−18, 2−34) were isolates of the same phage, and the third (2-38B) carried sequences contained within the other two.

Lysogen extraction

Lysogens harboring the appropriate phages were isolated (Huynh *et al.*, 1985), and extracts from uninduced and induced lysogen cultures were prepared as described by Singh *et al.* (1988), with the inclusion of protease inhibitors (5 μg/ml leupeptin, 2 μg/ml pepstatin, and 0.75 μg/ml aprotinin) in the extraction buffer. Equal amounts of protein from induced and uninduced extracts were analyzed in the gel mobility shift assay.

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Note added in proof

The sequence data reported here have been submitted to the EMBL/Genbank/DDBJ databases.