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Upstream and Parallel regulators of *Drosophila fushi tarazu* are Identified by a Double Interaction Screen

by

YAN YU

A dissertation submitted to the Graduate Faculty in Biomedical sciences, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The City University of New York

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ABSTRACT

Upstream and Parallel regulators of *Drosophila fushi tarazu* are Identified by a Double Interaction Screen

by

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fushi tarazu (*ftz*) gene is a homeobox containing segmentation gene required for the establishment of the segmental body pattern of the *Drosophila* embryo. Like other homeodomain proteins, the specificity of FTZ protein was hypothesized to be regulated by interacting with cofactors. A 323 bp *ftz* proximal enhancer (fPE) was shown to contain necessary information for *ftz* stripe formation and autoregulation. This 323 bp fPE was used to develop a yeast-based screen (Double Interaction Screen) for identifying factors that interact with FTZ to modulate its activity and factors that interact directly with binding sites in the 323 bp fPE to regulate *ftz* gene transcription.

FTZ-F1α and TTK were isolated in the screen, demonstrating the validity of the selection scheme. In addition, FTZ-F1β, Sloppy paired1 (Slp1) protein, and several novel DNA-binding proteins were isolated. We showed that Slp1, a *forkhead* domain protein, interacts with at least one site in the 323 bp fPE, and that the 323 bp fPE mediates repression of *ftz* by Slp1 protein.

Two potential FTZ Interacting Proteins, FIP1 and FIP2, were isolated. *FIP1* encodes FTZ-F1α. Our experiments

suggested that FTZ and FTZ-F1α are present in the same complex in vivo, and that the two proteins bind cooperatively to target DNA. *FIP2* is a novel gene. In situ hybridization revealed that *FIP2* mRNA was expressed uniformly at early embryonic stages, and later restricted to the primordial endoderm cells. Preliminary data also showed that FIP2 protein selectively interacts with some homeotic gene products, suggesting it may be a co-factor for these homeotic gene products.

Using the Double Interaction Screen with a "bait" of the 323 bp fPE, we isolated some previously identified *ftz* regulators, several novel *ftz* regulators, and more importantly, two FTZ-interacting proteins. The isolation of FTZ cofactors demonstrates that the functional specificity of FTZ is indeed regulated by other proteins. Furthermore, the 323 bp fPE provides an example of the complexity of gene regulation which involves activation, repression, and cooperation. This type of complexity determines the unique identities of each cell in the developing organism.

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I dedicate this thesis to my parents.

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CHAPTER 1. INTRODUCTION

1. General Background

Like other insects, the body of the fruit fly, *Drosophila melanogaster* is composed of serial repeated body segments, which differentiate into particular structures according to their position. The process of determination of segmentation and segment identity is established in the early embryo (Akam, 1987; Gergen et al., 1986; Ingham and Gergen, 1988; Scott and O'Farrell, 1986). The study of this process has been made possible by the identification of mutations in a large number of genes. These mutations either disrupt the pattern of repeated units, or the identity of segments. The majority of these genes have been identified by systematic searches for lethal mutations resulting in abnormal segmentation (Akam, 1987; Johnston and Nusslein-Volhard, 1992; Nulsslein-Volhard and Wieschaus, 1980; Nusslein-Volhard et al., 1984). Both maternal genes and zygotically active genes were identified in these screens. They were classified according to whether their mutant phenotypes depend on the genotypes of the mother or that of the progeny. These genes were grouped into three classes.

The first class is maternal coordinate genes, which is composed of four systems. Three systems determine the anterior-posterior axis, one system determines the dorsal-ventral axis (Nusslein-Volhard et al., 1987). The second class consists of zygotically active genes affecting segment

number and polarity. These genes have been subdivided into three classes according to their mutant phenotypes (Jurgens et al., 1984; Nusslein-Volhard and Wieschaus, 1980; Nusslein-Volhard et al., 1984; Wieschaus et al., 1984). Mutations in the gap genes (e.g. *hunchback*) cause deletions of continuous segments. Mutations in the pair-rule genes (e.g. *fushi tarazu*) cause deletions of alternative segments. Mutations in the segment polarity genes (e.g. *engrailed*) affect part of every segment. The third class, the homeotic genes, is necessary for determining segmental identity. Mutations in the homeotic genes cause transformation of one body part into another (Bateson, 1894). There are two clusters of homeotic genes in flies: the *Antennapedia* complex (ANT-C) controls the development of the head and the anterior thoracic segments, and the *Bithorax* complex (BX-C) specifies the thoracic and abdominal segments (Akam, 1987; Lewis, 1978).

These three classes of genes form a hierarchical control network in which the maternal genes regulate the activity of the gap genes; the gap genes regulate the expression of pair-rule genes; the pair-rule genes regulate the expression of segment polarity genes; and finally, both pair-rule genes and segment polarity genes control the expression of the homeotic selector genes. Also, interactions between genes in the same class have been found. This cascade of interactions was established by the epistatic studies, which demonstrate how a mutation in one gene affects the expression of another gene (Akam, 1987). For instance, studies of the *fushi tarazu*

(ftz) expression pattern in different gap gene mutant backgrounds suggested that all the gap genes affect *ftz* expression (Carroll and Scott, 1986). The same kind of studies also showed that *ftz* gene expression is affected by some pair-rule genes (e.g. *hairy*, *runt*, and *even-skipped*) (Carroll and Scott, 1986; Ingham and Gergen, 1988; Lawrence and Johnston, 1989). In addition, the pair-rule genes were suggested to fall into two classes: the primary pair-rule genes, and the secondary pair-rule genes (Ingham and Martinez-Arias, 1986), because the expression pattern of the secondary pair-rule genes (e.g. *ftz*) is affected by mutations in the primary pair-rule genes (e.g. *hairy*, *runt*, and *even-skipped*) , but not vice versa.

2. The *fushi tarazu* gene

2-1. Expression and function

One of the best characterized homeobox-containing genes is the pair-rule gene, *fushi tarazu* (*ftz*). Homozygous *ftz* mutant embryos die before hatching, and lack alternate body segments (Nusslein-Volhard et al., 1984; Wakimoto and Kaufmann, 1981). The *ftz* gene is located 30 kb to the left of the *Antennapedia* gene, in the ANT-C (Kaufman et al., 1980; Wakimoto et al., 1984). The gene was isolated by its cross-homology with a fragment of *Antennapedia* (Kuroiwa et al., 1984). *ftz* is one of the genes (e.g. *Antp*, *Ubx*) that the homeobox was originally defined from. The *ftz* expression pattern has been studied by in situ hybridization to *ftz* mRNA

and by histochemical staining of FTZ protein using polyclonal and monoclonal antibodies (Carroll et al., 1986; Duncan, 1986; Hafen et al., 1984; Krause et al., 1988; Lawrence et al., 1987; Weir and Kornberg, 1985; Yu and Pick, 1995). *ftz* mRNA is first detected at low levels in the whole embryo, and then accumulates as seven zebra stripes in the primordia of the even numbered parasegments (Hafen et al., 1984). The seven *ftz* stripes arise individually along the anterior-posterior axis of the embryo, and also develop differentially along the dorsal-ventral axis (Fig. 1; Yu and Pick, 1995). The seven *ftz* stripes persist throughout the cellular blastoderm and during gastrulation and disappear before the end of germband extension. *ftz* mRNA is later expressed in specific neuron precursor cells. The cells where *ftz* is normally expressed are the precursor cells of the region that are missing in *ftz*$^-$ embryos. Embryos also die when *ftz* is ectopically expressed (Struhl, 1985). This suggests that the correct spatial expression of *ftz* is necessary for normal development of embryos.

In common with other pair-rule genes, *ftz* is necessary for the correct spatial expression of segment polarity genes, for example *engrailed* (*en*) and *wingless* (*wg*) (Howard and Ingham, 1986; Ingham et al., 1988). The normal pattern of *en* expression is dependent on activity of the *ftz* gene. In *ftz*$^-$ embryos at the germ band extended stage only seven rather than fourteen bands of *en* transcript are expressed (Howard and Ingham, 1986). In *ftz*$^-$ embryos, most of the *wg* stripes

are significantly wider than in wild type spanning five cells rather than one cell (Ingham et al., 1988). In addition, *ftz* is necessary for the correct activation of some homeotic genes at certain stages, for example *Antennapedia* (*Antp*), *Ultrabithorax* (*Ubx*), and *Sex Combs Reduced* (*Scr*) (Ingham and Martinez-Arias, 1986; Duncan, 1986). In the late blastoderm of wild type embryos, *Scr*, *Antp*, or *Ubx* is expressed at highest levels in PS2 (parasegment 2), PS4, or PS6, respectively. However, in the same regions of *ftz*$^-$ embryos, those peak levels of transcripts (*Scr* in PS2, *Antp* in PS4, and *Ubx* in PS6) are abolished (Ingham and Martinez-Arias, 1986). Thus, *ftz* is necessary for the correct expression of homeotic and other segmentation genes.

2-2. cis-regulatory elements

The expression of the *ftz* gene is regulated primarily at the transcriptional level. To understand the transcriptional regulation of the *ftz* gene, cis-regulatory elements were identified by examining expression of the reporter gene, *lac-Z*, in P-element mediated germ line transformed embryos (Hiromi et al., 1985; Hiromi and Gehring, 1987). A 5' regulatory region of ~ 6 kb was shown to contain three cis-regulatory elements: A "zebra element" directs *lac-Z* fusion gene expression in seven stripes localized in the mesodermal primordia; a "neurogenic element" directs gene expression in neural precursor cells; and a "upstream element" which acts as an enhancer (Hiromi et al., 1985; Hiromi and Gehring,

1987). These cis-regulatory elements are necessary to rescue *ftz* mutant embryos. The upstream element directs lacZ fusion gene expression in seven stripes via a heterologous promoter. The upstream element was further divided into a distal enhancer, containing a mesodermally active element; and a proximal enhancer, containing both mesodermally and ectodermally active elements (Pick et al., 1990).

Stripe-specific cis-regulatory elements were found to direct the expression of *hairy* stripes (Howard and Struhl, 1990; Pankratz et al., 1990). Similarly, stripe-specific cis-elements were identified for the *even-skipped* (*eve*) gene (Goto et al., 1989; Harding et al., 1989). In particular, an *eve* stripe 2 element was studied extensively. These studies showed that *eve* stripe 2 is generated by positive regulation by *bicoid* and *hunchback* and negative regulation by *giant* and *Krüppel* (Small et al., 1992; Stanojevic et al., 1989). The positive regulation initiates the stripe and the negative regulation forms anterior and posterior borders of the stripe. DNA-binding and co-transfection experiments using the *eve* stripe 2 element suggested that the binding of *giant* and *Krüppel* interferes with the binding or activity of *bicoid* and *hunchback* (Small et al., 1991). Studies of *eve* stripe 2 provide evidence that each pair-rule stripe is generated individually by responding differently to the combination of cues from maternal and gap gene products. This may also apply to the generation of *ftz* stripes. Although for a long time people have been convinced that *ftz* stripes are

generated in a coordinated fashion -en masse - (Carroll, 1990; Dearolf et al., 1990), using digoxygenin labeled probes on whole mount embryos, we revealed that the *ftz* stripes are generated differentially along anterior-posterior axis (Yu and Pick, 1995). The order of stripe appearance is 1, 5, 2, 3, 6+7, and 4. Furthermore, a *ftz* stripe 5 element was recently identified which responded to mutations in some gap genes (Alonso and Pick, submitted). Thus, our results suggest that seven *ftz* stripes are likely to be generated by non-periodic cues provided by gap and other pair-rule gene products. However, all the *ftz* cis-acting elements identified so far appear to affect equally all seven stripe pattern, which suggests that the ~ 6 kb of *ftz* 5'-regulatory sequence contains multiple elements each of which is sufficient to direct seven striped expression. Therefore, the mechanism by which region-specific establishment of *ftz* stripes remains a mystery and requires further study.

2-3. Autoregulation

FTZ protein has been shown to bind DNA specifically (Percival-Smith et al., 1990), and to function as a transcriptional activator. The expression of reporter genes that contain an oligomerized FTZ binding site (CAATTA) was activated by FTZ protein in transient transfection assays in yeast and *Drosophila* cultured cells (Fitzpatrick and Ingles, 1989; Han and Levine, 1989; Jaynes and O'Farrell, 1988).

ftz autoregulation was first suggested by genetic studies (Hiromi and Gehring, 1987; Pick et al., 1990). These studies showed that wild type FTZ protein was required for the striped expression of *ftz* upstream element-*lacZ* fusion genes (Hiromi and Gehring, 1987; Pick et al., 1990). This suggested that the *ftz* gene product might regulate its own expression through the upstream element. In vitro studies showed that the upstream element is one of the direct targets of the homeodomain of FTZ protein. DNase I protection assays were carried out to identify FTZ protein binding sites (Pick et al., 1990). Purified FTZ homeodomain, expressed in *E.coli*, was used for this analysis. As a result, three classes of protected regions within the upstream element were identified (high, medium, and low affinity). However, the in vitro data does not prove a direct interaction between FTZ homeodomain and the upstream element in vivo. A genetic approach was used to test whether or not *ftz* autoregulation was direct (Schier and Gehring, 1992). This was done by combining genetic second-site suppresser mutations with in vitro DNA-binding specificity mutants. A 430 bp fragment of the proximal enhancer (between positions 2145-2574) was used as a *ftz* autoregulatory element (AE) (Schier and Gehring, 1992). The in vivo activity of AE was reduced with changes of putative FTZ-binding site CCATTA to GGATTA, a binding site for another homeodomain containing protein, Bicoid protein. This down-regulatory effect was specifically suppressed in vivo by the DNA-binding specificity mutant FTZQ50K (Percival-

Smith et al., 1990). FTZQ50K protein has higher DNA-binding specificity for the GGATTA site. This experiment provides in vivo evidence that FTZ protein mediates autoregulation by interacting with it's own upstream element.

2-4. Transacting factors

A combination of molecular and biochemical approaches have led to the identification of other *ftz* regulatory activities. Analysis of *ftz* expression in embryos with various mutant genetic backgrounds has revealed three groups of *ftz* regulators. The first group includes five maternal genes, *vasa*, *valois*, *stauffen*, *tudor* and *oskar*. Each of the five mutants affected *ftz* expression with a common feature of abnormality in the region between stripes 2 and 7 (Carroll et al., 1986). Another maternal gene product *bicoid*, has been suggested as a repressor to suppress *ftz* gene expression in the anterior region of the embryos (Frohnhoffer and Nusslein-Volhard, 1987; Vavra and Carroll, 1989). The second group includes four gap genes, *Kruppel*, *knirps*, *hunchback*, and *giant*, since mutations in each of these genes affected the normal *ftz* expression pattern (Carroll and Scott, 1986). The third class includes three pair-rule genes, *hairy*, *runt* and *even-skipped*. They regulate *ftz* expression by refining and maintaining the striped pattern, rather than establishing them (Ingham and Gergen, 1988; Yu and Pick, 1995). *hairy* represses *ftz* expression in the odd-numbered parasegments; *runt* and *even-skipped* positively regulate some of the *ftz*

stripes. The other pair-rule genes (except *ftz* which was discussed above and *slp1* which will be discussed later) and segment-polarity genes showed no effects on *ftz* expression.

Other trans-regulators of the *ftz* gene have been identified biochemically. *tramtrack* cDNA was cloned by screening a *Drosophila* expression library with oligonucleotides from the *ftz* upstream element and zebra element (Brown et al., 1991; Harrison and Travers, 1990). Tramtrack protein contains a zinc-finger DNA binding motif. FTZ-F1α was purified by DNA affinity chromatography using a DNA binding site within the zebra element (Ueda et al., 1990). The deduced amino acid sequence revealed that it is a member of the hormone receptor superfamily (Lavorgna et al., 1991). A related gene, FTZ-F1β/DHR39, was also isolated and shown to bind the same DNA sequences as FTZ-F1α does (Ayer et al., 1993; Ohno and Petkovich, 1992). A more extensive biochemical study was carried out to reveal the molecular basis of *ftz* proximal enhancer (part of the *ftz* upstream element, see above) functions. Ten sequence-specific DNA-binding complexes were identified to interact with nine footprinting sites in the proximal enhancer (Han et al., 1993). At least three DNA-binding proteins were purified with affinity chromatography, and their functions in regulating *ftz* transcription were studied (Han et al., in preparation).

However, among many candidates of genes encoding *ftz* regulators, orly *tramtrack*, *FTZ-F1*, *Adf-1* (England et al.,

1992; Harrison and Travers, 1990; Ueda et al., 1990; Han et al., in preparation) and *fEBP-1* (Han et al., in preparation) were cloned so far. This is perhaps due to the limitations of biochemical approaches or in vitro expression cloning methods. The biochemical methods (e.g. protein purification), for instance, result only in the identification of the apparent molecular mass of the proteins and cloning genes for these proteins is often a difficult process. Therefore, an alternative method seemed necessary to identify and clone more (if not all) the DNA-binding factors involved in transcriptional control of the *ftz* gene. I developed a yeast genetic system to isolate genes encoding DNA-binding transcription factors (see below).

3. The homeobox genes

The *Drosophila* homeotic genes (HOM-C) are organized as two gene clusters on chromosome III, the Antennapedia (ANT-C) and Bithorax (BX-C) complexes (Lewis, 1978; Scott et al., 1983). The ANT-C contains five genes including *Labial, proboscipedia, Deformed, Sex combs reduced,* and *Antennapedia;* the BX-C contains three genes including *Ultrabithorax, abdominal A, and abdominal B* genes (McGinnis and Krumlaut, 1992; Regulski et al., 1985). *Drosophila* homeotic genes share a highly conserved 180 bp sequence named the homeobox (McGinnis et al., 1984; Scott and Weiner, 1984). The homeobox encodes a polypeptide segment of 60 amino acids termed the homeodomain. The homeodomain consists of a helix-

turn-helix DNA-binding motif (Laughon and Scott, 1984). *Drosophila* also has a group of non-homeotic, homeobox-containing genes (e.g., *fushi tarazu, bicoid, even-skipped*). These homeobox-containing genes do not cause homeotic transformation when the gene is either mutated or misexpressed. Homeodomain-containing proteins have been shown to bind DNA specifically (Desplan and Theis, 1985; Desplan et al., 1988; Hoey and Levine, 1988; Johnson and Herskowitz, 1985) and function as activators and/or repressors of transcription (Biggin and Tjian, 1989; Fitzpatrick and Ingles, 1989; Han and Levine, 1989; Jaynes and O'Farrell, 1988; Winslow et al., 1989).

Each homeotic gene in the complex is expressed in a restricted region of the embryo during early development and determines the identity of that region in the larvae and adults (Akam, 1987; Duncan, 1986; McGinnis and Krumlaut, 1992). Gain-of-function mutations in the homeotic genes cause transformation of one body part into another. For instance, misexpression of the *Antennapedia* gene in the primordium of the antenna results in its development into a leg rather than an antenna (Wakimoto and Kaufmann, 1981); whereas, loss-of-function mutations in the *Ultrabithorax* gene lead to a transformation of the halteres into a second pair of wings (Lewis, 1978). Thus, genetic evidence indicates that each HOM-C gene is needed to specify the developmental fate of cells in a certain position.

Different HOM-C proteins have varied and specific functions despite their structural similarity. To study this functional specificity, chimeric HOM-C proteins were constructed and their functions were tested (Gibson et al., 1990; Kuziora and McGinnis, 1989; Mann and Hogness, 1990). The results of such studies provide evidence that some of the functional specificity of HOM-C proteins is determined by the small differences within or immediately adjacent to the homeodomain region. A tetrapeptide YPWM motif located just N-terminal to the homeodomain is conserved among most HOM-C proteins and their Hox counterparts (Burglin, 1994). The YPWM motif, however, does not appear to influence DNA binding specificity in vitro (Ekker et al., 1992). The YPWM motif has been suggested to play a role in interactions with other proteins (Vershon and Johnson, 1993), and is necessary for the biological activities of Hox proteins (Zhao et al., 1996). Besides the domains within the homeotic gene products, other factors must be taken account for the specificity of HOM proteins (see below).

Homeobox genes have been found in a variety of organisms, including yeast, frog, mouse and human where they are called *Hox* genes (Levine et al., 1984; McGinnis et al., 1984; McGinnis and Krumlaut, 1992; Scott et al., 1989). In both mice and humans, *Hox* genes are clustered into four complexes on different chromosomes (the Hox-A, Hox-B, Hox-C, and Hox-D clusters) (Breier et al., 1988; Duboule et al., 1986; Duboule and Dolle, 1989; Graham et al., 1988). The

equivalent *Hox* and *HOM-C* genes are arranged in the same linear order within their respective complexes. Individual genes of the *Hox* complexes are also expressed in the same anterior-posterior order as their structural homologs in the HOM-C (McGinnis and Krumlauf, 1992). The expression of *Hox* genes is most concentrated in the nervous system and somatic mesoderm (Duboule and Dolle, 1989; Graham et al., 1989). Like their *Drosophila* counterparts, *Hox* genes are expressed in a restricted region during early embryogenesis and specify the identity of that region (Holland and Hogan, 1988). Despite the fact that the structural similarity between HOM-C and Hox proteins is mainly limited to the homeodomain regions, some HOM-C and Hox proteins are functionally conserved. Human *HoxD4*, when ectopically expressed in developing fly cells, induced homeotic transformations similar to that of its *Drosophila* paralog *Deformed* (McGinnis et al., 1990). Mouse *HoxA5* when over-expressed in *Drosophila* embryos displayed *Sex combs reduced*-like transformations in larvae and adults (Zhao et al., 1993).

Gene-targeting techniques have offered a powerful tool to study the functions of *Hox* genes. In the mouse, null alleles of *HoxC8*, *HoxB4*, *HoxA2*, *HoxD3*, and *HoxD13* display some form of anterior transformation (Condie and Capecchi, 1993; Gendron-Maguire et al., 1993; Le Mouellic et al., 1992; Ramirez-Solis et al., 1993; Rijli et al., 1993), while null alleles of *HoxA5* and *HoxA11* cause posterior transformations (Jeannottee et al., 1993; Small and Potter, 1993). Loss-of-

function mutations of *HoxA1* and *HoxA3* have no detectable transformations, but display apparent phenotypes in the development of cranial and mesenchymal neural crest (Chisaka and Capecchi, 1991; Lufkin et al., 1991). These studies have led the determination of specific functions for *Hox* genes in specifying anterior-posterior identities of structures in early mouse development (McGinnis and Kuziora, 1994).

4. Functional specificity

The homeodomain containing proteins, including FTZ, achieve their effects by specific activation or repression of downstream genes. However, the mechanism through which they achieve specificity in this regulation is a mystery (Gehring et al., 1994; Hoey and Levine, 1988; Krumlauf, 1994). From in vitro studies, two interesting phenomena have been found. First, all the homeodomain containing proteins within the Antp-class are capable of binding to similar DNA sequences containing a core of ATTA (Desplan et al., 1988; Hoey and Levine, 1988; Laughon and Scott, 1984). Second, each of these proteins has multiple binding sites (Scott et al., 1989). The question that arises is how these proteins achieve diverse and specific regulatory effects?

In yeast, the different cell types are determined by the products of the MAT (mating-type) locus (Nasmyth and Shore, 1987). Two MAT locus products, $\alpha 2$ and a1 contain homeodomains (Laughon and Scott, 1984; Shepherd et al., 1984). These proteins do not act on their own to produce

cell-type-specific patterns of gene expression. Rather, they interact with a common protein, MCM1, that is present in all cell types (Keleher et al., 1988). MCM1 protein has binding sites in the regulatory regions of both the a-specific and the α-specific genes. In the a cell, MCM1 binds cooperatively with a1 to the target sequences and activates transcription of a-specific genes. In the α cell, MCM1 binds cooperatively with α2 in the upstream of a-specific genes and represses their transcription (Herskowitz, 1989; Philips et al., 1994; Vershon and Johnson, 1993). Thus, the specificity of these yeast homeodomain containing proteins is acheived by interacting with another protein.

In *Caenorhabditis elegans*, two genetically identified transcriptional regulators, UNC-86 and MEC-3, are required for proper expression of target genes in a subset of sensory neurons that respond to a touch stimulus (Way and Chalfie, 1988). UNC-86 is a POU (nameed for the family, Pit-1, Oct 1 and 2, and Unc 86) homeodomain protein (Finney et al., 1988) that is thought to regulate the transcription of many target genes including *mec-3* (Xue et al., 1992). MEC-3 is a LIM (Lin-11, Isl-1, and mec-3) homeodomain protein (Freyd et al., 1990; Karlsson et al., 1990; Way and Chalfie, 1988) that directs cellular differentiation and appears to specify neuron formation. UNC-86 alone is able to bind DNA and activate transcription, while MEC-3 fails to bind DNA or activate transcription on its own. Both UNC-86 and MEC-3 cooperatively bind to DNA and synergistically activate

transcription (Lichtsteiner and Tjian, 1995). Thus, the MEC-3 protein requires a partner, UNC-86, to form an active DNA binding complex capable of transcriptional activation. This situation is very similar to the FTZ/FTZ-F1α interaction which will be discussed later.

The POU domain has been subdivided into two regions termed the POU-specific box and the POU homeobox. The function of the POU-specific box is still unclear. Two *Drosophila* POU-domain proteins, I-POU and Cf1-a, are coexpressed in subsets of neurons during development. Because I-POU lacks two basic residues in the N-terminus of its homeodomain, it cannot bind DNA. However, it does form a stable heterodimeric complex with Cf1-a. This heterodimerization prevents Cf1-a from binding to DNA and, therefore, from transactivating the dopa-decarboxylase gene (Maurice et al., 1991). The inhibition by I-POU provides a potential mechanism by which gene activation is modulated by a homeodomain protein that does not bind DNA.

The first characterized cofactor of *Drosophila* homeotic gene product is Extradenticle (Exd). Exd was suggested by genetic studies to function in concert with certain HOM-C proteins to regulate target gene expression (Peifer and Wieschaus, 1990; Rauskolb et al., 1993). Mutations in *exd* caused homeotic transformations in thoracic and abdominal segments, but the expression of HOM-C genes was not altered. Exd has recently been shown to cooperatively interact with the Ultrabithorax and Abdominal A homeotic proteins, but not

with Antennapedia or Abdominal B. These interactions selectively increase the binding affinity of Ubx for a particular DNA target (Chan et al., 1994; Van Dijk and Murre, 1994). In vivo, Exd is required for Ubx regulated *decapentaplegic* gene (*dpp*) expression in the visceral mesoderm (Capovilla et al., 1994). This was shown by analyzing a *lacZ* reporter gene under the control of a 303 bp *dpp* enhancer element (*dpp303*) in *Drosophila* embryos (Capovilla et al., 1994). In *exd* mutants, the expression of *dpp* and the *dpp303-lacZ* reporter gene was greatly reduced, while Ubx expression was normal in *exd* mutant embryos (Chan et al., 1994). In addition, strong interaction between Ubx and Exd requires only the Ubx homeodomain and the YPWM motif (Johnson et al., 1995). There is also evidence that PBX1, a human homolog of *Drosophila* Exd, binds DNA cooperatively with certain murine Hox homeodomain proteins (Lu et al., 1995). The *PBX1* homeobox-containing gene was first identified as the t(1;19) chromosomal translocation in pre-B-cell acute lymphoblastic leukemia (Kamps et al., 1990; Nourse et al., 1990). The normal PBX1 protein, as well as its oncogenic derivative, E2A-PBX1, bind DNA cooperatively with Hox-B7, Hox-B8, Hox-C8, Hox-A5, and Hox-D4. In addition, in cotransfection assays, transactivation by E2A-PBX1 is repressed by Hox-B8, suggesting that Pbx1 may cooperate with Hox proteins to regulate target gene expression and contribute to normal differentiation in vertebrates.

5. The significance of finding FTZ-cofactors

Based on studies presented above, we suggest that the ability of the *Drosophila* homeodomain containing protein, FTZ, to regulate transcription is modified by other cofactors as well. *ftz* autoregulation is being used as a model system to identify *Drosophila* proteins capable of associating with FTZ protein and activating transcription.

As a developmentally important modulator, FTZ protein ought to control the expression of other genes, besides itself, for instance, other pair-rule genes, segment polarity genes, homeotic selector genes, and other target genes necessary for the differentiation of alternate body segments. Unfortunately, these genes have not yet been identified. One possible reason for that is that *ftz* protein is essential but not sufficient for regulating downstream genes. Perhaps a complex of FTZ with other proteins is necessary for either functional DNA-binding or transactivation or both. The identification of other components within the FTZ complex will be crucial to reconstitute the complex and to look for the real downstream target genes of FTZ. Therefore, finding FTZ accessory protein seems necessary to understand the transcriptional regulation of *ftz* gene expression and the role of FTZ function in normal development of *Drosophila* embryos. The elucidation of the action of *Drosophila* homeodomain-containing proteins will also help us to understand the mechanisms underlying the function of

homeodomain containing proteins involved in the development of higher organisms.

CHAPTER 2. MATERIALS AND METHODS

For Part I of Results:

1. In situ hybridization of ftz mRNA to whole mount embryos

The hybridization was carried out essentially according to the protocol of Tautz and Pfeifle (1989). Digoxygenin labeled probes were prepared using a Genius nonradioactive DNA labeling kit as described by the manufacturer (Boehringer Mannheim). The *ftz* probe was an ~0.5 kb BalI/SalI fragment of *ftz* cDNA generated from plasmid pFKΔX-1. Reactions containing 100 ng DNA, 2 µl hexanucleotide mixture, 2 µl dNTP labeling mixture, and 1 µl Klenow enzyme were incubated overnight at room temperature. Labeled DNA was precipitated and resuspended into 50 µl PBT (PBS plus 0.1% Tween 20). Probes were either stored at -20°C or used directly.

0-6 hour embryos were collected, dechorionated with 3% sodium hypochlorite for 1.5 minutes, rinsed with embryo wash solution (0.7% NaCl, 0.03% Triton X-100), followed by fixation in equal volume of 1X PBS/4% formaldehyde/50 mM EGTA and heptane for 20 minutes with rocking. Devitellinized embryos were rinsed with methanol and then ethanol. Embryos may be stored in ethanol at -20°C for few years. To prepare for hybridization, embryos (~20 µl volume) were rinsed with methanol and 50% methanol/50% PBS containing 4% formaldehyde, fixed in PBT/4% formaldehyde for 20 minutes, followed by

rinsing with PBT three times for 5 minutes with rocking. Embryos were then treated with 400 µl of 50 µg/ml proteinase K in PBT (diluted from frozen 100 mg/ml stock solution) for 4 minutes at room temperature. Prehybridization was done in Hybridization mix (50% formamide, 5X SSC, 100 µg/ml sonicated herring sperm DNA, 50 µg/ml heparin, and 0.1% Tween 20) for more than three hours at 45°C water bath without rocking. Hybridization was carried out with high stringency in 2X SSC at 52°C overnight without rocking. Digoxygenin labeled *ftz* probe was diluted at a 1:10 in Hybridization mix immediately before use and denatured at 95°C for 10 minutes. 10 µl of diluted probe was used to hybridize ~10 µl of embryos. After hybridization, embryos were washed extensively with Hyb. mix, 50% Hyb. mix/50% PBT, and five times PBT. Each wash was carried out for 20 minutes at 50°C. Embryos were then incubated with 400 µl of alkaline phosphatase coupled anti-digoxygenin antibody (supplied by the Genius nonradioactive DNA detection kit) at a 1:1000 dilution in PBT at room temperature for 1.5 hours. After several washes in PBT and levamisole solution (100 mM NaCl, 50 mM $mgCl_2$, 100 mM Tris-HCl, pH 9.5, and 0.1% tween 20), purple bluish color was developed in 500 µl levamisole solution containing 3.5 µl X-phosphate and 4.5 µl NBT (supplied by the Genius nonradioactive DNA detection kit). Embryos were normally stained for about 30 minutes and rinsed with several changes of PBS, and then mounted in 90% glycerol/PBS for photographing.

2. Plasmid preparation

2-1. The 323 bp fPE (*ftz* proximal enhancer) construct

The proximal enhancer of the *ftz* upstream element was subdivided into prox A and prox B, and the prox A was previously cloned into pHZ50PL (Pick et al., 1990). A DraI construct was derived from the prox A construct, which deleted a portion of prox A from the 5' end (Han et al., 1993). The DraI fragment was then cloned into the XbaI site of a Bluescript vector KS (KS-DraI). The 323 bp fPE DNA fragment was generated by polymerase chain reaction (PCR) using KS-DraI as template. The two PCR primers were, 5'-GATCTCTAGAGGTGACCTTCGATCGG-3' (located around the BstEII site of the *ftz* proximal enhancer), and 5'-AATACGACTCACTATAG-3' (annealed to KS polylinder region). The amplified 323 bp fPE was released as an XbaI fragment (one XbaI site was generated from the primer, the other was from KS the vector), and subcloned into the XbaI site of pHZ50PL (Hiromi and Gehring, 1987). This 323 bp fPE contains part of Prox A and part of Prox B (positions 2167-2490).

2-2. The 323-HIS3 reporter construct

The *HIS3* reporter gene contains the region from position -83 to the 3' UTR of the *HIS3* gene which includes a minimal promoter and the whole coding sequence (Struhl, 1985). To make the 323-HIS3 construct, three DNA pieces, a DdeI/XhoI

DNA fragment of the *HIS3* gene including its promoter and coding sequences, an XbaI DNA fragment of the 323 bp fPE, and a SacI/EcoRI DNA fragment of the TRP1 gene from plasmid D759 (Lawrence Marsh et al., 1984), were inserted into a PUC19 vector sequentially. The *HIS3* DNA was cloned into the SalI site by ligating one end, filling-in the other end with klenow, and re-ligating. The 323 bp fPE DNA fragment was inserted into the XbaI site. The TRP1 DNA was cloned into EcoRI and BamHI site by cohesive end ligation.

2-3. The 323-LacZ reporter construct

The 323 bp fPE was subcloned into a KS^+ vector as an XbaI fragment to generate the KS-323 construct. A BamHI-SalI fragment of the 323 bp fPE was released from KS-323, and inserted into a derivative of the *lacZ* expression vector, pLG669ZΔXho, which contains a basal promoter (*CYC1*) and lacks other regulatory sequences (Guarente and Ptashne, 1981). The cloning was done in three steps: (i) ligating the SalI site of the fragment to XhoI site of the vector, (ii) filling-in the other end with the large fragment of DNA polymerase I, Klenow enzyme, (iii) re-ligating. In order to create an integration vector, the 2μ region was deleted by cutting the plasmid with EcoRI, religating, and picking blue colonies in HB101 on LB plates (100 mm x 15 mm) containing 800 μg of X-gal in dimethylformamide. The URA3 marker in pLG669ZΔXho was replaced with a TRP1 marker: a HindIII fragment of the TRP1 gene was generated from partial digestion of plasmid, D759.

This TRP1-HindIII fragment replaced a URA3-HindIII fragment in the pLG669ΔXho construct.

2-4. The NP6-HIS3 reporter construct

Six copies of an engrailed binding site (NP6) was used as a FTZ binding site, since FTZ was able to interact with this site (Desplan and Theis, 1985). NP6 DNA was generated by annealing and ligating two synthetic oligonucleotides, each containing approximately three copies of the binding site. The sequence of one set of oligonucleotides was: 5'-CTAGATCAATTAAATGATCAATTAAATGATCAATTAAATGA and 5'-TCATTTAATTGATCATTTAATTGATCATTTAATTGATCATTTAATTGAT. The sequence of the other set of oligonucleotides was: 5'-TCAATTAAATGATCAATTAAATGATCAATTAAATGAT and 5'-CTAGATCATTTAATTGATCATTTAATTGA. After annealing, the two double stranded oligonucleotides were phosphorylated with T4 polynucleotide kinase and ligated with T4 DNA ligase. The final full length oligonecleotide containing six binding sites was then ligated into an XbaI site of pUCHIS3 (see above). The correct sequence and orientation of binding sites were confirmed by DNA sequencing.

2-5. The *ftz* expression construct

The yeast expression vector, pADANS (Colicelli et al., 1989), was used to provide an active ADH promoter for *ftz* gene expression, and a unique NotI site for cloning. The *ftz* cDNA was derived from pNHfI (a gift of Dr. M. Miller) as a

NotI fragment. In order to make the *ftz* expression construct, the BamHI fragment containing the ADH promoter and terminator was first cloned into a pYCP50 (Rose et al., 1987) vector to create a Ycp50/ADH plasmid. A NotI fragment containing the *ftz* cDNA was subsequently cloned into the Ycp50/ADH plasmid. The resulting construct contains an ADH promoter including an ATG codon, *ftz* cDNA, and a URA3 marker. This plasmid expresses FTZ protein missing the first two amino acids and having twenty non-FTZ amino acids derived from the vector at the N-terminus (they are, MSIPETQKGVIFYEACGRDP).

3. Germ-line transformation and immunohistochemical staining of *Drosophila* embryos

The P-element transformation vector pHZ50PL (Hiromi and Gehring, 1987), which contains regions of the upstream element fused to a basal promoter and the *E.coli lacZ* gene, was injected along with a helper plasmid p25.1WC into ry^{506} embryos according to Rubin and Sprading (1982). Following transformation into the germ line of *Drosophila*, multiple independent lines were established. This part of experiment was done by Dr. L. Pick. Fusion gene expression in embryos was monitored by immunohistochemical staining of the embryos (Gutjahr et al., 1993). 0-10 hour old embryos were collected, dechorionated using clorox, and fixed with 4% formaldehyde. The fixed embryos were incubated with anti β-galactosidase antibodies. After several washes, the embryos

were incubated with anti-rabbit IgG biotinylated antibodies, provided in the ABC kit (Vector Lab), ABC reagents were used to amplify the signal. A brownish color from immunoperoxidase reaction was developed in the presence of diaminobenzidine, $NiCl_2$ and H_2O_2. Stained embryos were photographed with a Ziess Axiophot using Kodak TMAX-100 film.

4. Yeast strain preparation

The original yeast strain, w3031A, was kindly provided by Dr. J. Hirsch. w3031A contains five auxotrophic markers. They are, leu2, ura3, ada1, trp1, and his3. Three reporter strains, w/323His3, w/323lacZ, and w/NP6His3, were made by integrating three reporter constructs individually into the TRP1 locus of the w3031A cell genome. 20 µg of plasmid DNAs were digested with StuI, a site located within the coding region of the TRP1 gene. The linearized DNA was then transformed into w3031A cells (see below). Transformants were selected on synthetic dropout (SD)-plates without tryptophan. The proper chromosomal integrations were confirmed with southern blotting (see below).

To make reporter strains expressing FTZ protein, 1 µg of *ftz* expression plasmid DNA was transformed into w/323His3, w/323LacZ, or w/NP6His3. Transformants were selected on SD-plates without uracil as the *ftz* expression plasmid contains a URA3 marker. The resulting strains were named w/323His3FTZ, w/323LacZFTZ, and w/NP6His3FTZ,

5. Southern blot using the Genius system

To confirm the proper integration of each reporter gene, Southern blotting was carried out using total yeast DNA. The procedure for the Southern blot followed the Genius system user's guide (Boehringer Mannheim).

Total yeast DNA was prepared (see methods below). 5 µl (~1 µg) was digested with restriction enzymes StuI. The digestion products were separated on 1 % agarose gel using 1X TAE buffer. Following denaturation and neutralization, the DNA was transferred from the gel to a nylon membrane (Boehringer Mannheim) overnight, using 20X SSC. DNA was fixed on the membrane by baking at +80°C for 1 hour. The membrane was prehybridized in 10 ml standard prehybridization solution (5X SSC, 1% blocking reagent, 0.1% N-lauroylsarcosine, and 0.02% SDS) at +65°C for 2 hours, then hybridized at +65°C overnight in standard prehybridization solution containing the labeled probe (100 ng *lacZ* probe, denatured at 95°C for 10 minutes before use). Probe was labeled with DIG-dUTP using random primers and Klenow enzyme according to the user's guide. At the end of hybridization, the membrane was washed twice, 5 minutes per wash, in 2X wash solution (2X SSC, 0.1% SDS) at room temperature, and washed twice, 15 minutes per wash, in 0.5X wash solution (0.5X SSC, 0.1% SDS) at room temperature. The membrane was then incubated with anti-DIG-alkaline phosphatase antibodies (Boehringer Mannheim) at a 1:5000 dilution in buffer (2% blocking reagent, 100 mM Tris-HCl, pH 7.5, and 150 mM NaCl) for 30

minutes. The final color was developed in 10 ml buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$) containing 45 µl NBT and 35 µl X-phosphate (Boehringer Mannheim).

6. Yeast transformation with plasmid DNA

Recipient cells, 30 ml of overnight liquid cultures, were pelleted, washed with 20 ml H_2O, and resuspended in 20 ml 0.1 M LiAc in TE. After shaking for 30 minutes at 30°C in a 50 ml Falcon tube, cells were re-pelleted and suspended in 2 ml 0.1 M LiAc in TE. 0.2 ml of cells were distributed to microfuge tube containing plasmid DNA (~1 µg) plus 5 µl carrier DNA (salmon sperm DNA, 5 mg/ml). The cells were incubated with DNA at 30°C for 30 minutes without shaking. 0.22 ml 60% PEG 3350 was then added and the cells were continuously incubated for 1 hour at 30°C without shaking. After heat shock at 42°C for 5 minutes, cells were pelleted, washed with H_2O, resuspended in 0.3 ml YPD media (containing Yeast extract, Peptone, and Dextrose), and plated onto selective SD-plates. Transformants grew after 2-3 days of incubation at 30°C.

7. β-galactosidase enzyme activity assay

The β-galactosidase enzyme activity assay in liquid culture was performed essentially as described in Current Protocols in Molecular Biology (Ausubel et al., 1992). 1.5 ml overnight cultured cells were centrifuged and resuspended in 200 µl of Z-buffer (60 mM $Na_2HPO_4.7H2O$, 40 mM $NaH_2PO_4 \cdot H2O$,

10 mM KCl, 1 mM $MgSO_4 \cdot 7H20$, 50 mM β-mercaptoethanol). Cells were broken using ~100 µl of acid-washed glass beads (Sigma) for 3 minutes by vortexing. After centrifuging briefly, 50 µl of suspension was transferred to another microcentrifugation tube containing 750 µl Z-buffer and 200 µl of 4 mg/ml o-Nitrophenyl β-D-Galactopyranoside (ONPG). The reaction was placed in a 30°C water bath overnight. 300 µl of 1 M Na_2CO_3 was added to stop the reaction. OD_{420} of the reaction solution was determined. The β-galactosidase activity was calculated with equation, $U=OD_{420}/OD_{595}$. OD_{595} is determined using Bio-red protein assay reagent. Values for β-galactosidase activity were assayed on at least two transformants, each assayed at least twice.

8. Construction of λACT cDNA library

λACT phage was supplied as an extrachromosomal 42 Kb plasmid lysogen by Dr. Stephen Elledge (Baylor College of Medicine). Phage DNA was purified by double banding on Cesium Chloride (CsCl) gradient (Maniatis et al., 1989) from 2 liters of culture. 50 µg of λACT DNA was digested with XhoI and treated with alkaline phosphatase (40 units) before ligation to cDNA.

0-6 hours embryos were collected from a population cage of *Drosophila melanogaster* (Oregon R). The embryos were dechorionated in 3% Clorox for 1.5 minutes and rinsed with water thoroughly. The embryos were then frozen in liquid nitrogen, and stored at -80°C. The RNA purification

procedure was described in Current Prototols in Molecular Biology (Ausubel et al., 1992). The whole experiment was done under RNase-free conditions (solutions were treated with Diethyl Pyrocarbonate (DEPC) and glassware was baked at 180°C overnight). About 5 g of embryos were dissolved in 30 ml of lysis buffer (4 M Guanidinium Thiocyanate (GTC), 25 mM Sodium Citrate, 0.5% N-Lauroyl Sarcosyl), plus 300 µl of β-mercaptoethanol. The embryos were then homogenized in a Yamata LH-21 homogenizer at the speed of 1500 rpm for approximately 2 minutes at 4°C, and centrifuged at 10,000 rpm for 5 minutes at 4°C in a corex glass tube. The supernatant was sheered several times by passage through a 18G1/2 needle, and dispensed into 10 ml quick seal ultramicrocentrifuge (5 ml) tubes that already contained 4.5 ml of solution (5.7 M CsCl, 0.1 M EDTA). Samples were spun at 40 K rpm at 18°C overnight in a VTI65 rotor. At the end of the centrifugation, the liquid was removed and the RNA pellet (at the bottom) was rinsed with 70% ethanol, resuspended in DEPC treated water, and precipitated with 3 M NaAc, pH 5.2/100% ethanol in a 1.5 ml eppendorf tube. The mRNA purification steps followed the instructions of an mRNA purification kit from Boehringer Mannheim. ~2 mg of total RNA was used to run through an oligo-(dT) column. Binding of poly(A)$^+$ RNA to oligo-(dT) cellulose was done in buffer containing 1 M NaCl, 20 mM Tris-HCl, pH 8, 1 mM EDTA, and 0.1 % N-lauroylsarcosine. Poly(A)$^+$ RNA was eluted with preheated (65°C) TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). RNA

concentration was measured at OD_{260}. ~34 µg of poly(A)$^+$ RNA was obtained.

The synthesis of cDNA was done according to the instructions of the ZAP-cDNA Synthesis Kit (Stratagene). First strand cDNA was synthesized in a reaction (50 µl) containing 10 µg of poly(A)$^+$ mRNA, 50 bp linker-primer which has an XhoI site in the middle and poly T sequences at the 3' end, and Moloney-Murine leukemia virus reverse transcriptase (50 units). Second strand synthesis was carried out in a reaction (400 µl) containing 5 units of RNase H and 100 units of DNA polymerase I. The synthesis of the first strand cDNA was monitored by doing a 5 µl reaction including 5 µCi α-^{32}P(dATP) in parallel. The synthesis of the second strand cDNA was monitored by including 10 µCi α-^{32}P(dATP) in the reaction. The double stranded cDNA was then phenol extracted and ethanol precipitated. cDNA ends were made blunt by treating with 10 units of Klenow enzyme in a 50 µl reaction volume at 37°C for 30 minutes. Approximately 4 µg (determined by checking cDNA on a 1 % agarose gel) of cDNA was ligated to 2 µg of XhoI adapters at 8°C for 14 hours. The sequence of the phosphorylated adapters was 5'-GGCCTTCGTGGCC-3' (top strand), and 5'-CGAGGCCACGAAGGCC-3' (bottom strand). Adapted cDNA was digested with XhoI to release the cloning sites. cDNA of 400 bp and longer was then selected using one Sephacryl S-400 spun column (Pharmacia). cDNA (100 ng) was ligated to 1 µg of CIAP treated λACT DNA in a volume of 4 µl at 12°C for 18 hours

with 4 units of T4 ligase. The ligation was packaged using Gigapack Gold packaging extract (Stratagene), according to the manufactures instructions. At the end of packaging reaction, 200 µl of SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$, and 0.01% gelatin) was added. 4 µl of 1:10 diluted extract was used to plate out and the number of plaques was counted. Total recombinants ($1X10^7$) were obtained. The phage library was amplified in XL1-Blue MRA cells with a method described in Molecular Cloning, A Laboratory Manual (Maniatis et al, 1989). Briefly, the phage library was plated at a density of about 10^5 plaques per 150mm plate for two plates, and the phage were grown for 7 hours at 37°C. The plate was overlaid with 10 ml SM buffer for 2 hours at room temperature with shaking. The supernatant (~ 8 ml) containing amplified phage library was collected and stored either in 7% Dimethyl Sulfoxide (DMSO) at -70°C for years or at 4°C for months. The amplified library has a titer of $8X10^9$/ml. A methylation restriction minus, $mcrA^-$ and $mcrB^-$ strain, XL1-Blue MRA, was used, because of the incorporation of 5' methyl C in the first strand cDNA synthesis.

9. Excision of pACT library from λACT

The phage library was converted to a plasmid library by plasmid excision. The excision was carried out in bacterial strain, BNN132, a kan^r lambda lysogen containing the *cre* gene. Infection of this strain with the library produces

Amp^r colonies that have quantitatively excised the plasmid via cre-mediated lox recombination (Elledge et al., 1991). About 10^8 phage were used to infect 2 ml of log phase BNN132 cells resuspended in 10 mM $MgCl_2$ for 30 minutes at 30°C. 2 ml LB was then added, cells were incubated with shaking for 1 hour at 30°C. The cells were plated onto five 150 x 15 mm LB/Amp plates. The lysogens were scraped directly from these plates after overnight incubation. These cells were resuspended in 3 liters of terrific broth (Maniatis et al., 1989), and grown to stationary phase. The plasmid library DNA was prepared and purified using CsCl density gradients by standard methods (Maniatis et al., 1989). It was now ready to transform into yeast cells.

10. Yeast transformation with library DNA

This protocol was provided by Dr. S. Elledge.

The reporter strain yeast cells were grown overnight in 200 ml liquid media. The overnight cultures were inoculated into 500 ml YEPD and continuously grown for another 3-4 hours to OD_{600}=0.5~0.8. Cells were then pelleted, washed with H_2O, and incubated for 30 minutes at 30°C in 50 ml LiSORB solution (1 M sorbital, 0.1 M LiAc in TE). Cells were centrifuged and resuspended in 1 ml LiSORB. Carrier DNA (4 µg sheared salmon sperm DNA) was then mixed with 40 µg of pACT library DNA, and incubated with 40 ml of yeast cells in LiSORB (prepared before) for 10 minutes at 30°C. After incubation, 1.8 ml of 40% PEG 3350 in 100 mM LiAc/TE was added, cells were further

incubated for 30 minutes at 30°C without shaking. After heat shocking at 42°C for 12 minutes, cells were allowed to recover for 3 hours in SD-his, leu liquid media by shaking at 30°C. After recovery, cells were pelleted and resuspended in 4 ml of SD-his, leu media. 5 µl was used to plate on SD-leu plates to determine the transformation efficiency. The rest of the cells were plated onto 10 plates (150 X 15 mm) containing 25 mM 3-AT (Sigma). Transformants grew after 5-7 days of incubation at 30°C. To make 3-AT plates, all the ingredients were mixed and autoclaved as usual, 3-AT powder was added when the media cooled down.

11. Isolation of total yeast DNA

1 ml of the saturated cell cultures were resuspended in 250 µl of 0.1 M EDTA and 14 mM β-mercaptoethanol. 12.5 µl of zymolyase (15 mg/ml) was added and cells were incubated at 37°C for 30 minutes. 50 µl of solution (0.25 M EDTA, 0.5 M Tris-HCl, pH 8.0, 2.5% SDS) was then added and incubated for another 30 minutes at 65°C. After adding 63 µl of 5 M potassium acetate and incubating on ice for 30 minutes, the suspensions were centrifuged. The DNA was precipitated by adding 720 µl ethanol into the supernatant. Subsequently, DNA resuspended in 120 µl TE buffer was treated with RNase A (12 µg) for 30 minutes at 37°C, and reprecipitated with 130 µl isopropanol. The final DNA pellet was resuspended in 20 µl TE. 5 µl of this DNA solution was used for southern

blotting, and 1 µl was used to transform *E.coli* for plasmid isolation.

12. Isolating plasmids of interest from yeast cells

1 µl of total yeast DNA (see methods above) was used to transform competent cell strain, XL1-blue, with a standard bacterial transformation procedure (Maniatis et al., 1989). Colonies that grew on plates containing 50 µg/ml ampicillin were picked. Plasmid DNA was prepared using the lysozyme/boiling method (Maniatis et al., 1989). Plasmids were analyzed by restriction enzyme digestion of mini-prep DNA extracted from *E.coli* cells.

For Part II of Results:

1. Sequence analysis

DNA was sequenced using dideoxy dNTPs (Sanger et al., 1977) and Sequenase 2.0 according to the manufacturer's specifications (USB). Two primers were used to initially sequence the insert of isolates. They were λACTF (5'-GATGATGAAGATACCCCACC) located 5' to the XhoI cloning site, and λACTR (5'-GGGGTTTTTCAGTATCTACG) located 3' to the XhoI cloning site. Sequence comparisons were searched with the GenBank/Non-redundant database.

2. Plasmid preparation

To express *slp1* protein without the Gal4 activation domain in w/323lacZ cells, a BglII fragment containing a full length *slp1* cDNA including its own ATG and 43 bp of 5' untranslated region generated from clone F55, was inserted into pHSS7 vector digested with BglII. A NotI fragment was then released and cloned into pADNS to express Slp1 under its own ATG.

A yeast *lacZ* expression vector containing a *CYC1* promoter and its upstream activation sequence (UAS), pNG22 (Wang et al., 1994), was used to make pNG224X. pNG224X contains four copies of the Slp1-site between the promoter and UAS. Four copies of the Slp1-site (19 bp for each site) was synthesized as a single oligonucleotide (Genset Corp) containing SalI sites at the ends for subcloning.

To express *ttk* protein without the Gal4 activation domain, a PvuI fragment containing full length *ttk* cDNA including its own ATG was generated from plasmid, NB408 (a gift of D. Read). This fragment was then filled-in with Klenow enzyme at both ends to create a blunt ended fragment. The blunt ended fragment containing *ttk* cDNA was subsequently ligated into pADNS vector digested with NotI and then blunt ended by filling-in with Klenow enzyme.

3. In vitro DNA binding assays

Bacterially expressed Slp1 protein prepared as described by Hoey and Levine (1988) was used in the gel-retardation and footprinting assays. To overexpress Slp1 protein in

bacteria, a plasmid, pET3d/slp1C (a gift of K. Cadigan), was transformed into BL21(DE3) cells. 5 ml of overnight culture from the transformed cells was added to 500 ml LB and grown for 2 hours. The expression of Slp1 was induced with 0.2 mM isopropyl thiogalactopyranoside (IPTG) at 37°C for 3 hours. The cells were collected by centrifugation, and resuspended in 20 ml Z-buffer (Treisman et al., 1989) (100 mM KCl, 25 mM HEPES, pH 7.8, 1 mM DTT, 0.1% NP-40, 20% glycerol, 1 mM PMSF, 2 mM benzamidine, 5 µg/ml leupeptin), containing 0.5 mg/ml lysozyme. They were disrupted by sonicating 6 times for 30 seconds repeatedly with a 5 mm probe at 3.5 output on ice. Cell lysates were centrifuged at 16K rpm for 15 minutes, and the pellet was resuspended in 2 ml Z-buffer. Protein extracts were denatured by incubating with 4 M Guanidine-HCl on ice for 30 minutes. The proteins were then renatured by dialyzing against Z-buffer without Guanidine-HCl overnight at 4°C. The dialyzed solution was spun briefly and supernatant was either stored at -80°C or used directly.

Gel retardation assays were carried out essentially as described by Han et al. (1993). Oligonucleotides with 5'-protruding ends were labeled with α-^{32}P-dCTP and Klenow enzyme. Binding reactions (25 µl) contained 25 mM Hepes, pH 7.8, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol, 1 µg polydI-dC and 20,000 cpm probe (~10 fmole DNA) were carried out for one hour on ice. The reactions were analyzed by electrophoresis through 4% polyacrylamide gels using 0.5X TBE as running buffer, followed by autoradiography.

A standard footprinting protocol (Ausubel et al., 1992) was used. One end labeled fragment was generated by labeling with α-^{32}P-dCTP at both 5'-protruding ends of the fragment, digesting off one labeled end, and gel-purifying single end-labeled fragment. DNA was purified by cutting the gel slice, electroeluting DNA out of the slice, and running through a Elutip column (Schleicher & Schuell). Binding reactions were as described for Slp1. At the end of the incubation, 2 µl of 50 U/ml DNaseI (Boehringer Mannheim) and 2 µl of solution containing 125 mM $MgCl_2$ and 62.5 mM $CaCl_2$ were added. DNase I digestion proceeded for 1 minute on ice, and was stopped by the addition of 35 µl 10 mM EDTA. The DNA was purified by phenol-chloroform extraction, followed by ethanol precipitation with 2 µl of 10 mg/ml tRNA. DNA samples were then separated on 5% polyacrylamide/8 M urea gels.

4. Examining 323-lacZ fusion gene expression in slp mutant embryos

To examine 323 bp fPE/*lacZ* fussion gene expression in *slp* mutant embryos, a *slp* mutant strain, Δ34B (a gift of K, Cadigan), was crossed to transformed flies carrying the fusion gene. The *slp* deletion in Δ34B fly is located on the second chromosome with a *Cyo* marker; the P[323/lacZ] in the transformant line is balanced over *TM3,Sb* on the third chromosome. *Cyo*, non-*Sb* progeny was mated and their embryos were collected for staining with rabbit anti-β-galactosidase antibodies ((Gutjahr et al., 1993)).

For Part III of Results:

1. Plasmid construction

To express amino acids 200-480 of FTZ-F1α for testing its ability to interact with FTZ, FTZ-F1α cDNA encoding this segment was inserted into the pACT vector (the same vector used for constructing the library used in these studies). As control, amino acids 580-800 of FTZ-F1α was also cloned into the same vector. Two pairs of primers were synthesized to generate the cDNA fragments encoding FTZ-F1α 200-480 or 580-800. After PCR, each product was digested with XhoI restriction enzyme (the XhoI site was derived from the primers) and subcloned into the XhoI site of pACT.

2. Bacterially expressed FTZ protein was used to raise an anti-FTZ antibody

To express high levels of FTZ protein in *E.coli*, pGEMF1 (Krause et al., 1988) was transformed into BL21(DE3). The following procedure is adapted from Maniatis et al., 1989. The induction was done by adding IPTG to a final concentration of 2 mM into 500 ml culture of OD_{600}=0.2. After growing at 37°C for an additional 2 hours, cells were pelleted and resuspended in 10 ml lysis buffer (50 mM Tris-HCl, pH 8, 1 mM EDTA, 100 mM NaCl, and 1 mM PMSF). 80 µl of lysozyme (10 mg/ml) was then added to lyse the cells. After

stirring for 20 minutes at room temperature, 10 µg of deoxycholic acid (a detergent) was added while stirring continuously. When the lysate became viscous, 50 µg of DNase I was added to digest the DNA. The cell lysate was then centrifuged for 15 minutes at 4°C. The pellets contained purified inclusion bodies. The inclusion bodies were then washed sequentially with 5 ml of, (i) lysis buffer containing 1% Triton X-100, (ii) lysis buffer containing 1.5 M NaCl, (iii) lysis buffer containing 3 M NaCl. For each washing step, the pellet was dissolved and centrifuged, the supernatant was decanted. Finally, the protein pellet was resuspended in 400 µl of cold PBS buffer and stored at -70°C. Proteins were analyzed by 10% SDS-PAGE gel, followed by Coomassie blue staining (Fig. 19).

To raise an antibody, a preparative 8% SDS-PAGE was used to isolate large amounts of FTZ protein (about 2 mg). After electrophoresis, the gel was stained with 1% Commassie blue (R-250) in dH_2O for 10 minutes, followed by destaining with water. A gel slice containing FTZ protein was excised and sent to a company (HRP, inc. Denver Philadelphia) for making a rabbit polyclonal antibody.

3. Purification of anti-FTZ antibody

Bacterially expressed FTZ protein 3 mg (total protein concentration) was dissolved in 5 ml of buffer containing 0.1M $NaHCO_3$ pH, 8.3, 0.5 M NaCl, and 6 M Guanidine HCl. The protein was coupled to CNBr-activated Sepharose 4B

(Pharmacia) by rotating overnight at 4°C. Protein coupled Sepharose (~0.8 grams) was further treated by: (i) blocking with 0.2 M glycine, pH 8 for 2 hours at room temperature, (ii) washing with alternating pH buffers: 0.1 M acetate buffer, pH 4/0.5 M NaCl and 0.1 M Tris, pH 8/0.5 M NaCl, and (iii) equilibrating with PBS/0.5 M NaCl. 10 ml of buffer was used for each washing. Binding of anti-serum (4 ml of second bleed serum) and protein coupled Sepharose beads was carried out in PBT/0.5 M NaCl overnight at 4°C with rotating. The resulting Sepharose was packed into a column, washed with PBT/0.5 M NaCl. Bound antibody was eluted with 0.25 mM glycine, pH 3.5/4 M $MgCl_2$. The elution volume was 0.5 ml per each fraction (Dr. M. Frash, personal communication). Fractions 3-6 with protein concentrations of 1 µg/ml-0.3 µg/ml were pooled and dialyzed against 0.4X PBS/0.5 M NaCl overnight at 4°C. Purified antiserum was stored either at 4°C or -20°C in 50% glycerol. However, purified antiserum stored in 50% glycerol at -20°C seems to lose its activity very quickly.

4. Co-immunoprecipitation of FTZ and FTZ-F1α using *Drosophila* embryonic nuclear extract

The co-immunoprecipitation experiment was done essentially according to the protocol described in "Molecular cloning: A laboratory manual" (Maniatis et al., 1989). Protein A Sepharose (Pharmacia) was precoated with antiserum: 100 µl of protein A Sepharose was incubated with either 100

µl preimmuserum (containing ~10 µg of protein) or 300 µl purified anti-FTZ serum (containing ~10 µg of protein) overnight at 4°C with rotation. 100 µl (~2 mg) 0-9 hour *Drosophila* embryo nuclear extract (#9413, provided by M. Landrigan) was precleared with 20 µl preimmuserum coated protein A Sepharose overnight at 4°C with rotation. Sepharose was collected by centrifugation for 20 seconds and used as control (Fig. 21, lane 2). The supernatant containing precleared nuclear extract was subsequently incubated with 20 µl of anti-FTZ antibody coated protein A Sepharose by rotating at 4°C for 2 hours. Sepharose was collected by spinning for 20 seconds and washed twice with 1 ml NET-gel buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% NP40, 1 mM EDTA pH 8, 0.25% gelatin, and 0.02% sodium azide). 20 µl SDS-PAGE loading buffer was added to the final beads and heated at 95°C for 3 minutes before loading on a 10% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane for two hours in a transfer buffer (25 mM Tris-HCl, pH 8, 190 mM glycine, and 20% methanol). The membrane was blocked overnight in 20 ml TTBS buffer (0.1% Tween 20, 100 mM Tris-HCl, pH 7.5, 0.9% NaCl) containing 15% BSA. A 1:1000 dilution of anti-FTZ-F1α antibody was used for the first antibody incubation, and a 1:2000 dilution of anti-mouse IgG was used for the second antibody incubation. Both antibody incubations were carried out at room temperature in 10 ml TTBS for 1 hour. Color was generated with a peroxidase ABC kit (Vector Lab.).

5. Cooperative binding of FTZ-F1α and FTZ to DNA

Both FTZ and FTZ-F1α proteins were prepared as described for Slp1. To express FTZ-F1α protein, an expression plasmid pJC20FTZ-F1 (provided by Dr. C. Wu) which expresses FTZ-F1α without the first 200 amino acid was used. pGEMF1 was used for producing FTZ protein. Probes were end labeled using α-^{32}P-dCTP and klenow enzyme, as they all contain cohesive restriction site at both ends. The sequence of o-F1F is 5'-TCGAGCGGCATCCTTGACTTTGATTGGTGCCGC (top strand), the sequence of o-m1F1F is 5' GTAACCGGCATATCTGACTTTGATTGGTGCCG (top strand), the sequence of o-m2F1F is 5' GTAACCGGCATCCTTGACTTTGCGATGTGCCG (top strand).

6. Sequencing of *FIP2* cDNA

The full length FIP2 cDNA was sequenced using an Exo III/Mung Bean Nuclease Deletion kit (Stratagene). The FIP2 insert was subcloned into a Bluescript KS vector to generate plasmids, KSFIP2#4 and #9, which contain FIP2 inserted opposite orientations. CsCl preparations of both KSFIP2 plasmid DNAs were used for making deletions. Both KSFIP2 DNAs were digested with restriction enzyme SpeI and filled-in with α-thio-dNTP to create a protected end, and deletion started from EcoRV digestion end towards the cDNA insert. Deletions were made in 25 µl of Exo III buffer (100 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, and 20 µg/ml tRNA) containing 5 µg of double-digested DNA, 10 mM β-mercaptoethanol, and 100 units

of Exo III for 15 minutes at room temperature. After heating at 68°C for 15 minutes to inactivate the Exo III, 15 units of mung bean nuclease was added and the reaction was carried out for 30 minutes at 30°C. The DNAs were precipitated and further treated with Klenow enzyme to make blunt ends. This Klenow reaction increased the efficiency of the following ligation significantly. Mini-prep DNAs were used for sequencing with the KS forward primer.

7. Northern and Southern analysis of the *FIP2* gene

A deletion fragment of the *FIP2* gene (about 1 kb containing the middle portion of the cDNA) was end labeled with α-^{32}P-dCTP using klenow enzyme. This probe was used in both Southern and Northern blot analysis.

20 µg of *Drosophila* genomic DNA (provided by W. Han) was digested with different restriction enzymes and DNA was separated on an 1% agarose gel, denatured in 0.5 N NaOH and 1.5 M NaCl for 1 hour, renatured in 1 M Tris-HCl, pH 8 and 1.5 M NaCl for 1 hour, and transferred to a nitrocellulose membrane in 20X SSC solution overnight. Prehybridization was carried out at 50°C for 2 hour in prehybridization solution (6X SSC, 20 mM sodium phosphate buffer pH 6.4, 0.1% SDS, 0.1 mg/ml sonicated salmon sperm DNA, 1X Denhardt's solution, and 50% formamide). Hybridization was done in the hybridization solution with 10^7cpm (~10 nmole DNA) labeled probe at 50°C for 18 hour. The filter was washed with 1X SSC/0.1% SDS

twice for 15 minutes each at 50°C, and 0.25X SSC/0.1% SDS twice for 15 minutes each at 50°C.

mRNA isolated from 0-6 hour embryos was prepared as described under "library construction". 3 µg of mRNA poly A^+ was separated on a 1% formaldehyde agarose gel. mRNA was blotted to a nitrocellulose membrane in 20X SSC overnight. Prehybridization and hybridization were carried out as for Southern blot. The filter was washed as described for Southern analysis.

8. Expression and purification of FIP2 protein in bacterial cells

FIP2 DNA was inserted into a pET30b vector to create an expression plasmid. pET30b-FIP2 expressed FIP2 protein is a histidine and S-protein tagged fusion protein. Protein was induced as described for making FTZ antibody. Four bands detected by SDS-PAGE were highly induced by 2 mM IPTG. The identity of these bands as products of S-tagged fusion proteins was confirmed with an S-Tag Western blot kit (Novogene). The blot was prepared as usual (described before), blocked in 20 ml TBST (0.1 M Tris-HCl, pH 8, 1.5 M NaCl, 0.1% Tween 20)/1% gelatin for 15 minutes at room temperature, and incubated with alkaline phosphatase conjugated S-protein (1:5000 dilution in TBST) for 15 minutes at room temperature. After several washes with TBST, the target bands were visualized with 60 µl streptavidin-alkaline

phosphatase and 60 µl NBT/BCIP in a 20 ml AP buffer (supplied by the kit).

One of the induced bands was transferred to a PVDF membrane and microsequenced. Sequencing result showed that the first six amino acids from the N-terminal were M-H-H-H-H-H. This band (~2 mg protein) was excised and sent to a company (HRP) for making anti-FIP2 antibody. FIP2 fusion protein was also purified with a HiTrap kit (Pharmacia) according to the instructions.

CHAPTER 3. RESULTS

Part I. A Double Interaction Screen for detecting protein-DNA and protein-protein interactions

1. A 323 bp *ftz* proximal enhancer contains information necessary for generating seven *ftz*-like stripes

The cis-regulatory elements of the *ftz* gene have been very well characterized (Dearolf, 1989b; Dearolf et al., 1989a; Han et al., 1993; Hiromi and Gehring, 1987; Hiromi et al., 1985; Pick et al., 1990; Topol et al., 1991). A large 5' region was shown to contain three individual cis-acting elements (Fig. 2): A zebra element contains an active promoter and also directed partial stripe expression; a neurogenic element directed expression in neural precursor cells; and an upstream element acted as an enhancer of stripe expression. The upstream element was further subdivided into distal and proximal enhancers by deletion analysis (Pick et al., 1990). The proximal enhancer directs *lacZ* fusion gene expression in seven stripes both in the mesoderm and ectoderm via a heterologous *hsp70* minimal promoter in transgenic fly embryos. The proximal enhancer contains two separate regulatory units: Prox A, a mesodermally restricted stripe element, and Prox B, which when combined with Prox A, directs fusion gene expression as stripes in both mesodermal and ectodermal primordial (Pick et al., 1990). I have continued the deletion analysis of the *ftz* proximal enhancer. A 323 bp

fragment of the *ftz* proximal element is a small region containing part of Prox A and part of Prox B (Fig. 2) that directs expression in seven *ftz*-like stripes when fused to a *lacZ* reporter gene in transgenic flies (Fig. 3). We therefore used this 323 bp *ftz* proximal enhancer (323 bp fPE) to identify *ftz* trans-regulators employing a genetic screen which will be described below.

Previous genetic experiments demonstrated that wild-type *ftz* gene product is required for expression of enhancer-*lacZ* fusion genes in seven stripes, and each individual enhancer appears to be a target for autoregulation by the *ftz* gene product (Hiromi and Gehring, 1987; Pick et al., 1990; Schier and Gehring, 1992). DNA binding studies in vitro have identified a number of binding sites for purified FTZ homeodomain in the upstream element. The 323 bp fPE contains 5 binding sites for FTZ homeodomain. Taking together the results from genetic studies and biochemical studies, we have one of the few regulatory elements demonstrated to interact with a homeodomain protein in vivo. Thus, we used this 323 bp fPE as a natural FTZ binding site to identify proteins that interact with FTZ protein.

2. A new strategy for isolating DNA-binding proteins that regulate *ftz* expression and FTZ interacting proteins

The new scheme that we developed is a yeast-based assay to detect both protein-protein and protein-DNA interactions

in vivo. It was named a Double Interaction Screen. The strategy of the Double Interaction Screen is modified from the yeast two-hybrid system developed by S. Fields (Fields and Song, 1989) to detect protein-protein interactions. The 323 bp fPE described above was used as the regulatory element in our screen.

Our Double Interaction Screen has several basic components: (i) a *HIS3* reporter gene containing the 323 bp fPE fused to the yeast *HIS3* gene with its own basal promoter (Fig. 4), (ii) a *lacZ* reporter gene containing the 323 bp fPE fused to the *lacZ* gene with a yeast *CYC1* basal promoter (Fig. 4), (iii) a hybrid expression library, constructed by fusing a Gal4 transcriptional activation domain to *Drosophila* cDNAs, and (iiii) An expression plasmid containing *ftz* cDNA under the control of the yeast *ADH1* promoter (Fig. 5). Hybrid proteins that either recognize the 323 bp fPE sequence directly or that interact with FTZ protein are expected to induce expression of reporter genes. The detection of increased levels of transcription of the *HIS3* reporter gene was accomplished by cell growth in the absence of histidine and in the presence of different concentrations of 3-aminotriazole (3-AT) (Fig. 6). 3-AT is a competitive inhibitor of the *HIS3* gene product, IGP dehydratase. Thus cells expressing more His3 can survive in higher concentrations of 3-AT. The levels of transcription were also analyzed by detecting *lacZ* gene expression using β-galactosidase activity assays (Fig. 7).

The yeast strain w3031A (MATa leu2-3,112 trp1-1 ura3-1 ada2-1 his3-11,15) was used as the host. Two reporter plasmids each containing a TRP1 marker were introduced into w3031A cells individually by genomic integration to create reporters, w/303his3 and w/303lacZ. The proper integration of these reporter genes was confirmed by Southern analysis using Digoxygenin (DIG) labeled *lacZ* probes, since both reporter constructs contain sequences of the *lacZ* gene (see Material and Methods). For the Southern blot, genomic DNAs isolated from yeast cells were digested with StuI restriction enzyme (StuI was also used to make site-specific genomic integrations) before hybridizing to the *lacZ* probe. Digestion of genomic DNA from cells carrying integrated reporter genes should result in a band corresponding to the size of the reporter plasmid. As shown in Figure 8, genomic DNA extracted from cells carrying the *323-lacZ* reporter construct (lane 1), the *323-HIS3* reporter construct (lane 2), and the *NP6-HIS3* reporter construct (lane 3), each contains a band of predicted size on the blot, while DNA extracted from the host cell shows no detectable band (lane 4).

The yeast cells carrying the *HIS3* reporter, w/323his3, are able to grow in the absence of histidine, indicating background levels of activation of the reporter gene by yeast proteins. Since FTZ protein alone is able to activate transcription in yeast (Fitzpatrick and Ingles, 1989), it was expected that levels of expression would increase when the *ftz* expression plasmid was introduced into the reporter

cells. As shown in Figure 9, w/323hisFTZ cells carrying the 323 bp fPE-*HIS3* reporter gene and the *ftz* expression plasmid grew on synthetic dropout (SD)-his, -ura plates containing no more than 1 mM 3-AT. Consistent with this, cells carrying an 323 bp fPE-lacZ reporter gene and the *ftz* expression plasmid, w/323lacZFTZ, expressed low levels of β-galactosidase (~5 units). Thus, activation of reporter gene expression by FTZ alone was much weaker than expected. This allowed us to screen a *Drosophila* cDNA expression library in the presence of FTZ using 3-AT concentrations higher than 1 mM (e.g. 25 mM 3-AT) and subsequently screening for β-galactosidase activities higher than 5 units (Fig. 10).

Two explanations of the weak activation by FTZ were considered: 1) FTZ protein was not expressed or active in the reporter cells, and 2) FTZ is not sufficient to activate transcription through native target elements. To rule out the possibility that FTZ is not synthesized or active in yeast cells, another reporter was constructed which contained concatamerized *engrailed* binding sites (NP6) (Desplan and Theis, 1985), since FTZ binds strongly this site. FTZ binds to the same site. As shown in Figure 11, FTZ activated transcription very strongly via the NP6 sites allowing for growth of cells in 25 mM 3-AT. This observation verifies that FTZ is expressed and functions as a transcriptional activator in yeast. It also supports the idea that FTZ requires co-factors to activate transcription via its native binding element.

Overall, in our screen system, we constructed a natural environment where a native DNA-binding sequence was used instead of a Gal4 binding site. Thus interactions which are based on DNA-binding of this native element can be detected (Fig. 12B). In addition, since FTZ is a DNA-binding protein, and the 323 bp fPE used in the screen contains several FTZ binding sites, we can isolate proteins that interact with FTZ in the same screen. Two types of FTZ-interacting cofactors can be isolated: 1) cofactors that do not bind DNA themselves, but do interact with FTZ (Fig. 12C), and (2) cofactors that bind to DNA and interact with FTZ (Fig. 12D)

3. Construction of the Gal4 activation domain tagged *Drosophila* cDNA library

A 0-6 hour *Drosophila* embryonic cDNA library was generated for the Double Interaction Screen. To construct the library, a λ phage vector, λACT (activation domain), containing sequences encoding the Gal4 activation domain was used (Durfee et al., 1993). λACT has the ability to generate large cDNA libraries with a high percentage of inserts, has a large insert size capacity, and can be simply converted from a phage to a plasmid using cre-lox mediated site-specific recombination (Elledge et al., 1991). pACT, the plasmid excised from λACT, contains the ColE1 origin of replication and Amp^R gene for replication and selection in *E. coli*, and LEU2, 2μ origin, and the ADH1 promoter sequences for selection, replication, and expression in *Saccharomyces*

cerevisiae. The ADH promoter drives expression of a hybrid protein consisting of the SV40 large T antigen nuclear localization signal and sequences encoding the activation domain of Gal4 fused to the cDNA. A polylinker is created between activation domain sequences and *ADH* terminator sequences, containing an XhoI site into which cDNAs are inserted.

A *Drosophila* cDNA library was constructed in λACT (Fig. 13, see methods for details of library construction). *Drosophila* RNA was prepared from Oregon R 0-6 hour embryos, using the GTC/CsCl method. Poly(A)$^+$ RNA was selected with an oligo-dT column (Boehringer). cDNA was made using a cDNA synthesis kit from Stratagene as described in methods. Double stranded cDNAs were ligated to adapters to give XhoI sites at both ends. cDNAs longer than 400 bp were ligated into λACT phage arms. The library contained $1X10^7$ total recombinants with >90% inserts. It was then amplified in XL1-Blue MRA cells, a methylation restriction minus strain. The phage library was converted into a plasmid library by excision, and plasmid DNA was prepared in large quantities for transformation into yeast.

4. Isolation of potential *ftz* trans-regulators and FTZ cofactors in a same screen

To screen for DNA-binding factors and FTZ interacting proteins, the Gal4 activation domain tagged embryonic cDNA library was transformed into reporter yeast strain w/323his3

using a method described in Durfee et al. (1993). A total of $5X10^6$ transformants were plated on ten (150 X 15 mm) SD-his, leu, ura plates containing 25 mM 3-AT. The number of transformants was determined by plating aliquot of cells (e.g. 5 µl) transformed with cDNA library on an SD-ura, leu plate and counting the number of colonies after two days of incubation and calculating the total transformants. Approximately 100 positives were recovered on SD-plates containing 25 mM 3-AT after incubation at 30°C. Among them, some were "strong positives" as they grew into colonies within 3-5 days of incubation; some were "weak positives" as they grew into colonies after 7 days of incubation. All the positive colonies were patched onto a fresh SD-his, leu, ura plate containing 25 mM 3-AT and stored at 4°C for further studies.

To test whether the phenotype observed in the original screen was reproducible and dependent on the introduced library containing plasmids, two kinds of plasmid dependency tests were carried out. The first one was to test the growth of positive cells selected from the original screen in the presence of 25 mM 3-AT after cells lost the plasmids. The "loss of plasmid" was done by growing the cells under non-selective conditions (e.g. in YPD media) overnight in 2 ml cultures, and for each individual clone making duplicates on SD-leu or -ura plates. Cells lost the library plasmid grew only on YPD but not on SD-leu plates, these cells were tested for their growth on SD-his plates containing 25 mM 3-AT. The

second one was to isolate plasmids from the positive cells, re-transform them back into fresh w/323his3 cells, and examine the growth of these cells in the presence of 25 mM 3-AT. Among the 67 positives that have been tested so far (Fig. 14), 40 clones were plasmid-dependent after the first plasmid dependency test, that is they did not grow on SD-his plates containing 25 mM 3-AT after their plasmids were lost. Among these 40, only 18 clones were plasmid-dependent after the second plasmid dependency test, that is their isolated plasmids allowed for the growth of w/323his3 reporter cells on SD-his, leu plates containing 25 mM 3-AT after re-transformation. Therefore, it is my experience that the second test is more stringent than the first one. The other 22 plasmids that failed to make w/323his3 cells grow in the presence of 25 mM 3-AT after re-transformation were also considered as false positives.

The 18 plasmid-dependent positives were then tested to determine whether their ability to activate transcription were dependent upon interacting with FTZ. The FTZ expression plasmid was lost under non-selective growth conditions (media containing uracil) as described for plasmid-dependent test. The resulting cells were tested for their growth on SD-leu, his plates containing 25 mM 3-AT. At the same time, all 18 plasmids isolated from plasmid-dependent clones were transformed individually into w/323His3 cells and w/323His3FTZ cells. Growth of these transformants on either SD-leu, his (in the absence of FTZ) or -leu, his, ura (in the

presence of FTZ) plates containing 25 mM 3-AT were compared. For 15 plasmids, their transformants grew the same on both plates, suggesting they were FTZ-independent clones. Two clones only survived at 25 mM 3-AT in the presence of FTZ, suggesting they were FTZ-dependent. One clone, C11, grew alone on SD-leu, his plates containing 25 mM 3-AT but grew much better with FTZ on SD-leu, his, ura plates containing 25 mM 3-AT. Furthermore, C11 grew at 50 mM 3-AT in the presence of FTZ, but barely survived at the same concentration of 3-AT in the absence of FTZ. Thus, C11 was also considered as a FTZ-dependent clone. Therefore, both FTZ-independent clones, expressing potential DNA-binding proteins that regulate *ftz* gene transcription, and FTZ-dependent clones, expressing potential FTZ cofactors, were isolated.

After His3 selection, positive clones were partially sequenced (discussed below). Their abilities to activate transcription were re-assayed with *lacZ* reporter cells. Plasmids from the 18 positive clones were isolated by transforming yeast total DNA containing positive cDNAs into bacterial XL1-blue competent cells. Plasmids recovered from ampicillin resistant colonies were then transformed into w/323lacZ yeast cells. Liquid β-galactosidase activity assays were used to detect the level of *lacZ* expression. As summarized in Table 1, for ten His3 positive clones, β-galactosidase activity was higher than control levels (~4.3 units); for eight His3 positive clones, β-galactosidase activity was the same as control cells. However, for reasons

discussed below, all 18 clones were considered to be real positives and were subjected to further studies.

Those clones that were His3 positive and had β-galactosidase units at control levels including, FTZ-F1α isolates (F9, C11), FTZ-F1β isolate (F51), and novel isolates (F2, F14, F78, Cd, and Cs), share a common feature. All eight cDNAs were inserted in reverse orientation in the pACT vector. It has been shown that yeast transcription can be initiated from non-conventional promoters (e.g. poly dA-dT or random sequences) (Singer et al., 1990; Struhl, 1985). cDNAs inserted in the reverse orientation in pACT were presumably transcribed from initiation sites within the *ADH1* terminator sequences. However, we presume that this transcription initiation event is less efficient than transcription initiated from the strong *ADH1* promoter. As a result, lower levels of the corresponding proteins would be produced. The negative results of β-galactosidase assays could be due to the differential sensitivity of the two reporter gene promoters (*HIS3* and *CYC1*) to the level of activator protein expression. Our confidence in classifying these clones as real positives came from the fact that two out of eight such clones contained cDNAs for FTZ-F1α (see part II of results). Two FTZ-F1α cDNAs inserted in the forward orientation in pACT were isolated in the same screen as well. These cDNAs, inserted in the forward orientation in pACT, were double positive, whereas, the cDNAs inserted in the reverse orientation were His positive and had β-galactosidase units

about 4.5 (Table 1). This leads us postulate that those cDNAs which were His3 positive but gave control levels of β-galactosidase units are "real" if their cDNAs were inserted in the reverse orientation in pACT. However, we cannot absolutely rule out the possibility that some of these positives encode proteins that interact with the *HIS3* promoter rather than the 323 bp fPE. To rule out these false positives, we would have to fuse those cDNAs in frame with the pACT activation domain and test their ability to activate *lacZ* gene expression.

In summary, a total of 67 positives isolated from the primary His3 selection were further analyzed. 40 isolates were positive after the first plasmid-dependency test; 18 isolates were positive after the second plasmid-dependency test. Among the 18 His3 positive clones, ten were lacZ positive and eight expressed lacZ at control levels. Of these, 3 were FTZ-dependent (all lacZ negative) and 15 were FTZ-independent (5 lacZ negative, 10 lacZ positive).

Part II. Positive and negative regulators of *ftz* gene transcription were identified in the Double Interaction Screen

1. FTZ-F1 and TTK were isolated from the screen

The 15 FTZ-independent clones were partially sequenced using primers located in the polylinker of pACT. As

summarized in Table 2, database searches showed that three clones (F1, F9, and F74) contained inserts that match *FTZ-F1α* cDNA; six clones (F18, F19, F51, F56, F59, and F90) contained inserts that match *FTZ-F1β* cDNA; one clone (F53) contained an insert that matches *ttk* cDNA; one clone (F55) contained an insert that matches *slp1* cDNA; four clones (F2, F14, F70, and F78) had no similarities to any known *Drosophila* genes listed in the GenBank. All the *FTZ-F1α* isolates were derived from different clones and contained truncated forms of the cDNA missing between 1300 bp and 1500 bp from the translation start site. All the *FTZ-F1β* isolates were also derived from different clones and contained short forms of the cDNA missing between 1200 bp and 1800 bp from the translation start site. The *slp1* isolate contained the full length cDNA. The *ttk* isolate contained a truncated cDNA missing 861 bp from the translation start site.

Thus, we have isolated cDNAs encoding four previously identified transcription factors: FTZ-F1α and β, TTK, and Slp1 in my double Interaction Screen. FTZ-F1α was originally identified as a specific DNA-binding protein interacting with the *ftz* zebra element (Ueda et al., 1990). It was suggested that FTZ-F1α is a transcriptional activator of the *ftz* gene, since transgenic embryos carrying zebra element-lacZ constructs with mutated FTZ-F1 binding sites showed a decrease of β-galactosidase activity in stripes 1, 2, 3, and 6 (Ueda et al., 1990). Three binding sites for FTZ-F1α were identified previously in our lab in the 323 bp fPE (Han et

al., 1993). TTK was identified as a transcription factor binding to the *ftz* zebra element and the *ftz* upstream element. It was thought to act as a repressor of *ftz* gene expression (Harrison and Travers, 1990), because overexpression of TTK under the heat shock promoter suppressed endogenous *ftz* stripe expression in *Drosophila* embryos (Brown and Wu, 1993; Read, 1992). TTK also binds to the *even-skipped* (*eve*) promoter and autoregulatory region (Brown et al., 1991; Read et al., 1990). Han et al. (1993) identified five TTK binding sites within the 323 bp fPE. Therefore, the isolation of two known *ftz* regulators, *FTZ-F1α* and *ttk*, validates the Double Interaction Screen, in which the 323 bp fPE has been used as a "bait".

2. One clone encodes Sloppy paired 1 (Slp1) protein

Sequence analysis demonstrated that clone F55 is *slp1* (Grossniklaus et al., 1992). *slp* was originally identified in a screen for embryonic lethal mutations affecting pattern formation (Nusslein-Volhard et al., 1984). The *slp* locus is composed of two structurally related genes (*slp1* and *slp2*). Both *slp* proteins have a *fork head* domain, that is also present in the *Drosophila* fork head protein and in the rat HNF-3 family of transcription factors (Lai and Darnell, 1991; Weigel and Jackle, 1990). The *fork head* domain contains a winged helix DNA binding motif (Clark et al., 1993).

It has been demonstrated that Slp plays a very important role in maintaining segment polarity gene expression (Cadigan

et al., 1994a). Genetic studies also suggested that Slp could repress the expression of two pair-rule genes, *ftz* and *eve* (Cadigan et al., 1994b; Cadigan et al., 1994a). In *slp* mutant embryos, *ftz* and *eve* were ectopically expressed. *ftz* was expressed in fourteen stripes during late gastrulation instead of the normal seven stripes; *eve* stripes were broader than normal after stage 8. However, genetic studies could not address the question of whether Slp regulates *ftz* and *eve* expression directly or indirectly. In addition, it had never been shown that Slp can bind to DNA specifically despite the fact that the protein contains a putative DNA-binding motif.

3. Slp1 protein binds to a region of the 323 bp fPE specifically in vitro

To examine the specific interaction between Slp1 and the 323 bp fPE, recombinant Slp1 protein was expressed in *E. coli* (Studier et al., 1990). An expression plasmid, PET3d-slp1c (provided by Dr. K. Cadigan), containing full length *slp1* cDNA was transformed into BL21(DE3) cells, and T7 RNA polymerase-directed expression was induced by addition of IPTG. Denatured and then renatured protein extract was used directly for in vitro DNA-binding assays as described in methods.

DNase I footprinting was performed to define Slp1 binding sites within the 323 bp. ^{32}P-labeled 323 bp fPE was incubated with protein extract expressing *slp1* protein; the reaction was then subjected to DNaseI digestion, and analyzed

on 8% polyacrylamide/8 M urea sequencing gels. As shown in Fig. 15A, one region of about 19 bp was protected from DNase I digestion. The footprint site was confirmed on the other strand (data not shown). The footprinted region is located between positions 2227 and 2246 of the *ftz* upstream element (sequence: 5'-TCTTCGATGTCAACACACC-3', top strand).

To further confirm the interaction between Slp1 and the footprint site, an oligonucleotide (Slp1-site) containing the protected sequence was synthesized and used for gel retardation assays. As shown in Figure. 15B, a single complex was formed following incubation of the labeled probe with bacterially expressed Slp1 protein (lane 1). The presence of Slp1 protein in the DNA-protein binding complex was tested by including anti-Slp1 antibodies in the binding reaction. As shown in lane 3, the retarded band was supershifted by anti-Slp1 antibody but not by preimmune serum (lane 2) or by an unrelated antibody (lane 4), demonstrating that Slp1 protein is present in the DNA-protein complex.

The binding specificity of Slp1 protein to the Slp1-site sequence was further analyzed. As shown in Fig. 15C, Slp1 protein bound to Slp1-site generating a single retarded band, as shown before (lane 2). Protein extract prepared from *E. coli* cells without expression vector was used as a control. No shifted band was detected with control protein (lane 1). Different amounts of specific competitor (Slp1-site) at a 1:1, 1:10, 1:50, and 1:100 molar excess were used in lanes 3, 4, 5, and 6 respectively. The binding was inhibited with a

1:50 molar excess and was totally abolished at a 1:100 molar ratio of the slp1-site. Similar amounts of two non-specific competitors: a FTZ-binding site and a FTZ-F1-binding site were used in lanes 7-10, and 11-14 respectively. The sequence of oligonucleotide FTZ-site is 5'-AGCTTGACAGGAGCAATTA for the top strand, and the sequence of oligonucleotide FTZ-F1-site is 5'-AGCTTATCCTTGACTTA for the top strand. The binding was not strongly affected even at a 1:100 molar ratio of either non-specific competitors. The gel shift competition along with the footprinting analysis, demonstrate that Slp1 behaves as a sequence-specific DNA binding protein. Moreover, we have identified at least one potential native target within the 323 bp fPE through which Slp1 might regulate *ftz* gene expression in vivo.

4. Slp1 protein represses transcription in yeast cells

The use of a Gal4 activation domain tagged library in the Double Interaction Screen theoretically should have allowed us to isolate both transcriptional activators and repressors. As expected, at least two potential repressors were isolated from the screen, one is *ttk*, the other is *slp1*.

As summarized above, Slp1 was thought to be a repressor of *ftz* (Cadigan et al., 1994b; Cadigan et al., 1994a), since *ftz* was ectopically expressed as fourteen stripes during late gastrulation in *slp* mutants and since over-expression of *slp* proteins dramatically decreased *ftz* seven stripe expression. To test whether Slp1 protein is capable of transcriptional

repression, an expression plasmid containing full length *slp1* cDNA under the control of an *ADH1* promoter was constructed and introduced into w/323lacZ cells. Liquid β-galactosidase assays of the yeast cells expressing different forms of Slp1 protein were carried out to detect the level of *lacZ* gene expression driven by the 323 bp fPE. As shown in Figure 16, w/323lacZ cells expressing Slp1 fused to the Gal4 activation (Slp/GAD) produced β-gal activity at a level that is ~3 fold higher than that of the base line of w/323lacZ cells. w/323lacZ cells expressing Slp1 without fusion to the Gal4 activation domain produced β-galactosidase activity at levels ~2.5 fold lower than that of w/323lacZ cells.

To further confirm this repression, a derivative of *lacZ* construct pNG22 (Wang et al., 1994), pNG224X, containing four copies of the Slp1-site (Fig. 16B, bottom) was used in the same β-galactosidase liquid assays. pNG224X plasmid together with either the Slp1 expression plasmid or the empty vector (pADNS) were co-transformed into w3031A yeast cells. Transformants were obtained and their β-galactosidase expressions were tested. As shown in Figure 16B, the expression of Slp1 protein reduced β-galactosidase units ~4 folds compared to the control. In addition, Slp1 did not change the *lacZ* expression directed by the pNG22 plasmid, suggesting the repression described above is dependent upon the Slp1-site. Taken together, these results demonstrate that Slp1 protein has ability to repress transcription via either the 323 bp fPE or the Slp1-site in yeast cells.

5. Slp1 repression of *ftz* gene expression is mediated by the 323 bp fPE in *Drosophila* embryos

Our experiments so far have suggested that, (1) *slp1* protein can bind to at least one region of the 323 bp fPE specifically in vitro, and (2) *slp1* protein represses transcription directed by the 323 bp fPE in yeast cells. To address whether or not Slp1 can repress transcription mediated by the 323 bp fPE in *Drosophila* embryos, we examined *lacZ* expression driven by the 323 bp fPE in *slp* mutant embryos (Fig. 17). The 323 bp fPE directed expression in seven *ftz*-like stripes when it was fused to the *lacZ* reporter gene in transgenic embryos (Fig. 17A). A null *slp* mutant line, Δ34B (Cadigan et al., 1994a), in which both *slp* genes (*slp1* and *slp2*) are deleted, was crossed to flies carrying the 323 fPE/*lacZ* fusion gene. As shown in Figure 17B, 17C, and 17D, ectopic expression of the *lacZ* reporter gene was observed repeatedly in the mutant embryos. The percentage of embryos with mutant phenotypes was as predicted. Extra *lacZ* stripes (indicated by arrows) appeared between normal *ftz/lacZ* stripe in *slp* mutant embryos, as expected. The number of ectopic stripes varied from one to seven, and the level of these stripes also differed from embryo to embryo. However, the extra stripe between *ftz/lacZ* stripe 3 and 4 was always expressed and was the strongest one.

The effect described above provides in vivo evidence that Slp1 directly represses *ftz* gene expression, and that this repression may be mediated in part by the 323 bp fPE.

6. TTK activates *ftz* transcription in yeast cells

Two forms of TTK protein resulting from differential splicing have been identified (Read, 1992). One form is 69 kD and the other is 88 kD. Genetic studies have suggested that the 69 kD TTK is a transcriptional repressor of the *ftz* gene, since its overexpression partially suppressed *ftz* stripe expression in transgenic *Drosophila* embryos (Brown and Wu, 1993; Read, 1992). The 69 kD form of TTK was isolated from our library screen as a fusion protein containing the Gal4 activation domain. To test whether or not TTK protein alone acts as a direct repressor of *ftz* via the 323 bp fPE, we expressed the full length 69 kD form cDNA with and without the Gal4 activation domain (GAD) in w/323lacZ cells. As shown in Figure 18, w/323lacZ cells expressing TTK fused with GAD produced ~4 fold higher levels of β-galactosidase than w/323lacZ cells alone. Unexpectedly, w/323lacZ cells expressing full length TTK produced β-galactosidase activity at levels similar to w/323lacZ cells expressing TTK/GAD fusion protein, which is four times higher than the base line. Thus, TTK has the potential to activate transcription via the 323 bp fPE. This interesting observation raises the possibility that TTK might be a transcriptional activator

despite the fact that genetic studies suggested it was a repressor.

Part III. Two potential FTZ co-factors were identified in the Double Interaction Screen

1. FTZ-F1α is a potential FTZ-Interacting Protein (FIP1)

In the category of FTZ-dependent clones, three cDNAs (C11, Cs, and Cd) were isolated from the screen (Table 2). Partial sequencing of the clones revealed that one of them (C11) contains sequence of a known gene which encodes a member of the nuclear hormone receptor family protein, FTZ-F1α. The C11 isolate contained a full length FTZ-F1α cDNA including some 5′ untranslated region. The isolation of FTZ-F1α as both FTZ-dependent and -independent from the Double Interaction Screen, suggested that FTZ-F1α not only interacts with the *ftz* upstream element as a transcriptional activator but also physically interacts with FTZ protein. The physical association between FTZ and FTZ-F1α may influence the functional activity of FTZ protein.

In this section, several lines of evidence will be presented to confirm the interaction between FTZ and FTZ-F1α proteins. Possible outcomes of this interaction with respect to FTZ function are discussed.

1-1. FTZ and FTZ-F1α proteins are co-expressed in *Drosophila* embryos

If FTZ and FTZ-F1α interact in vivo, they must be expressed in the same cells at same stage of development. 0-10 hour embryos were stained immunohistochemically, using our Anti-FTZ antiserum generated as described in the method. As reported by others (Carroll and Scott, 1985; Karr and Kornberg, 1989), staining results showed that FTZ protein was nuclear localized and was expressed as seven zebra stripes at the cellular blastoderm stage (Fig. 20D). FTZ expression began at early cellular blastoderm stage and persisted through mid-gastrulation (Fig. 20E). After germband extention, FTZ was re-expressed in the precursors of neuronal cell (data not shown).

We also stained 0-10 hour embryos with anti-FTZ-F1α antibody (provided by Dr. C. Wu). As shown in Figure 20A and 20B, FTZ-F1α protein is also nuclear localized and highly expressed throughout the embryo. FTZ-F1α expression was detected in the syncytial blastoderm stage, cellular blastoderm stage, and gastrulation. To test whether FTZ-F1α is also maternally expressed, unfertilized eggs were collected and stained with anti-FTZ-F1α antibody. Uniform expression of FTZ-F1α was detected in the unfertilized eggs (Fig. 20C) demonstrating that the protein is maternally expressed.

In summary, FTZ-F1α expression emerges before FTZ appears and decays after FTZ disappears. FTZ and FTZ-F1α

overlap temporally and are co-expressed in all cells where FTZ is expressed.

1-2. FTZ-F1α was co-immunoprecipitated with FTZ from *Drosophila* embryonic nuclear extract

A co-immunoprecipitation experiment was carried out to confirm the physical interaction between native FTZ and FTZ-F1α proteins. Purified anti-FTZ antibody was used for the following experiment. Antiserum was affinity purified through CNBr-activated Sepharose 4B (Pharmacia Biotech) column coupled to recombinant FTZ protein (see Materials and methods for details).

0-10 hour *Drosophila* embryonic nuclear extract was precleared with preimmuserum coupled protein-A Sepharose beads (Pharmacia Biotech). The precleared nuclear extract was then incubated with purified anti-FTZ antibody coupled protein-A Sepharose beads. Immunoprecipitates were recovered by brief centrifugation and were subjected to SDS-PAGE. The proteins on the gel were subsequently transferred to a nitrocellulose membrane and blotted with anti-FTZ-F1α polyclonal antibody. If FTZ and FTZ-F1α were present in the same complex in the nuclear extract, a band representing FTZ-F1α would be detected in this blot. As shown in Figure 21, an ~110 kD band corresponding to the size of FTZ-F1α protein was detected in the lane in which immunoprecipitate from the anti-FTZ antibody was loaded (lane 1), while no band was detected with immunoprecipitate from preimmuserum (lane 2).

The coimmunoprecipitation of FTZ-F1α with FTZ from *Drosophila* nuclear extracts strongly suggests that the proteins are present in the same complex in vivo.

1-3. FTZ-F1α facilitates the binding of FTZ to its target DNA sequence

To understand the molecular basis of FTZ and FTZ-F1α interaction, gel retardation assays were carried out to study the in vitro DNA-binding properties of FTZ with or without FTZ-F1α. Bacterially expressed FTZ and FTZ-F1α proteins were used in these assays. A 33 bp DNA fragment (o-F1F) from the 323 bp fPE (Fig. 22, positions 2391 and 2420 of the upstream element) was used for the following gel shift assay. o-F1F contains a FTZ-F1 binding site adjacent to a medium-affinity FTZ binding site ((Han et al., 1993; Pick et al., 1990)). The results are shown in Figure 23A. Control protein extract did not give any shifted band (lane 1). FTZ-F1α bound to o-F1F strongly, generating a single retarded band (lane 2). FTZ did not bind to o-F1F at a detectable level under these conditions (lane 3). However, in the reaction in which both FTZ and FTZ-F1α proteins were included, an additional band with slower mobility than the FTZ-F1α/DNA complex appeared (lane 4). This complex is presumed to be a result of a FTZ/FTZ-F1α/DNA triple interaction. This triple interaction complex, and the FTZ-F1α/DNA complex were both abolished by inclusion of anti-FTZ-F1α antibody in binding reactions (lane 5), but not by preimmuserum (lane 6), which confirmed the

presence of FTZ-F1α in both shifted bands. Furthermore, the binding specificity was demonstrated by competition analysis. Both complexes were competed by a 100 fold molar excess of specific competitor o-F1F (lane 7), but not by a 100 fold molar excess of a non-specific competitor (Slp1-site, lane 8).

To demonstrate the presence of FTZ protein in the triple complex, FTZ was depleted from the extract by immunoprecipitation with anti-FTZ antibody. Protein-A Sepharose beads (~50 µl) were precoated with either preimmuserum (50 µl) or anti-FTZ serum (50 µl, second bleed) overnight. The mixture of FTZ and FTZ-F1α proteins (3 µg of each) was then incubated with 1) preimmuserum coated beads, 2) anti-FTZ coated beads, or 3) uncoated beads for two hours. After the incubation, supernatants from 1), 2) and 3) were collected and used directly for gel-shift assay. The results are shown in Figure 23B. FTZ and FTZ-F1α together gave two binding complexes as shown before (lanes 1 and 5). The protein mixture incubated with anti-FTZ beads failed to generate the triple complex with o-F1F (lane 2), while the incubation with either preimmuserum coated or uncoated beads had no effect on the formation of the triple complex (lanes 3 and 4). Thus, FTZ protein is present in the triple complex, as the triple complex cannot form after FTZ has been depleted from the reaction.

1-4. The triple interaction of FTZ/FTZ-F1α/DNA requires both FTZ and FTZ-F1 binding sites

To test whether or not the interaction of FTZ and FTZ-F1α is dependent upon DNA binding, oligonucleotides containing point mutations in either the FTZ-F1α binding site (o-m1F1F) or the FTZ (o-m2F1F) binding site were synthesized and their abilities to form the triple complex were tested in gel retardation assays (Fig. 24). o-m1F1F changes the core of the FTZ-F1α binding site, AGGA, to ATAG (GG are two methylation protected sites, Han et al., 1993); o-m2F1F changes the core of the FTZ binding site, ATTG, to CGAT. Radioactively labeled o-m1F1F did not interact with FTZ-F1α, FTZ, or both proteins (lanes 1, 2, and 3, respectively). Radioactively labeled o-m2F1F interacted with FTZ-F1α (lane 4) since it still retains a wild-type FTZ-F1 binding site, but o-m2F1F did not interact with FTZ, nor did it form the triple complex with both proteins (lanes 5, and 6) as o-F1F formed two complexes with FTZ and FTZ-F1α(lane 7).

Thus, mutations in either the FTZ or FTZ-F1α binding site abolished formation of the triple FTZ/FTZ-F1α/DNA complex. We suggest two possible explanations for these results: 1) the formation of a FTZ/FTZ-F1α protein complex is dependent upon the binding sites for both proteins; 2) the FTZ/FTZ-F1α complex targets DNA sequences that are different from either protein alone, and an overlapping FTZ and FTZ-F1α binding sites makes a good target for this protein complex.

1-5. FTZ and FTZ-F1α bind DNA cooperatively

To test whether or not FTZ and FTZ-F1α bind to DNA cooperatively, gel retardation assays were performed. As shown in Figure 25A, the reaction including 1.5 μg FTZ and 3 μg FTZ-F1α produced two DNA-binding complexes with the lower complex (representing FTZ-F1α binding) stronger than the higher complex (representing FTZ/FTZ-F1α binding) (lane 1). When the amount of FTZ protein was increased to 7 μg, the lower complex (FTZ-F1α/DNA) was almost completely abolished and the higher complex (FTZ/FTZ-F1α/DNA) formed (lane 2). Further increases in the amount of FTZ (30 μg, lane 3; 75 μg, lane 4) with 3 μg FTZ-F1α generated only the higher complex, whereas, 75 μg FTZ alone did not result any specific binding (lane 5). A weak binding complex at a lower position in lane 5 resulted from the binding of control protein to o-F1F (not shown). This data suggests that the FTZ/FTZ-F1α/DNA binding can compete off the FTZ-F1α/DNA binding, and the FTZ/FTZ-F1α DNA-binding is stronger than the sum of each DNA-binding alone (compare lane 1+5 to 4).

In addition, at concentrations at which either FTZ or FTZ-F1α alone failed to interact with DNA, the two together resulted a triple DNA-binding complex. As shown in Figure 25B, 0.3 μg FTZ-F1α and 75 μg FTZ can form a triple DNA-binding complex (lane 4), while neither 0.3 μg FTZ-F1α (lane 1) nor 75 μg FTZ bound DNA alone (Fig. 25A, lane 5). Increasing amounts of FTZ-F1α together with 75 μg FTZ resulted in more triple DNA-binding complexes (lanes 5, 6),

that are much stronger than the binding of FTZ-F1α alone at the same concentrations (lanes 2, 3). Therefore, the results presented in Figure 25A and 25B demonstrate a cooperative binding of FTZ and FTZ-F1α to a particular target DNA.

Bacterially expressed FTZ and FTZ-F1α were used for all the in vitro DNA-binding experiments described. To test the expression level and purity of both proteins, extracts expressing either FTZ-F1α or FTZ were analyzed on a 10% SDS-polyacrylamide gel. As shown in Figure 26A, protein extracts expressing FTZ-F1α and FTZ were separated in lanes 1 and 2 respectively. Control protein extracts were loaded in lane 3. FTZ-F1α was highly expressed as its native size (indicated by an arrow in lane 1). Unfortunately, no detectable FTZ protein band was apparent as its native size in lane 2, suggesting either FTZ was not expressed or expressed at low levels. To confirm the proper expression of FTZ in bacterial cells, Western blot was performed on crude extracts from FTZ expressing cells. A band corresponding the size of full length FTZ protein (~ 55kD) was clearly detected in lane 1 (Fig. 26B), but not in lane 2 (Fig. 26B) where control extracts were loaded. Thus, both FTZ-F1α and FTZ proteins were expressed in crude extracts used for DNA-binding assays. Since both proteins are impure, and since both were subjected to denaturing agents, we do not have an accurate estimate of the amount of active protein present in binding reactions. Therefore, the amounts of total protein

added to binding reactions do not indicate the amount of active DNA-binding species.

1-6. FTZ alone is sufficient to bind to its high-affinity site but not to its medium-affinity site

The following strategy was used to demonstrate that the FTZ protein used in these experiments was able to bind to DNA.

There are three classes of FTZ homeodomain binding sites that were identified within the *ftz* upstream element using a DNase I protection assay (Pick et al., 1990). Different DNA sequences were protected against DNase I digestion at different concentrations of FTZ homeodomain. Accordingly, they were grouped into high-affinity sites, medium-affinity sites, and low-affinity sties. High-affinity sites required 26-78 ng FTZ homeodomain, medium-affinity sites required 10 times more FTZ homeodomain, and low-affinity sites required 20 times more FTZ homeodomain (Pick et al., 1990).

We observed that full length FTZ protein did not bind to an oligonucleotide (o-F1F) containing a medium-affinity site (Fig. 23). However, the same FTZ protein bound avidly to an oligonucleotide (o-2, Han et al. 1993) containing a high-affinity site (Fig. 27). In these shift experiments, both oligonucleotides were radioactively labeled and incubated with bacterially expressed full length FTZ protein. 1.5 µg of the protein extract gave weak shifted bands with labeled o-2 (lane 1), and 6 µg protein extract gave strong bands

(lane 2). However, 30 μg (lane 5) and even 150 μg (lane 8) of protein extract containing FTZ protein did not result in significant retarded band on native 5% acrylamide gels with labeled o-F1F. Thus, the binding of FTZ protein to its high-affinity site is at least 100 fold stronger than the binding of FTZ to its medium-affinity site, if there is any binding at all. The multiple bands in lanes 1, 2, and 4 could be a result of protein aggregation, since they were abolished by anti-FTZ antibody (lane 3), but not by preimmuserum (lane 4). Control protein extract expressing no FTZ protein gave a nonspecific band when incubated with labeled o-F1F (lanes 5, 6, 7, and 8). This nonspecific band was not affected by either anti-FTZ antibody and preimmuserum (lanes 6 and 7).

Although the full length FTZ alone only binds to o-2 containing a high-affinity binding site (Fig. 27), not to o-F1F containing a medium-affinity binding site (Fig. 23), it does bind to o-F1F in the presence of FTZ-F1α (Fig. 23). These observations support a hypothesis that different FTZ target elements are regulated differently by FTZ; some are activated by FTZ alone and some require other proteins assistance (e.g. FTZ cofactors). This differential response may account for the divergent functions of FTZ protein, for instance, functions involved in *ftz* autoregulation and functions involved in regulating FTZ downstream target genes expression.

1-7. A region of FTZ-F1α between amino acid positions 200 and 470 may be required for its interaction with FTZ

FTZ-F1α was isolated as both a FTZ-dependent and -independent activator from the Double Interaction Screen. However, the FTZ-dependent FTZ-F1α clone consists of full length cDNA, while the FTZ-independent FTZ-F1α clones contain truncated versions. These three clones each encode proteins with different N-termini, amino acid 451 for F1 clone, amino acid 478 for F9 clone, and amino acid amino acid 466 for F74 clone. Thus, each is missing at least 1350 nucleotides of 5' parts of FTZ-F1α cDNA.

A truncated FTZ-F1α protein missing the first 200 amino acids was produced in *E. coli* (provided by Dr. C. Wu). This truncated protein was used in all in vitro DNA-binding experiments described above. Therefore, this truncated FTZ-F1α protein retains its ability to bind DNA cooperatively with FTZ.

Two conclusions can be drawn from the above facts: 1) the first 470 amino acids are likely to be required for interacting with *ftz* protein, since only FTZ-F1α protein containing that region was FTZ-dependent, 2) the first 200 amino acids of FTZ-F1α are dispensable for the formation of the triple (FTZ/FTZ-F1α/DNA) interaction complex, and are therefore probably not required for its interaction with FTZ. Therefore, it is reasonable to predict that a 270 amino acid region (between amino acid positions 200 and 470) of FTZ-F1α

might be involved in direct protein-protein interactions with FTZ.

To test this hypothesis, a cDNA encoding amino acids 200-480 of FTZ-F1α (F1-280) was subcloned into the pACT vector, and its ability to interact with FTZ was tested in yeast using the HIS3 selection strategy. A fragment of FTZ-F1α between amino acids 580 and 800 (F1-220) which does not contain any defined functional domain was also cloned into pACT and used as a control. F1-280 or F1-220 construct was cotransformed with FTZ expression plasmid into w/323His3 cells, and the resulting transformants were tested for their growth in the presence of 10 mM 3-AT. Unfortunately, neither F1-280 nor F1-220 survived at 10 mM 3-AT, suggesting that these two regions of FTZ-F1α protein cannot interact with FTZ on their own. Thus, it seems likely that region between amino acids 200-480 of FTZ-F1α is necessary but not sufficient to mediate the interaction between FTZ and FTZ-F1α. Perhaps the DNA-binding domain of FTZ-F1α is also required for the interaction with FTZ, since the DNA-binding results with mutant o-F1F (Fig. 25) suggested that the formation of the triple complex required the presence of both DNA-binding sites.

2. A second FTZ-Interacting Protein (FIP2) is a novel protein

A second FTZ-dependent clone (Cs) was also analyzed. Its partial cDNA sequence shares no homology to any *Drosophila* gene in the GenBank. Thus, *FIP2* encodes a novel protein. Sequence analysis of the third FTZ-dependent clone (Cd) revealed that it is a *Drosophila* homolog of acylCoA dehydrogenase. We think it is likely to be a false positive of the screen; therefore, will be mentioned only in the discussion.

Molecular Characterization of the *FIP2* gene and its interaction with FTZ are under investigation. Some preliminary results are summarized in this section.

2-1. Characterization of the *FIP2* gene

a). Sequence analysis of the *FIP2* cDNA

To obtain full length cDNA sequence of the *FIP2* gene (between 3100-3500 bp in length), the Exo III/Mung Bean nuclease system (Stratagy) was used to make sequential deletions of FIP2 cDNA. An Exo III/Mung Bean Nuclease Deletion kit (Stratagene) was used to produce unidirectional nested deletions. Exonuclease III progressively digests the 3′ end of double stranded DNA (5′ restriction overhang or blunt end), but does not digest the 3′ end of a single stranded DNA (3′ restriction overhang) or 5′ restriction overhang that has been filled-in with α-thio dNTPs. Therefore, a uniform set of nested deletions can be made in a unidirectional manner. After the Exo III digestion, mung

bean nuclease is used to digest away the remaining overhang, producing two blunt ends, which can then be ligated and transformed in *E. coli* competent cells.

The FIP2 insert was released from the pACT vector and subcloned into Bluescript KS vector. Deletions were carried out according to the manufacturer's instructions of the Exo III/Mung Bean deletion kit (Stratagene). Mini-prep DNA plasmids were used directly for double stranded DNA sequencing. Different length of deletions were sequenced using the KS forward primer (Promage), and the resulting sequences were lined up based on overlapping regions (Fig. 28A). With this strategy, a 3124 bp of FIP2 cDNA sequence was resolved. Sequence of the FIP2 cDNA sequences confirmed by sequencing from both strands is shown in Figure 28B.

Partial sequence of the FIP2 cDNA is presented in Figure 29. The cDNA has an putative ATG start codon, a TAA stop codon, and a poly A tail. It is very likely that the cDNA does not include its 5' ATG codon, as it begins with an open reading frames that is in frame with the first sequenced ATG. The FIP2 gene encodes a putative ~70 kD polypeptide. Protein database searching revealed that FIP2 protein may conatin several helix turn helix structures (between amino acids 40-120, 1275-1335, 1335-1380, and 2100-2250), suggesting its ability to interact with DNA.

b). Southern and Northern blots of the *FIP2* gene

Southern blot analysis of *Drosophila* genomic DNA was performed to detect the existence and copy number of the *FIP2* gene in the genome. The probe was a 1 kb *FIP2* fragment containing the first one third of the cDNA (between nucleotides 1 and 1144). *Drosophila* genomic DNA was digested with several restriction enzymes. After argarose gel electrophoresis and transfer to a nitrocellulose membrane, hybridization and washes were carried out as described in Materials and Methods. As shown in Figure 30A, one band was detected in lanes 1, 2, 3, 4, 5, and 7, suggesting that the *FIP2* gene is a single copy gene in the *Drosophila* genome. Two bands (3 kb and 0.6 kb) were detected in lane 6, indicating there is an internal SacI site within the *FIP2* gene.

Northern blot analysis of 0-6 hour *Drosophila* embryo mRNA was performed with the probe used for the Southern blot. As shown in Figure 30B, a single band of approximately 3600 bp was detected. The position of *FIP2* mRNA in the Northern blot is in agreement with the size of the FIP2 clone (between 3100-3500 bp), which indicates that the isolated *FIP2* sequence is close to the full length cDNA.

c). Expression pattern of the *FIP2* gene in *Drosophila* embryos

The expression pattern of the *FIP2* gene in Drosophila embryos was revealed by in situ hybridization using either digoxygenin (dGG) labeled DNA or RNA probes. For making the DNA probe, a ~3500 bp FIP2 DNA insert fragment was released

from pACT and labeled with dGG-UTP using a Genius nonradioactive DNA labeling kit (Boehringer Mannheim). For making the RNA probe, FIP2 cDNA was subcloned into a Bluescript KS vector. Sense and anti-sense probes were prepared using T7 and Sp6 RNA polymerases, respectively. Embryos hybridized with the DNA and RNA probes revealed identical expression patterns. However, staining using the RNA probe resulted in much stronger signals. The staining result using dGG-labeled FIP2 DNA probe is shown in Figure 28.

0-10 hour Oregon R embryos were collected, fixed, and incubated with labeled probe as described above. FIP2 RNA expression starts at very early stages of embryo development (it is probably maternally expressed), and is expressed throughout the embryo at that time (Fig. 31A). At cellular blastoderm stage, the FIP2 mRNA is mostly restricted to the yolk (Fig. 31B). FIP2 expression then disappears for several hours. The expression resumes at about 6 hours of development, in the precursor cells of the endoderm (Fig. 30C). Expression is maintained along the invaginating endoderm as development proceeds (Fig. 30D, E, and F).

d). Expression of FIP2 recombinant protein

Recombinant FIP2 protein was expressed in bacterial cells. The expression plasmid used for FIP2 was pET30b (Novogene), which expresses Histidine-tagged and S protein-tagged fusion proteins. A FIP2 cDNA fragment generated from

the pACT vector was subcloned into pET30b vector; the resulting plasmid expressed N-terminal His-tagged and S-tagged FIP2 fusion protein when transformed into a proper host strain (BL21). T7 RNA polymerase directed expression of the fusion protein was induced in the presence of 2 mM IPTG. FIP2 fusion protein was expressed in inclusion bodies, not in the supernatant. High level of fusion protein expression was detected as three major bands (Fig. 32A, lanes 2 and 3, indicated by arrows), probably a result of proteolysis. The largest band among four had the expected size of FIP2 fusion protein (~75 kD). In this experiment, control protein was made in the absence of IPTG induction. Low levels of leaky expression without IPTG was also observed (Fig. 32A, lane 1). The identities of three bands as fusion proteins were confirmed using the S-protein detection system (Fig. 32B). The detection system is based on the interaction of the 15aa S-tagged peptide with ribonuclease S-protein (Kim and Raines, 1993). Thus S-tagged fusion protein can be detected using S-protein conjugated with a color generating enzyme (alkline phosphatase) in the presence of correct substrates (X-phosphate and NBT). The result of the S-protein detection assay is shown in Figure 31B. Three fusion protein bands were detected in lanes 2 and 3 in which 1 μg and 4 μg of protein was loaded respectively; no visible band was detected in lane 1 in which 2 μg control protein was loaded.

To generate antibody against FIP2 protein, the smallest fusion protein band at ~55 kD was chosen, because of its high

level of expression. To further confirm the identity of this band as a histidine-tagged protein, about 20 μg of protein sample was separated on a 10% acrylamide SDS-gel, and was transferred onto a PVDF membrane. The PVDF membrane was then stained very briefly with Commasie blue and the corresponding band was excised and sent to be sequenced in Mount Sinai Medical School Protein Core Facility. After the protein was confirmed to be a histidine fusion protein, the same band was excised in a large quantity (2 mg) and sent to a company (HRP) to raise an anti-FIP2 antibody.

e). Purification of FIP2 recombinant protein

FIP2 was isolated as a potential FTZ-interacting protein, however, it could still interact with DNA directly. To test the ability of FIP2 protein to interact with DNA, bacterial expressed FIP2 recombinant protein was further purified.

FIP2 was expressed as a histidine-tagged fusion protein (see above), and can, therefore, be purified through a Ni^{2+}-coated sepharose column. The rational is that a stretch of histidine amino acids (six histidines) can be retained on sepharose beads pre-bound with Nickel. Cell lysates expressing high levels of FIP2 fusion protein were sonicated, the pellet was recovered, denatured in 8 M urea, and loaded onto a $NiSO_4$ pre-coated sepharose column (Pharmacia). Fusion protein was then eluted with high concentrations of salt (0.5 M NaCl). The purification procedure was done according to

the instructions of the Histrap Chelating Kit (Pharmacia). pET30b plasmid was transformed into BL21 cells, and IPTG induced cell lysates were purified in parallel as control. Histidine-fusion protein was eluted from a Ni^{2+}-column loaded with lysate expressing FIP2 gene. Fractions of flow through, wash, and eluate were analyzed on a 10% SDS-polyacrylamide gel (Fig. 33). Fractions of control protein were loaded in lanes 1-3; Fractions of FIP2 expression extract were loaded in lanes 4-7. Three bands containing His-tag were preferentially eluted from the Ni^{2+}-column (lane 6 and 7), and they were not eluted from the same column in which control protein lysates were loaded (lane 3). After Ni^{2+}-column purification, histidine-tagged fusion proteins were enriched, but were obviously not very pure. Partial purified FIP2 protein was then renatured through dialysis to be used for future studies.

2-2. FIP2 has the potential to interact with other homeodomain proteins

It was recently shown that *Drosophila extradenticle* (*exd*) encodes a homeodomain protein that acts as a co-factor for a selected number of *Drosophila* homeotic proteins (Chan et al., 1994; Van Dijk and Murre, 1994). Exd cooperatively interacts with Ultrabithorax (Ubx), but not Antennapedia (Antp). This interaction selectively increases the affinity of Ubx for a DNA target. Exd was also shown to be able to interact with Abdominal-A (Abd-A).

To test the possibility that FIP2 is also a co-factor for homeotic gene products, we conducted a functional interaction assay. The 323 bp fPE was used as a DNA target for HOM-C proteins to activate transcription. The rationale of using the 323 bp fPE as a target is that all HOM-C proteins have the potential to bind the same site which FTZ binds. Yeast expression plasmids expressing homeotic gene products, including Labial (Lab), Deformed (Dfd), Sex combs reduced (Scr), Antp, Ubx, Abd-A, and Abd-B, were transformed individually into w/323His3 cells. In addition, these plasmids were also transformed into w/323His3FIP2 cells expressing FIP2 protein. The abilities of these cells to grow in the presence of 25 mM 3-AT were tested. None of the transformed cells expressing only the homeodomain protein survived at 25 mM 3-AT (Fig. 34, middle). However, cells expressing Dfd, Scr, Ubx, and Abd-A were able to grow at 5 mM 3-AT (Fig. 34, left). In contrast, w/323His3FIP2 cells transformed with either Lab or Antp plasmids were able to survive at 25 mM 3-AT (Fig. 34, right). To show that all the homeodomain proteins were expressed in yeast cells and they were able to bind the same site that FTZ binds, each homeotic gene expression plasmid was transformed into the w/NP6His3 reporter cell, and the transformants were tested for their growth in the presence of 25 mM 3-AT. All the transformants survived on SD-plates containing 25 mM 3-AT (Fig. 35), demonstrating that these homeodomain proteins were expressed in the yeast cells and that they could activate

transcription. Thus, our results suggest that FIP2 may selectively interact with Lab or Antp proteins, and enhance the abilities of these proteins to activate transcription via a particular DNA target.

CHAPTER 4. DISCUSSION

1. A Double Interaction Screen strategy

A yeast genetic system (the Double Interaction Screen) was developed and employed to isolate FTZ interacting proteins and potential DNA-binding transcription factors that regulate *ftz* gene expression. The Double Interaction Screen was modified from the yeast two-hybrid system for detecting protein-protein interactions (Chien et al., 1991; Fields and Song, 1989). The yeast two-hybrid screen was designed based on the unique property of a yeast transcriptional activator Gal4. Gal4 consists of two physically separable functional domains: one acts as the DNA-binding domain, the other functions as the transcriptional activation domain; both domains are required for normal function. Thus, in yeast cells expressing a reporter gene under the control of an enhancer containing Gal4 DNA-binding sites, a protein-protein interaction can be identified through reconstitution of the two Gal4 functional domains each fused with a protein of interest. A major modification we made for the Double Interaction Screen is that a native *ftz* regulatory element was used to direct reporter genes expression, instead of a Gal4 DNA-binding site. We hope that such modifications will lead us to isolate transcriptional regulators that function through authentic binding sites in vivo. The use of the yeast Gal4 activation domain fused cDNA library provides an environment for isolating transcriptional repressors and

factors that have weak DNA-binding activity and/or transactivation activities.

For the *HIS3* reporter gene, the *HIS3* promoter was used to drive *HIS3* gene expression. The promoter contains 83 base pairs upstream of the *HIS3* coding region. This basal promoter gives some low level of *HIS3* gene expression which is sufficient to allow cell growth without exogenous histidine, but not in the presence of 3-AT (Struhl et al., 1989; Struhl, 1985; Struhl and Hill, 1987). The w/323His3 reporter cell, however, was able to grow on plates containing 1 mM 3-AT. The ability of w/323His3 cells to grow in the presence of 3-AT probably resulted from increased expression of the *HIS3* reporter gene due to interactions between yeast transcription factors and the 323 bp fPE, leading to the stimulation of the *HIS3* gene expression. This background expression did not cause a problem for the selection strategy since the level of *HIS3* induction was not high. In general, this type of basal level expression can be overcome by using smaller regulatory elements.

Nevertheless, for the purpose of reducing background expression, another reporter gene was made which contains six-copies of the minimal binding site for FTZ protein (NP6) (Desplan and Theis, 1985) instead of the 323 bp fPE. This largely eliminates the possibility of interacting with yeast proteins. However, it also eliminates the chance of isolating some types of FTZ-cofactors, which themselves must interact with unique DNA-binding sites that are different

from FTZ binding sites, in order to interact with FTZ. w/NP6His3 cells failed to grow on plates without histidine, but w/NP6His3FTZ cells grew without histidine. Moreover w/NP6His3FTZ survived in the presence of 25 mM 3-AT. This result confirms the synthesis of FTZ protein in the yeast cells; and reproduces the binding of FTZ protein to NP6 sites in yeast cells, demonstrated by others (Fitzpatrick and Ingles, 1989).

The big advantage of the *HIS3* selection is its efficiency and sensitivity. Since a surival selection is used, a large number of cDNA clones can be screened at the same time. In addition, the use of 3-aminotriazole (3-AT) provides a semi-quantitative assay to measure the level of transcription by simply looking at cell growth. 3-AT is a competitive inhibitor of the *HIS3* gene product (Kishore and Shah, 1988). The level of *HIS3* gene expression is reflected by resistance of cell growth to 3-AT. The fact that *his3*$^-$ cells require low levels of exogenous *HIS3* gene expression in order to survive in media without histidine, makes this selection very sensitive such that weak interactions can be detected. 3-AT selection also provides an environment in which transcriptional repressors can be directly isolated in a screen using lower concentrations of 3-AT.

Positives isolated in the His3 selection scheme were re-assayed for their β-galactosidase activity. This step could eliminates His$^+$ revertants and plasmids bearing the *Drosophila HIS3* gene (Durfee et al., 1993). It could also

eliminate false positive clones that activate transcription through basal promoter sequence. Because the two different reporter gene promoters, *HIS3* and *CYC1*, share no common DNA sequence other than the TATA box, this class of false positives will be largely avoided from the screen. However, for the particular screen we carried out, no false positives were ruled out by assaying for β-galactosidase activity because of reasons discussed in the results.

2. Isolation of FTZ-F1 and TTK from the screen validates the selection scheme

Several known genes were isolated in our screen as FTZ-independent clones. First, FTZ-F1α, a member of the steroid hormone receptor superfamily (Lavorgna et al., 1991) was isolated repeatedly (Table 2). This protein was originally identified based on its interaction with the *ftz* zebra element (Ueda et al., 1990). Binding site dependent expression of the *ftz/lacZ* fusion gene in transgenic embryos suggested that FTZ-F1α is a potential regulator of *ftz* gene expression (Ueda et al., 1990). However, several other proteins were shown to bind the same FTZ-F1α sites (Han et al., 1993), which weakens the point that FTZ-F1α is a direct activator of *ftz* gene expression. At least, FTZ-F1α is capable of binding to its target site and activating transcription. Three binding sites for FTZ-F1α were identified in the 323 bp fPE using *Drosophila* nuclear extract (Han et al., 1993), which makes the 323 bp fPE a potential

target of FTZ-F1α. Therefore, FTZ-F1α was expected to be isolated from the screen. Similarly, FTZ-F1β was isolated repeatedly in our screen. The FTZ-F1β gene encodes a member of the steroid hormone receptor superfamily which is most closely related to FTZ-F1α. It has been reported that FTZ-F1β binds the same sequences as FTZ-F1α (Ohno and Petkovich, 1992), although binding to FTZ-F1α sites in the 323 bp fPE was not detected using *Drosophila* nuclear extracts (W. Han and L. Pick., unpublished). Until now there has been no data suggesting FTZ-F1β can activate transcription. The screen of FTZ-F1β as a FTZ-independent clone indicates that FTZ-F1β can interact with the 323 bp fPE and activate transcription, since one clone containing *FTZ-F1β* cDNA inserted in reverse orientation to the pACT vector was isolated. When cDNAs are transcribed from the initiation signal from the *ADH1* terminator sequence, the resulting gene products are no longer fused with the Gal4 activation domain. Therefore, from our screen data it is likely that FTZ-F1β is able to activate transcription, but whether it regulates the *ftz* gene transcription in vivo needs to be further elucidated.

The second known FTZ-independent clone isolated in our screen was *tramtrack* (*ttk*). The *ttk* gene encodes a zinc finger containing protein that has been shown to bind to regulatory elements of the *ftz* and *eve* genes (Brown et al., 1991; Harrison and Travers, 1990; Read et al., 1990). At least five binding sites for TTK were identified in the 323 bp fPE using *Drosophila* nuclear extracts (Han et al., 1993).

It has been proposed that TTK might be a repressor of *ftz* gene expression, since *ftz* stripes were suppressed when TTK was ectopically expressed in the embryos. Thus, *ttk* was also expected to be isolated in our screen. The third known cDNA isolated is *slp1*, which is another potential transcriptional repressor of the *ftz* gene, suggested by genetic studies. Slp1 had never been shown to bind DNA specifically even though it contains a DNA-binding motif. We showed that Slp1 can interact with DNA and repress transcription (discussed below).

We also isolated several novel cDNAs as FTZ-independent clones. They are likely to encode potential *ftz* regulators, as *FTZ-F1* and *TTK* were isolated in the same screen. These clones are being analyzed by other members of the laboratory. In summary, a number of upstream regulators of the *ftz* gene have been isolated in the Double Interaction Screen, including some identified previously by genetic and/or biochemical studies. Thus, we have developed a effective method to isolate DNA-binding proteins using an authentic binding-site, and the same method has also been successfully used to isolate co-factors (discussed below).

3. Sloppy paired 1 protein is a putative direct repressor of *ftz* gene

The *sloppy paired* (*slp*) locus consists of two related genes, *slp1* and *slp2* (Grossniklaus et al., 1992). Both proteins contain a fork head DNA-binding domain which was

first identified in the *Drosophila* protein fork head (Weigel and Jackle, 1990). One role of the *slp* genes is to maintain proper expression of *wingless* gene. Slp protein is also involved in the repression of *ftz*, *eve*, *en*, and *hh* genes (Cadigan et al., 1994b; Cadigan et al., 1994a). In the case of *ftz*, expression of fourteen *ftz* stripes arose during late gastrulation in *slp*$^-$ embryos and *ftz* expression was repressed in embryos overexpressing *slp* with a heat shock promoter. Unlike Slp1, Slp2 function seems not to be involved in *ftz* repression, as suggested by genetic studies (Cadigan et al., 1994b)

Our data showed that Slp1 binds to at least one site within the 323 bp fPE specifically (Fig. 15). Slp1 has the ability to repress transcription in yeast cells (Fig. 16). We also provided genetic evidence suggesting that Slp1 can repress *ftz/lacZ* fusion gene expression in embryos (Fig. 17). However, this data does not address whether the in vivo repression is dependent upon the Slp1-binding site within the 323 bp fPE. Therefore it does not prove that such repression resulted from direct DNA-binding. To address this question, we made transgenic flies carrying the *lacZ* gene fused to the 323 bp fPE in which Slp1-binding site was deleted. Unfortunately, extra stripes were not seen in these transgenic embryos (data not shown), which raises two possibilities about Slp1 function in vivo. The first is that the repression of *ftz* by Slp1 is not mediated directly by the binding of Slp1 to its target site in the 323 bp fPE. The

second is that Slp1 regulates *ftz* expression by interacting with more than one site in the 323 bp fPE, and these binding sites are functionally redundant. We favor the second possibility, as several "minor" Slp1 binding-sites were identified in the DNA-binding assays. The Slp1-site described before is referred to as "major" site from now on. Additional weaker footprinting sites were observed with higher concentrations of Slp1 protein extract in footprint assays (data not shown); the binding of Slp1 to its "major" site (Fig. 15, lane 1) was partly competed by several non-related oligonucleotides (e.g. o-1, o-9 sequences described in Han et al., 1993) in the gel retardation assays, suggesting that Slp1 has ability to interact with other DNA targets with lower-affinity. Therefore, it is reasonable for us to postulate that those low-affinity binding sites, in addition to the high-affinity site ("major" site), are responsible for mediating the in vivo repression by Slp1. It will be necessary to delete all the Slp1-binding sites simultaneously to observe a change in gene expression in vivo.

All the Slp1-binding sites including "major" and "minor" sites share no significant sequence homology to each other, and they are not similar to the consensus binding-site (Costa, 1994) of HNF-3 family members. Rat hepatocyte nuclear factors 3 (HNF3) family proteins encode a 110 amino acid polypeptide which shares a high degree of identity to the fork head DNA-binding domain of *Drosophila* proteins. The

fork head domain contains a winged helix DNA-binding motif (Clark et al., 1993). In addition, Slp1 did not bind to a synthetic oligonucleotide containing a 30 bp sequence from the 323 bp fPE which is the closest sequence to the HNF-3 consensus (60% identity) in the 323 bp fPE. This is not surprising since the homology between HNF-3 family and Slp1 protein within the fork head domain is only about 45%, even though they all encode winged helix motifs (Kaufmann and Knochel, 1996). The loose binding specificity observed in vitro for Slp1 proteins may indicate that Slp1 actually requires other proteins to function specifically in vivo.

The 323 bp fPE driven *lacZ* fusion gene was ectopically expressed in *slp* mutant embryos (Fig. 17). However, the detected phenotype was rather weak, as a full extra *ftz/lacZ* stripe was never observed. This suggests that the 323 bp fPE comprises only a small part of the Slp1 responsive element, other Slp1 responsive elements outside the 323 bp fPE are required for complete repression. Thus, the repression via the 323 bp fPE is just a portion of the repression mediated by the whole *ftz* upstream element, as the 323 bp fPE composes only about one ninth of the *ftz* regulatory element.

It is possible that the in vivo ectopic expression of *ftz/lacZ* fusion gene in *slp*$^-$ embryos resulted from *ftz* autoregulation, and not from a direct effect of Slp1 repression. Since *ftz* expression was shown to be autoregulated through the *ftz* proximal enhancer (Pick et al., 1990), and in *slp*$^-$ embryos, wild type *ftz* is over-expressed

as fourteen stripes (Cadigan et al., 1994b; Cadigan et al., 1994a). However, a disagreement is that the patterns of extra *ftz* stripes and *ftz/lacZ* stripes observed in *slp*$^-$ embryos were different, as extra *ftz* stripes were in the posterior portion of every even-numbered parasegment (Cadigan et al., 1994b), while extra *ftz/lacZ* stripes appeared in the middle of every even-numbered parasegment. In order to test whether this repression is caused by *ftz* autoregulation, we need to examine the *323/lacZ* fusion gene expression in *ftz*$^-$*/slp*$^-$ double mutant embryos. If the same pattern of extra *ftz/lacZ* stripes appear in double mutant embryos, we can then exclude the possibility that *ftz* autoregulation resulted in the ectopic expression of *ftz/lacZ* fusion gene.

4. Tramtrack protein is a potential trans-activator in yeast

TTK was isolated as a DNA-binding protein based on its interaction with the *ftz* upstream element (Harrison and Travers, 1990). The expression patterns of *ttk* and *ftz* do not overlap. *ttk* is expressed maternally throughout the embryo and is then re-expressed at the time when *ftz* stripes decay (Harrison and Travers, 1990), suggesting that TTK might be a repressor of *ftz* gene expression. Evidence supporting a repressive role of TTK for *ftz* expression was obtained when TTK was expressed ectopically under the control of a heat shock promoter. Repression of both *ftz* and *eve* stripes was observed (Brown and Wu, 1993; Read, 1992). In addition,

mutation of TTK binding sites in the *ftz* zebra element resulted in early activation of the *ftz-lacZ* reporter gene, suggesting that TTK represses early expression of *ftz* via these binding sites (Brown et al., 1991).

We have found that both TTK-GAD fusion protein and full length TTK protein strongly activate transcription via the 323 bp fPE in yeast cells (Fig. 18). This surprising result suggests that TTK has intrinsic potential to activate transcription. This result also raises several interesting possibilities about TTK function in vivo. First, the genetically identified repression of *ftz* by TTK may be indirect. The repression would be explained by TTK activating the expression of a real repressor of *ftz*. This function can be tested by examining whether the deletion or mutations in TTK-binding sites affect *lacZ* fusion gene expression in the embryos. Second, TTK may be a dual function protein that can both activate and repress transcription depending upon its interactions with other co-factors. To study this function, one approach would be to isolate cofactors, and assay their cooperative activation or repression functions with TTK. However, it is necessary to first repeat the same transcription assays carried out in yeast cells using *Drosophila* cell cultures, and ask whether or not the activation is an artifact in yeast cells which does not happen in flies.

5. Two potential FTZ cofactors were isolated

As was expected, both FTZ-dependent and FTZ-independent clones were isolated using the Double Interaction Screen. Among the FTZ-dependent clones, three potential FTZ cofactors were identified. FIP1 proved to be FTZ-F1α, FIP2 encoded a novel protein, and FIP3 seemed to be a false positive.

FTZ-F1α was isolated three times as a FTZ-independent activator (F1, F9, F74). Those clones survived at 25 mM 3-AT and their growth were not dependent upon the presence of FTZ expression plasmid. These isolates all contained truncated forms of FTZ-F1α cDNA. FTZ-F1α was also isolated as a FTZ-dependent clone (C11), because its growth was dependent upon the presence of FTZ expression plasmid. C11 in the presence of FTZ grew better in 25 mM 3-AT than C11 in the absence of FTZ. In addition, C11 in the presence of FTZ grew in up to 50 mM 3-AT while C11 in the absence of FTZ barely survived in 50 mM 3-AT. The C11 isolate contained a full length of FTZ-F1α cDNA.

Three possible mechanisms could account for the survival difference between C11 in the presence and absence of FTZ: 1) FTZ or FTZ-F1α binds to its own DNA site without physical protein-protein interaction, causing an additive effect; 2) a direct interaction between FTZ and FTZ-F1α is involved, causing a synergistic effect; 3) a combination of 1) and 2). To distinguish between these possibilities, a series of in vitro and in vivo experiments were carried out to elucidate the nature of the functional interactions between FTZ and FTZ-F1α (discussed below).

FIP2 is a novel FTZ cofactor. Extensive studies were carried out to reveal the molecular properties of the gene and its biology functions (discussed below).

Partial sequence of FIP3 gene done by a rotation student, H. Xu, revealed that it is a *Drosophila* homolog of human acylCo-A dehydrogenase gene. The human AcylCo-A dehydrogenase gene product is a mitochondrial flavoenzyme that catalyzes the initial step in fatty acid β-oxidation (Kelly et al., 1987). Therefore the *Drosophila* homolog of human AcylCo-A dehydrogenase is likely not a nuclear protein. FTZ, however, is expressed exclusively in the nucleus, as its role is in regulating transcription. Thus, FIP3 cannot be a cofactor for FTZ since they are expressed in different cell compartments. The reason FIP3 was isolated as a FTZ-dependent clone from the Double Interaction Screen is not clear. One possible explanation is that the nuclear localization signal in pACT artificially directs cytoplasmic proteins into the nucleus. AcylCo-A dehydrogenase is an enzyme which may bind (or stick) nonspecifically to proteins. Thus, when acylCo-A dehydrogenase was mistakenly expressed together with FTZ, it interacted with FTZ nonspecifically and resulted in a higher level of reporter gene expression.

6. FTZ-F1α not only interacts with the *ftz* upstream element but also interacts with FTZ protein

FTZ-F1α was isolated as a FTZ-dependent clone suggesting a cooperation between FTZ and FTZ-F1α. For the first time,

we revealed molecular evidence of such protein-protein interaction. We showed that FTZ and FTZ-F1α proteins are present in the same protein complex in embryo nuclear extract (Fig. 21), and that FTZ and FTZ-F1α cooperatively interact with a target DNA (Fig. 23, 25). Very recently, a female sterile mutation in which FTZ-F1α expression was impaired has been generated (A. Guichet et al., Abstract 185B, 37th Annual *Drosophila* Research Conference, San Diego, California). Such mutations exhibited a *ftz*⁻ cuticle phenotype in that the even-numbered segments were deleted, while *ftz* expression was not obviously affected. Embryos derived from homozygous mutant females lacked *engrailed* expression in even-numbered parasegments, suggesting FTZ was not functioning normally to regulate even-numbered *engrailed* stripes. These genetic data, together with our biochemical results, demonstrate that FTZ-F1α and FTZ act in parallel to regulate the proper development of even-numbered parasegments. The fact that *ftz* expression was not altered in FTZ-F1α mutant embryos suggested that other upstream regulators of *ftz* could replace FTZ-F1α to activate transcription.

FTZ-F1 homologs have been isolated in the mouse and other species (Ueda and Hirose, 1991; Ellinger-Ziegelbauer et al., 1994). One mouse homolog is the embryonal long terminal repeat-binding protein (ELP). It binds and suppresses transcription mediated by the virus long terminal repeat (Tsukiyama et al., 1992). Mouse steroidogenic factor 1 (SF-1) is a key regulator of steroid hydroxylase gene expression,

both ELP and SF-1 arise from the same gene by alternative promoter usage and splicing (Ikeda et al., 1993). SF-1 null animals die by postnatal day 8, and lack adrenal glands and gonads, suggesting that SF-1 is essential for sexual differentiation and for formation of the primary steroidogenic tissues (Luo et al., 1994). However, the molecular mechanisms by which mouse FTZ-F1 functioning is not entirely clear. Studies of *Drosophila* FTZ-F1 will likely to provide information about mouse FTZ-F1 as they might be functionally conserved. Since a FTZ homolog has never been identified in mouse, it is possible that mouse FTZ-F1α can cooperatively interact with Hox gene products and specify their regulatory functions.

7. One demonstration of a cooperation between a nuclear hormone receptor and a homeodomain protein

The homeodomain proteins display a high degree of functional diversity, while having weak DNA-binding specificity on their own (Desplan et al., 1988; Gehring et al., 1994; Hoey and Levine, 1988; Krumlauf, 1994). To account for their functional specificity in vivo, it has been suggested that differential interactions with cofactors enhance the DNA-binding and/or regulatory specificity of homeodomain proteins (Krumlauf, 1994; Lawrence and Morata, 1994). In the last few years, an increasing number of cofactors have been identified, including Exd/PBX, SRF, and Swi5p. *Drosophila* Exd cooperatively interacts with Ubx and

Abd-A (Chan et al., 1994; Van Dijk and Murre, 1994). A human homeodomain protein which is closely related to *Drosophila* Paired protein called Phox1 interacts directly with serum response factor (SRF) and enhances the DNA-binding activity of SRF to the serum response element (Grueneberg et al., 1992). The cooperative DNA-binding of a yeast zinc finger protein Swi5 and a homeodomain protein Pho2p to the HO promoter is required for full transcription of the yeast *HO* gene (Brazas et al., 1995), which encodes a protein initiating mating type switching in yeast (Kostriken et al., 1983).

Interestingly all the cofactors identified so far are DNA-binding proteins, including FTZ-F1α which belongs to the nuclear hormone receptor superfamily of transcriptional regulators. This suggests that a protein-DNA interaction is more stable and specific when proteins bind DNA as a dimers. When proteins bind DNA as a dimers, three levels of interaction (one level of protein-protein and two levels of protein-DNA interactions) are involved, it is therefore, more stable than a single leveled protein-DNA interaction. Further, the binding specificity increases when proteins bind DNA as a dimers, since only DNA sequences containing binding sites for both proteins are targets.

The nuclear hormone receptors are a group of transcriptional regulators activated upon hormone-binding (Mangelsdorf and Evans, 1995). Dimerization of receptors plays a major role during the activation. Either homo- or

hetero-dimerization of the receptors is required for proper DNA-binding (Mangelsdorf and Evans, 1995; Tsai and O'Malley, 1994; Umesono and Evans, 1989). Homodimers usually bind to inverted repeats sites, and heterodimers often bind to direct repeats sites. In contrast, most of the orphan receptors are thought to bind DNA as monomers because their binding sites are configured as nonrepeats. FTZ-F1α is an orphan receptor and binds to DNA as a monomer (Kastner et al., 1995). However, FTZ-F1α also can act as a heterodimer since it forms a complex with a homeodomain protein, FTZ. Because FTZ is not a nuclear hormone receptor, the binding site for FTZ/FTZ-F1α dimer shall not be configured as a repeated sequence.

Several corepressors and coactivators of nuclear receptors have been identified recently: corepressors including SMRT (for silencing mediator for RARs and TRs) and N-CoR (for nuclear receptor corepressor) (Chen and Evans, 1995; Horlein et al., 1995) that interact with unliganded receptors and keep them in inactive forms. Coactivators including Trip1 (for thyroid-hormone-receptor interacting protein) and TIF1 (for transcriptional mediators or intermediary factors) (Lee et al., 1995; vom Baur et al., 1995; Le Douarin et al, 1995) interact with several hormone receptors and induce their transcriptional activation functions. In addition, a functional interference between octamer transcription factor OTF-1 (Oct 1) and glucocorticoid receptor (GR) has been described (Kutoh et al., 1992). Such interference was mediated by a direct interaction between

OTF-1 and GR leading to the inhibition of OTF-1-dependent DNA-binding. The homeodomain of OTF-1 was involved in this interaction. OTF-1/GR interaction was the only example of functional interference between a homeodomain protein and a nuclear hormone receptor, until FTZ-F1α was shown to be a partner of FTZ. We demonstrated a cooperation between a homeodomain protein (FTZ) and nuclear hormone receptor (FTZ-F1α). Since FTZ-F1α is probably activated by an signal molecule, we hypothesize that some functions of FTZ are regulated by such signals and that makes FTZ different from other homeodomain proteins in terms of functional regulation.

8. Dual regulation of FTZ-F1α for FTZ

Gene transcription is generally controlled by cis-regulatory elements and the availability of trans-regulators. For example, the 323 bp fPE contains one high-affinity FTZ homeodomain binding site, three medium-affinity FTZ homeodomain binding site, and one low-affinity FTZ homeodomain binding sites (Pick et al., 1990). Similar combinations of different affinity binding sites are likely to be present in the enhancers of FTZ target genes as well. These different affinity FTZ binding sites together with the right combination of co-factor binding sites, may be a key issue to specify divergent functions of FTZ protein in regulating gene expression.

In the 323 bp fPE, the high-affinity binding site which is not close to a FTZ-F1α binding site interacts strongly

with FTZ protein in vitro (Fig. 27). A medium-affinity binding site, for instance, next to a FTZ-F1α binding site does not interact with FTZ protein alone, but it interactes with FTZ/FTZ-F1α dimer (Fig. 23). We hypothesize that FTZ has at least two types of target DNA elements: 1) simple target DNA (e.g. the high-affinity binding site) that can be activated by FTZ alone, and 2) compound target DNA (e.g. o-F1F site) that can only be activated by FTZ together with its cofactor (e.g. FTZ-F1α). The different types of target DNA elements in combination with their cognate binding proteins may account for the complexity of FTZ function.

Besides being a co-factor of FTZ, FTZ-F1α on its own is a transcriptional activator (Ueda et al., 1990), as it was isolated as a FTZ-independent clone. There are several other FTZ-F1α binding sites in the 323 bp fPE which do not overlap with any FTZ binding sites. These single FTZ-F1α binding sites interact with FTZ-F1α protein specifically (Han et al., 1993), and mediate transcriptional activation in yeast cells (W. Han and L. Pick unpublished). Therefore, FTZ-F1 protein may have dual functions in terms of regulating target genes that have no FTZ-binding sites, and cooperating with FTZ to regulate FTZ target gene expression.

A possible model to explain such dual function of FTZ-F1α is summarized in Figure 36. In the situation (Fig. 36A) in which a single medium-affinity FTZ binding site is present, FTZ is unable to bind this site effectively, even when FTZ-F1α is present. Thus the expression of reporter

gene is not activated. In the situation (Fig. 36B) in which a single FTZ-F1α binding site is present, FTZ-F1α binds to this site resulting in an activation of the reporter gene expression (+). The presence of FTZ protein will not change the level of activation, since there is no FTZ binding site available. In situations where both binding sites are present, but only FTZ-F1α protein is available (Fig. 36C), the binding of FTZ-F1α to its target DNA results in the same level of activation (+). However, when both proteins are available (Fig. 36D), through cooperative interaction between the two proteins, higher levels of activation are achieved (++). The high level (++) of activation may be required for turning on FTZ downstream target genes, while the low level (+) of activation may just be enough for turning on FTZ-F1α downstream target genes. Thus, we conclude that FTZ-F1α regulates FTZ at two levels: it activates *ftz* transcription and it modulates FTZ activity.

9. FIP2 is a novel FTZ-interacting protein which may selectively interact with some homeotic gene products

A second potential FTZ interacting protein was also isolated. The cDNA shares no significant homology to any gene in the GenBank. Protein sequence deduced from FIP2 cDNA shows no similarity to any protein in the database, except it contains several putative helix turn helix motifs. In *Drosophila* embryos, *FIP2* mRNA is expressed uniformly at a very early stage of development (Fig. 31), but is not

expressed in the somatic cells where *ftz* is expressed during cellular blastoderm. Later, *FIP2* mRNA is restricted to the endoderm precursor cells where *ftz* is not expressed. Thus, it seems that *ftz* and *FIP2* mRNAs are not co-localized in the embryos. However, it is still possible that the two transcripts are co-expressed in the beginning of cellular blastoderm, as *ftz* mRNA is expressed uniformly at low levels during that time (Yu and Pick 1995; Dearolf et al., 1990). Also, the FIP2 protein expression pattern may differ from mRNA pattern. FIP2 protein could turn out to be expressed in the somatic cells and overlap with FTZ protein expression. Double staining embryos with anti-FTZ and anti-FIP2 antibodies will definitively reveal the pattern of FIP2 expression and its co-localization with FTZ.

Our preliminary data suggests that FIP2 has the potential to interact with some homeotic gene products. In our yeast assays, FIP2 interacted strongly with Lab, FTZ, and Antp, weakly with Ubx, and Abd-A, but not with Dfd, Scr, and Abd-B (Fig. 34). Consistent with the possible interaction between FIP2 and Lab, *lab* is expressed in the endoderm precursor cells (Diederich et al., 1989) which overlaps with FIP2 expression pattern. At present, we do not know the molecular basis of these interactions. We do know that the homeodomain is not sufficient to mediate the interactions between homeotic gene products and FIP2, as FTZ homeodomain alone did not interact with FIP2 allowing cell growth at 25 mM 3-AT (data not shown).

However, we cannot exclude the possibility that FIP2 may be a non-specific interacting partner of FTZ, as currently we do not have biochemical data to reveal an in vitro interaction between FTZ and FIP2, nor do we have genetic evidence to suggest the biological function of such interaction. Therefore, future studies such as those described for FTZ-F1α are required to demonstrate whether or not FIP2 is a real cofactor of FTZ, and what is the molecular mechanism underlying the FTZ/FIP2 interaction.

CHAPTER 5. FIGURES AND LEGENDS

Table 1. Analysis of positive clones

18 positives including 15 FTZ-independent (F) and 3 FTZ-dependent (C) were isolated. Results of 3-AT selection and β-galactosidase units, and orientations of cDNAs inserted in the pACT vector are summarized in the table.

Positive Clones	Orientation in pACT	*HIS3* Selection	β-gal units*
F1	Forward	Positive	108
F2	Reverse	Positive	4.2
F9	Reverse	Positive	4.1
F14	Reverse	Positive	4.1
F18	Forward	Positive	24
F19	Forward	Positive	30
F51	Reverse	Positive	4.0
F53	Forward	Positive	20
F55	Forward	Positive	13
F56	Forward	Positive	28
F59	Forward	Positive	32
F70	Forward	Positive	40
F74	Forward	Positve	102
F78	Reverse	Positive	4.2
F90	Forward	Positive	25
C11	Reverse	Positive	4.3
Cd	Reverse	Positive	4.2
Cs	Reverse	Positive	4.5

F: FTZ-independent clones
C: FTZ-dependent clones
*: Control levels=4.3

Table 2. Results of screen for FTZ-independent clones

15 FTZ-independent clones were analyzed and results are summarized in the table.

Positive Clones	Sequence	Function
F1, F9, F74	*ftz-F1α*	DNA-binding & activation of transcription
F18, F19, F51, F56, F59, F90	*ftz-F1β*	DNA-binding
F53	*ttk*	DNA-binding & repression or activation* of transcription
F55	*slp1*	DNA-binding & repression of transcription
F2	novel	ND
F14	novel	ND
F70	novel	ND
F78	novel	ND

* see results (Fig. 18) and discussions

ND not determined

Figure 1. *ftz* mRNA pattern is revealed by in situ hybridization

The expression of *ftz* mRNA was analyzed by in situ hybridization using Digoxigenin labeled DNA probe. Photographs of stained whole mount embryos at different stages of development from early cellular blastoderm to late gastrulation are shown in panels **(A)** through **(L)**. Seven *ftz* stripes arose individually in a non-linear order along the anterior-posterior axis of the embryos. *ftz* mRNA expression began as an overall pattern through out the embryos **(A)**, then emerged as stripes. The order of stripe appearance along the anterior-posterior axis was 1, 5, 2, 3, 6+7, and 4 **(A-G)**. At the peak level, *ftz* mRNA was expressed as seven strong zebra stripes **(H, I)**. The embryo in **(I)** is tilted to reveal the invagination of the ventral furrow. *ftz* expression persisted through gastrulation **(J)** and then decayed. Stripe seven was the last one to decay **(K)**. Later, *ftz* mRNA reappeared in neuron precursor cells **(L)**. Embryos are positioned anterior left and dorsal side up, except **(I)** which is a ventral view of an embryo.

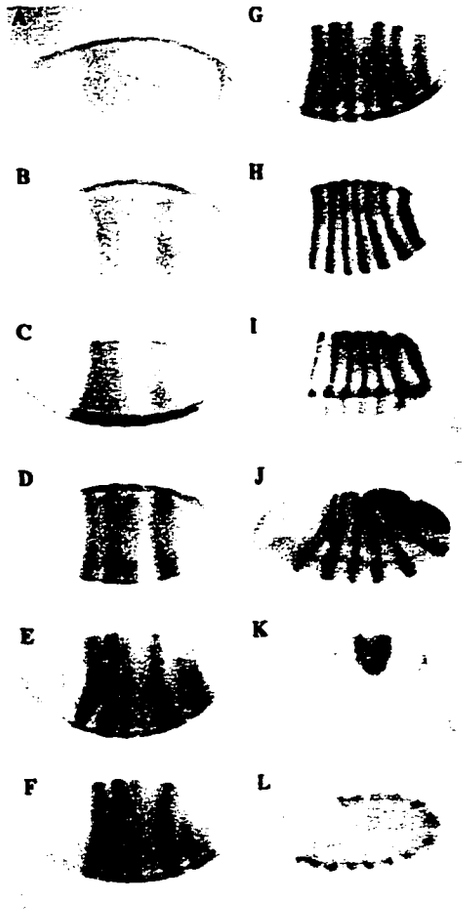


Figure 2. The 323 bp *ftz* proximal enhancer (323 bp fPE) element

The ~6 kb 5' regulatory region of the *ftz* gene containing three cis-elements: the zebra element, the neurogenic element, and the upstream element. The upstream element was divided into the distal and proximal enhancers, and the proximal enhancer was subdivided into prox A and prox B. A 323 bp *ftz* proximal enhancer (fPE) was derived from the proximal enhancer, which contains part of prox A and prox B. The *ftz* upstream element is from position 1 - 2574. The proximal enhancer is from position 1502 - 2574. The 323 bp fPE is from position 2167 - 2490.

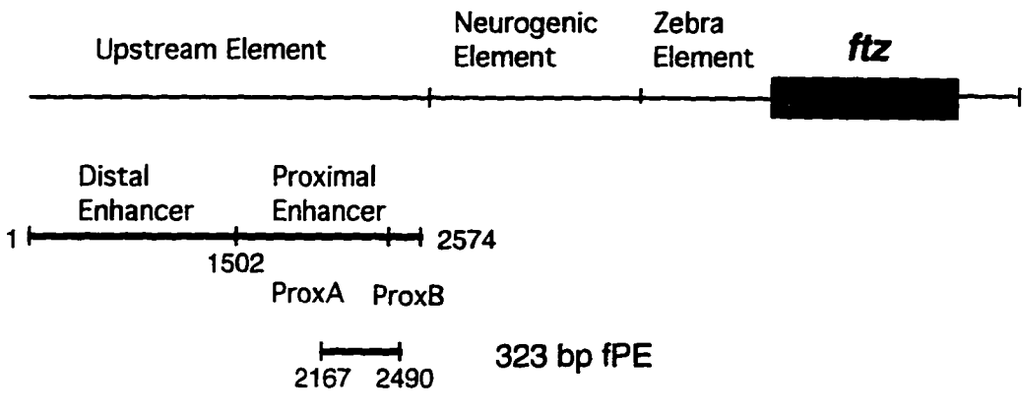


Figure 3. The expression of the 323 bp fPE-*LacZ* fusion gene

Photographs of embryos stained with anti-β-galactosidase antibodies are shown. Embryos are positioned anterior left and dorsal side up. **(A)** Transgenic embryo carrying the *ftz* upstream element/lacZ fusion gene (Hiromi et al., 1987); **(B)** Transgenic embryo carrying the 323 bp fPE/lacZ fusion gene. Both embryos expressed the *lacZ* fusion genes as seven stripes, however, stripes in **(A)** are stronger than stripes in **(B)**. This suggested that the 323 bp fPE contains all the information for positioning stripe expression, but does not contain all the information to generate high levels of expression.

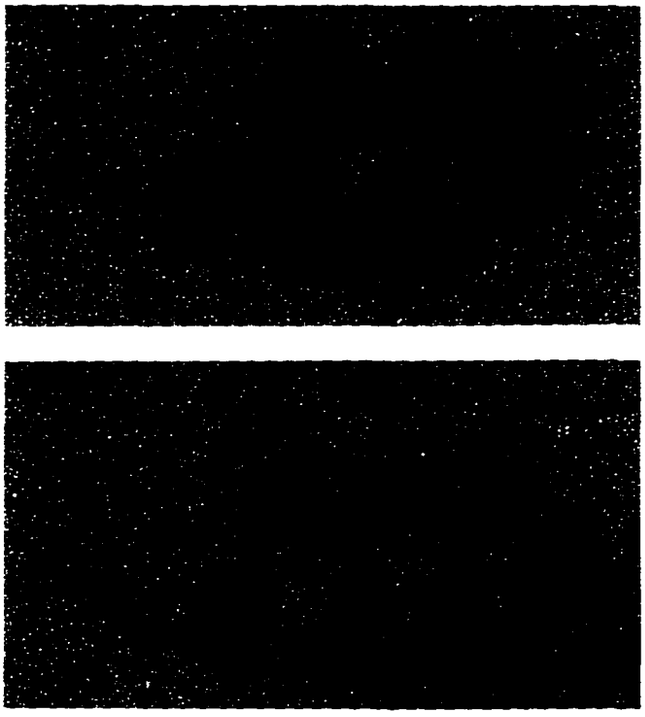
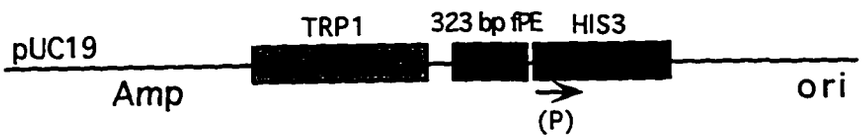


Figure 4. Maps of two reporters, 323-HIS3 and 323-lacZ

Schematic maps of reporter genes used in the yeast Double Interaction Screen. **(A)** The 323-HIS3 reporter construct. Three DNA fragments containing: the HIS3 coding region and its basal promoter, the 323 bp fPE, and the TRP1 gene, were sequentially cloned into the polylinker of pUC19 vector as described in methods. The 323 bp fPE was cloned immediately adjacent to the *HIS3* promoter. (Amp) ampicillin resistance gene, (ori) *E. coli* replication origin, and (P) the *HIS3* promoter. **(B)** The 323-lac-Z reporter plasmid. This plasmid was modified from pLG669ΔXhoI as described in methods: a URA3 marker was replaced by a TRP1 marker, the 2μ sequence was deleted in order to create a integration vector, the 323 bp fPE was cloned immediately adjacent to the *CYC1* promoter (P). Drawings are not to scale.

323HIS3



323lacZ

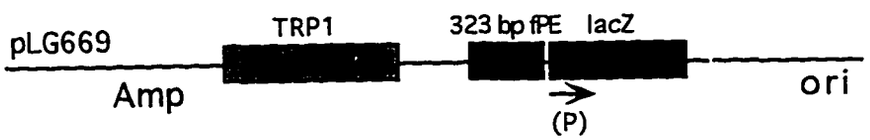


Figure 5. The FTZ expression vector

The plasmid backbone of the *ftz* expression plasmid is pYCp50. A BamHI fragment containing the ADH promoter and terminator from pADANS was inserted into pYCp50 to create a expression vector carrying a URA3 marker. *ftz* cDNA was cloned into the NotI site between the ADH promoter (including ATG) and terminator. (ori) *E. coli* replication origin, (ARS1) yeast replication origin, (CEN4) centramere sequences, (URA3) a yeast selection marker.

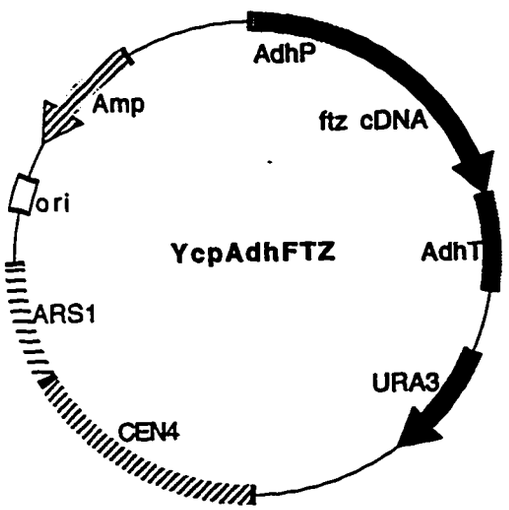


Figure 6. The His3 selection of the Double Interaction Screen

In the His3 selection, the reporter strain (w/323His3) was made by chromosomal integration of 323-HIS3 into the genomic TRP1 locus. **(A)** Reporter cells grew on a SD-his plate containing 1 mM 3-AT. **(B)** FTZ protein made reporter cells grow a little better on SD-plates containing 1 mM 3-AT, but not at any higher concentrations of 3-AT. **(C)** Because wild-type cells can grow in 60 mM 3-AT, selection in the presence of a cDNA library can be carried out in the presence of 25 mM 3-AT.

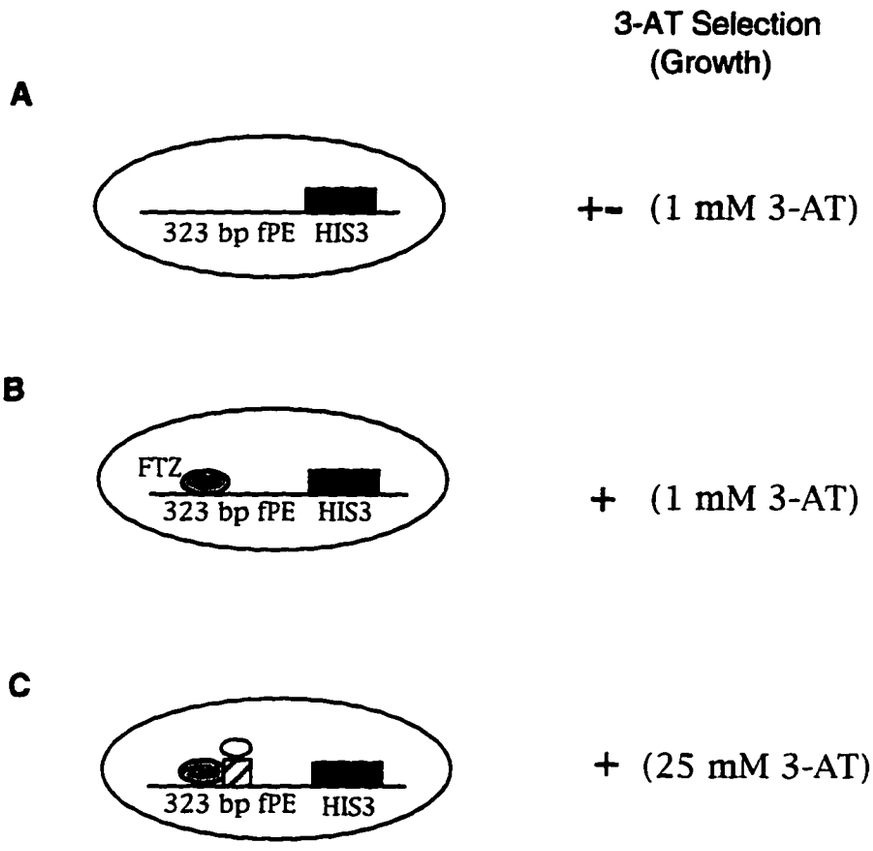
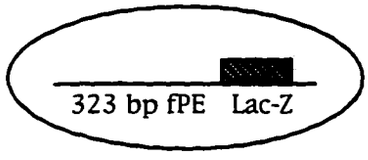


Figure 7. Positives were re-assayed for β-galactosidase activity

The level of *lac-Z* expression was determined by measuring the enzyme activity of β-galactosidase. **(A)** Reporter strain w/323LacZ gave ~5 units of β-galactosidase activity. **(B)** After expressing FTZ, w/323LacZFTZ still gave ~5 unit of β-gal activity. **(C)** Positive clones should have higher levels of β-galactosidase activity.

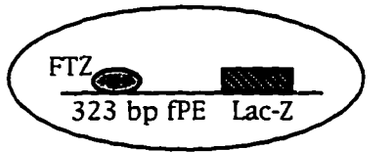
β-galactosidase activity

A



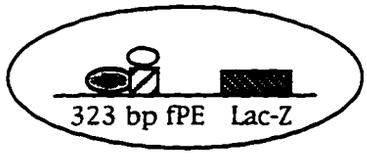
~5 units

B



~5 units

C



>5 units

 cDNAs tagged with GAL4 activation domain

Figure 8. Southern blot of reporter integrations

The proper genomic integration of reporter genes was confirmed by Southern blot analysis as described in Methods. DNAs were digested with StuI. Lane 1, DNA from w/323lacZ cells; lane 2, DNA from w/323His3 cells; lane 3, DNA from w/NP6His3 cells; lane 4, DNA from w3031A cells. No signal was detected in the control lane. Bands of approximately 9, 5, and 5 kb were detected for the 323-lacZ, 323-His3, and NP6-His3 reporter genes, demonstrating unique integration sites for each reporter. The hybridized band in each lane is indicated by an arrow. The blot was probed by DIG-labeled *lacZ* DNA probes. Probe DNA (1.1 kb) contains the first third of the coding sequence of the *lacZ* gene. A lighter band in lane 2 is probably a result of partial digestion of yeast genomic DNA.

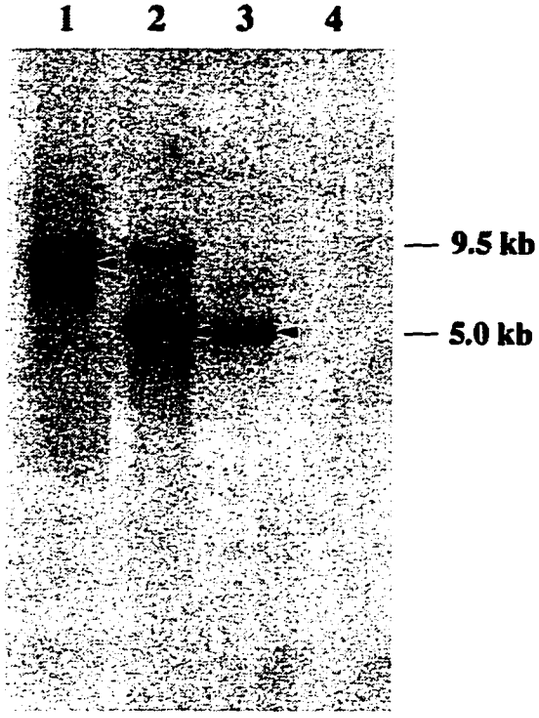


Figure 9. Growth of the positive clone (F2) and w/323His3 cells

Growth of one positive clone (F2) is compared to that of the w/323his3 reporter and wild-type $His3^+$ cells. The position of the three strains is shown schematically on the top panel. Growth on an SD-his plate is shown on the left, growth on an SD-his plate containing 25 mM 3-AT is shown on the right. w/323his3 cells grew on SD-his, but not on SD-his with 25mM 3-AT. A positive clone isolated in the screen, F2, and wild-type HIS3 cells grew on SD-his plate as well as SD-his plate containing 25 mM 3-AT.

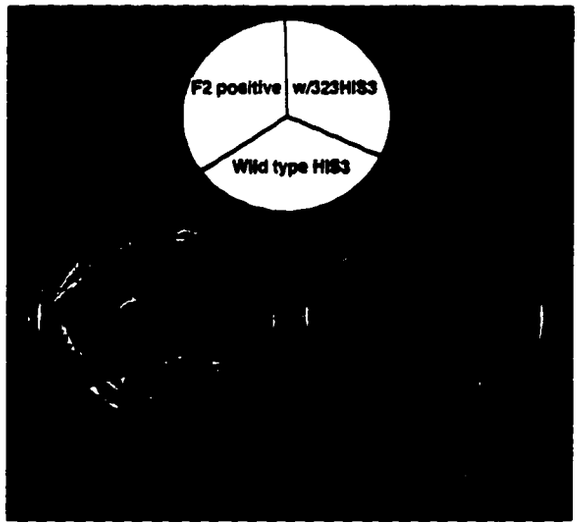
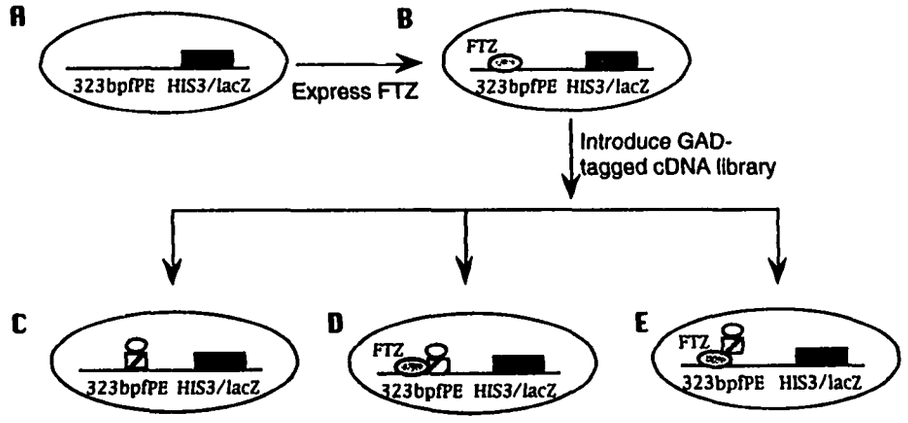


Figure 10. General scheme of the Double Interaction Screen

The scheme of the Double Interaction Screen is illustrated. **(A, B)** w/323His3 and w/323His3FTZ reporter cells grew on SD-plates containing 1 mM 3-AT. w/323lacZ and w/323lacZFTZ reporter cells produced ~5 units of β-galactosidase activity. **(C)** DNA-binding proteins were isolated by screening a Gal4 activation domain tagged *Drosophila* cDNA library on SD-plates containing 25 mM 3-AT. β-galactosidase activity levels were higher than 5 units for most of these positives. **(D, E)** FTZ-interacting proteins were isolated in the same screen. Their transcriptional activities were dependent upon the presence of FTZ protein. In **(D)**, co-factors interact with DNA and with FTZ protein; in **(E)**, co-factors interact with FTZ protein but not with DNA.



	His3 Selection (Growth in 3-AT)	***lacZ* expression** (β-gal activity)
B	+(1mM 3-AT)	<5 units
C, D, E	+(25mM 3-AT)	>5 units

cDNAs tagged with GAL4 activation domain (GAD)

Figure 11. FTZ activated transcription strongly via six copies of a synthetic binding sites

The ability of FTZ to activate transcription via the 323 bp fPE and via a synthetic binding site was compared. **(A)** w/NP6His3 cells transformed with the FTZ expression vector grew readily on SD-his plates containing 25 mM 3-AT plates, while cells without the FTZ plasmid did not. **(B)** w/323His3 cells transformed with the FTZ expression vector grew on SD-his plates but not in the presence of 25 mM 3-AT.

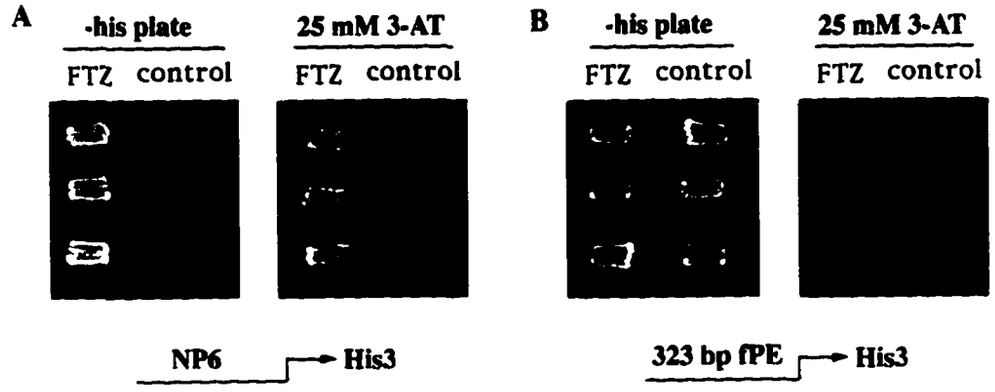


Figure 12. Three types of factors can be isolated in the Double Interaction Screen

Different types of transcription factors can be isolated using the Double Interaction Screen. **(A)** The cis-element of interest (e.g. the 323 bp fPE) gives either no or very low levels of background expression. **(B)** Factors that interact with the 323 bp fPE increase the expression of the reporter gene. For isolating cofactors (e.g. FTZ-cofactors), a *ftz* expression plasmid is introduced into the reporter cells. By screening a library, two types of cofactor can be isolated: **(C)** Factors don't bind DNA themselves, but interact with FTZ protein, **(D)** factors bind to DNA, and their binding sites are near FTZ binding sites. In all cases, the resulting yeast cells will have increased levels of reporter gene expression.

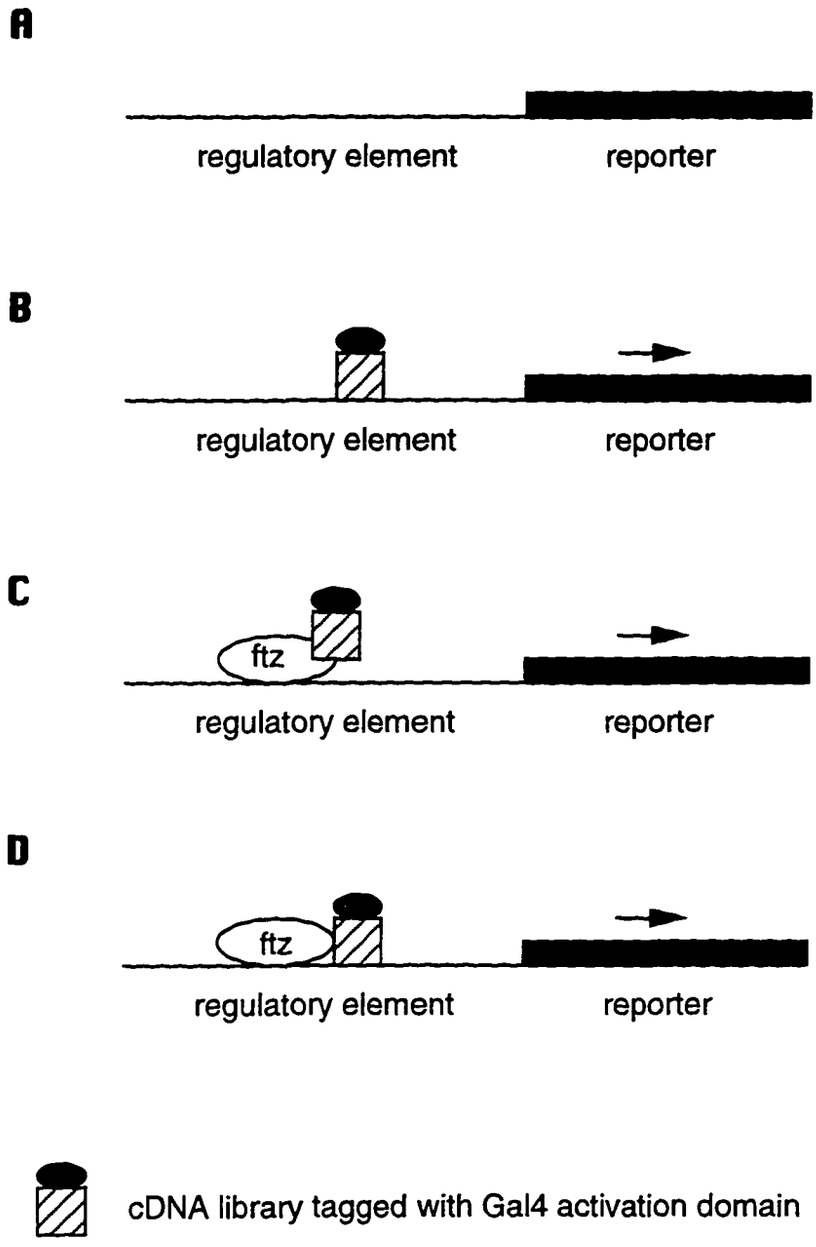


Figure 13. Flow chart for λACT library construction

Steps used to construct a Gal4 activation domain tagged *Drosophila* embryonic cDNA library are outlined (for details, see methods).

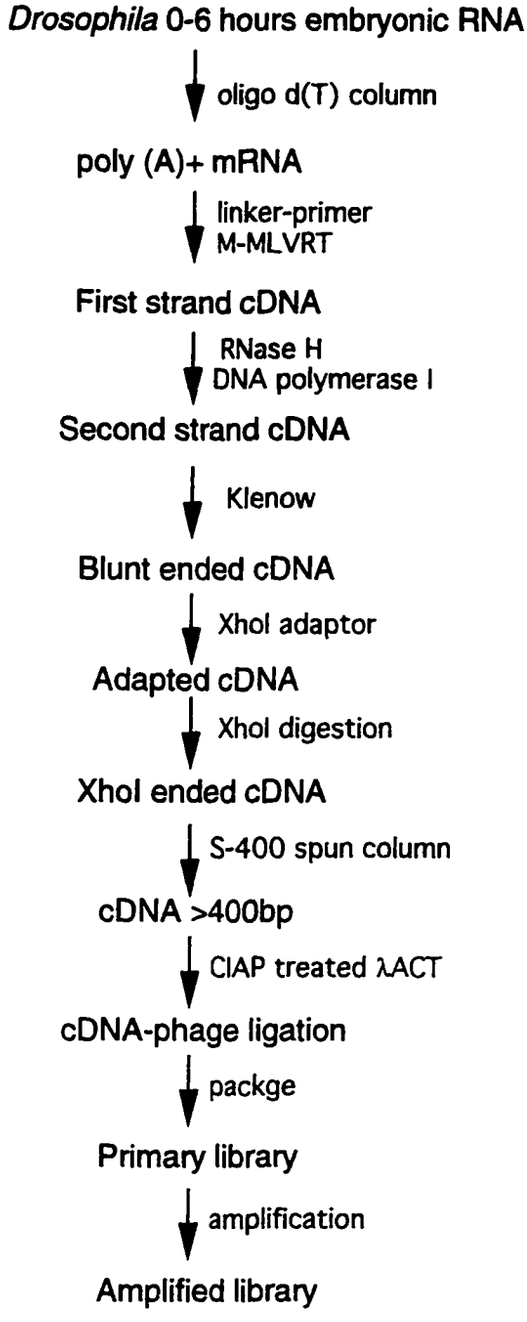


Figure 14. Screening data

$5X10^6$ library transformants were screened with His3 selection. About 100 colonies survived on SD-his, leu, ura plates containing 25 mM 3-AT. Among the 67 clones analyzed so far, the growth of 18 positives was dependent upon the introduced library plasmids, and 49 were not. Of these 18 "real" positives, the growth of 15 positives was independent of FTZ expression while 3 were dependent on FTZ expression. Thus, 15 candidate DNA-binding proteins and 3 potential FTZ interacting proteins were isolated from the Double Interaction Screen.

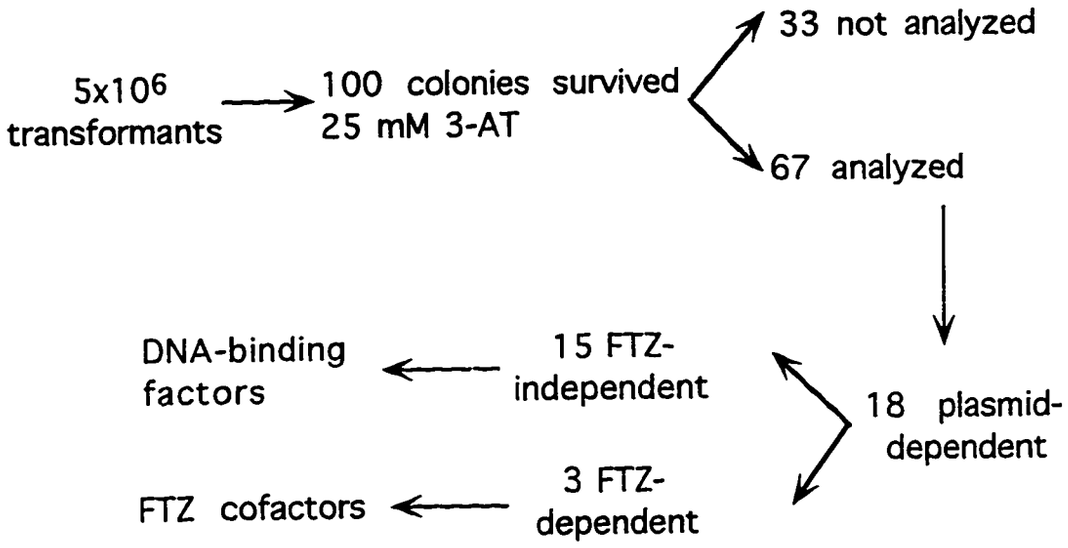


Figure 15. Slp1 protein binds to a novel site in the 323 bp fPE.

The 323 bp fPE was ^{32}P labeled and used for DNaseI footprinting. An autoradiograph of a 6% sequencing gel is shown in panel **(A)**. Lane 1, 8 µg of *E.coli* cell extract without Slp1 protein; lanes 2, 3, and 4, 5 µg, 7 µg, and 9 µg of *E.coli* cell extract containing Slp1 protein; lane 5, no protein. Sequence of the fragment was determined by a Maxam-Gilbert sequencing G+A reaction (not shown). The sequence of the protected region is indicated. **(B)** An oligonucleotide corresponding to the footprinted site (Slp1-site) was synthesized, and used for gel retardation assays. Lane 1, 5 µg of protein extract containing Slp1 protein; lanes 2, 3, and 4, 5 µg of Slp1 protein extract and 1 µl of preimmuserum, 1 µl of anti-Slp1 antibody, and 1 µl of an unrelated antibody, respectively. Arrows from bottom to top indicate respectively the free probe, the shifted bands, and a supershifted band appearing in every lane containing antiserum. **(C)** lane 1 and 2, labeled Slp1-site incubated with control protein extract and protein extract containing Slp1; in lanes 3-6, 1 ng, 10 ng, 50 ng, and 100 ng of unlabeled Slp1-site were included in the binding reaction; in lanes 7-10, 1 ng, 10 ng, 50 ng, and 100 ng of an unlabeled FTZ binding site (within the 323 bp fPE, for sequence, see results) were included in the binding reaction; in lane 11-14, 1 ng, 10 ng, 50 ng, and 100 ng of an unlabeled FTZ-F1 binding site (within the 323 bp fPE, for sequence, see results) were included in the binding reaction.

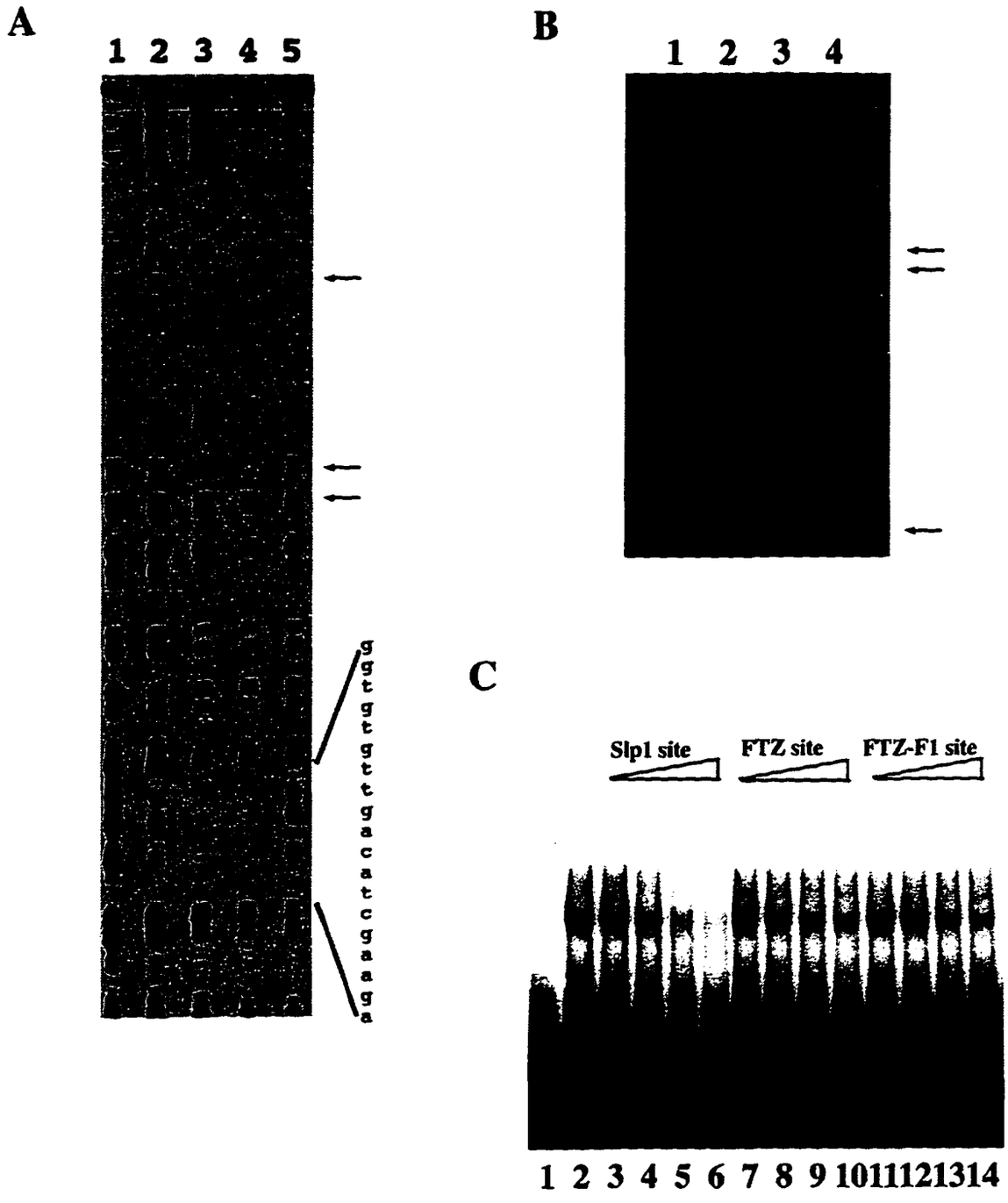


Figure 16. Slp1 represses transcription in yeast cells.

Liquid β-galactosidase assays were carried out to determine the levels of transcription regulated by forms of Slp1 proteins in different reporter cells. **(A)** w/3231acZ cells transformed with Slp1/GAD or Slp1 expression plasmids, or no plasmid as a control for basal level expression. Slp1/GAD activated, while Slp1 repressed *lacZ* gene expression. A yeast vector (pNG22) expressing the *lac-Z* gene under the *CYC1* promoter, and a derivative of this construct (pNG224X) containing four copies of Slp1-binding site were used as reporter genes. **(B)** Slp1 failed to repress transcription mediated by the *CYC1* promoter in the pNG22 plasmid. In contrast, Slp1 was able to repress transcription mediated by four copies of the Slp1-binding site in the pNG224X plasmid. For each construct, at least five individual transformants were assayed twice. Vertical axis indicates the mean values of β-galactosidase units. Error bars represent standard deviations from 10-12 measurements. β-galactosidase values for extracts from the transformants of Slp1/GAD, Slp1, and no plasmid were 13.5 ± 1.4, 1.63 ± 0.34, and 4.32 ± 0.50. β-galactosidase values for extracts from the transformants of Slp1-pNG22, empty vector-pNG22, Slp1-pNG224X, and empty vector-pNG224X were 17.1 ± 0.65, 16.8 ± 1.2, 1.4 ± 0.22, and 5.7 ± 0.8.

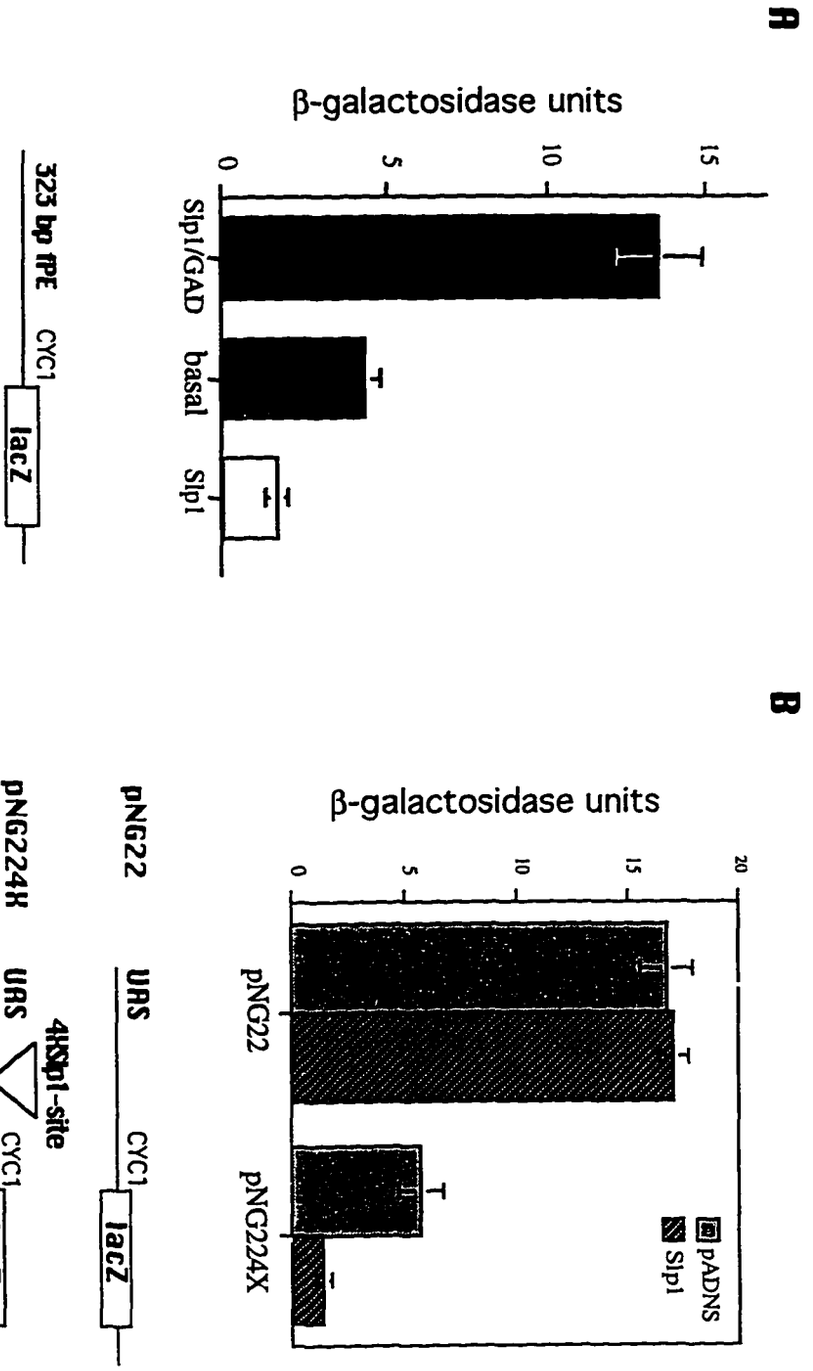


Figure 17. Slp1 represses *ftz* gene expression in vivo via the 323 bp fPE.

The expression of a 323 bp *ftz* proximal enhancer-*lacZ* fusion gene, P(323-*lacZ*), was monitored at the germ band extension stage with anti-β-galactosidase antibodies. **(A)** Seven *ftz*-like stripes were seen in wild type embryos carrying P[323-*lacZ*]. **(B)**, **(C)**, and **(D)** Extra stripes were seen in *slp* mutant (Δ34B) embryos carrying P[323-*lacZ*]. The intensities and numbers of extra stripes varied from embryo to embryo, but the extra stripe between *ftz/lacZ* stripes 3 and 4 was always observed. The extra stripes are indicated by arrows. Embryos are oriented as anterior left and dorsal up, except, **(B)**, which is a ventral view of the embryo.

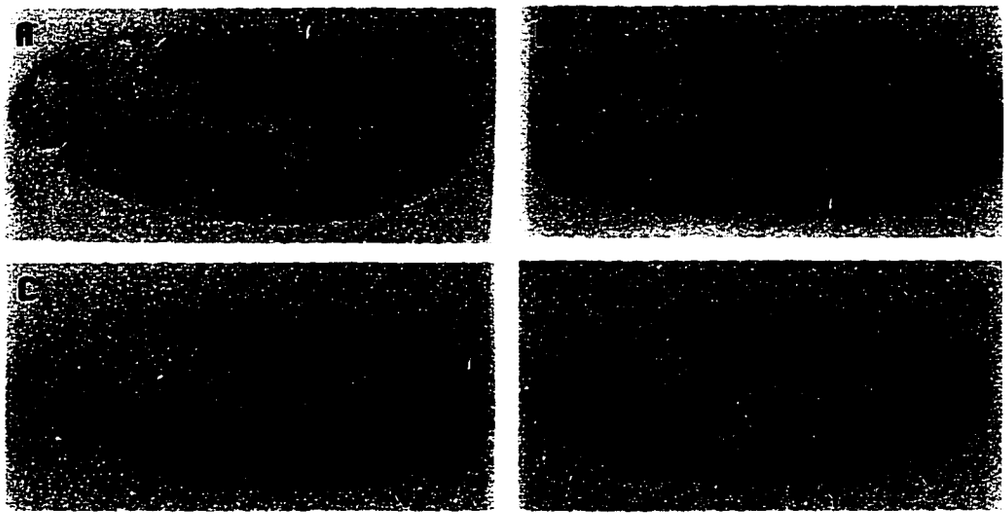


Figure 18. TTK activates transcription via the 323 bp fPE in yeast cells

w/3231acZ cells were transformed with TTK/GAD or TTK expression plasmids, or no plasmid as a control for basal level expression. Liquid β-galactosidase assays were carried out to determine the level of expression. For each construct, at least five individual transformants were assayed twice. Vertical axis indicates the mean values of β-galactosidase units. Error bars represent standard deviations from 10-12 measurements. β-galactosidase values for extracts from the transformants of TTK/GAD, TTK, and no plasmid were 19.75 ± 0.71, 20.3 ± 0.45, and 4.24 ± 0.27.

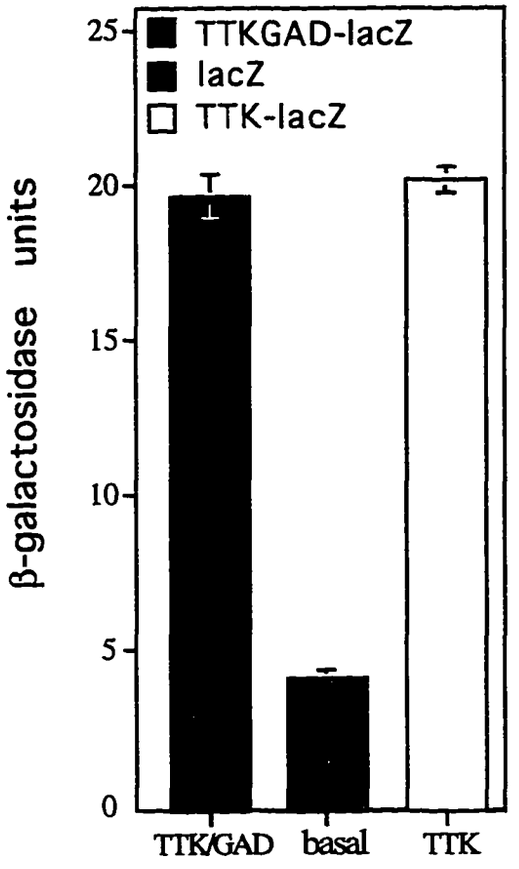


Figure 19. SDS-PAGE of partially purified FTZ protein for making antibody

FTZ protein was induced and partially purified from *E. coli* as described in the methods. Washed protein pellet was mixed with 3x SDS-PAGE sample buffer (Maniatis, 1989) and loaded on the 10% SDS-polyacrylamide gel. After electrophoresis, the gel was stained with 0.1% Coomassie-Blue (R-250). Lane 1, ~200 µg of protein extracts from uninduced cells; lane 2, 3, and 4 are the protein extracts from induced cells; the amount of protein loaded is 20 µg, 35 µg, and 50 µg, respectively; Lane 5, standard low range protein markers (Bio Rad), the size of the markers is indicated. The band corresponding to full length FTZ protein is indicated by an arrow on the left. Another major band at the lower position is probably a degradation product of FTZ protein, since it is not present in the control.

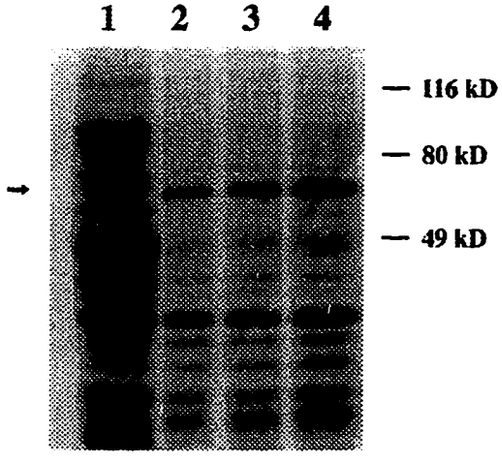


Figure 20. FTZ and FTZ-F1α are co-expressed in the embryo

0-10 hour Oregon R embryos were stained with anti-FTZ-F1α antibody **(A, B, C)** and anti-FTZ antibody **(D, E)**. Cellular blastoderm stage embryos are shown in **(A)** and **(D)**. Germ-band extention stage embryos are shown in **(B)** and **(E)**. **(C)** is an unfertilized embryo stained with anti-FTZ-F1α antibody. Embryos are oriented as anterior left and dorsal sides up.

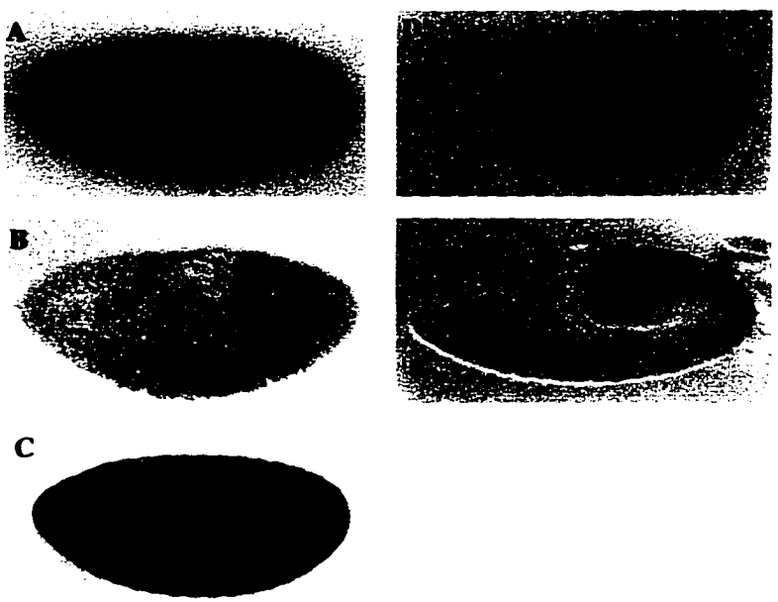


Figure 21. FTZ and FTZ-F1α are co-immunoprecipitated from embryo nuclear extract

0-10 hour *Drosophila* embryo nuclear extract was used for the co-immunoprecipitation experiment. Briefly, embryo nuclear extract was precleared with Protein-A beads coated with preimmuserum, and incubated with Protein-A beads coated with anti-FTZ antibody. The beads were collected and proteins were analyzed on a 10% SDS-polyacylamide gel. The gel was transferred to a nitrocellulose membrane which was blotted with Anti-FTZ-F1α antibody. Lane 1, nuclear extract precipitated with purified anti-FTZ antibody, and lane 2, nuclear extract precipitated with preimmuserum. The FTZ-F1α band is indicated by an arrow. The other four bands that are present in both lanes are nonspecific. Molecular weights are marked according to the low range protein markers from Bio-Rad.

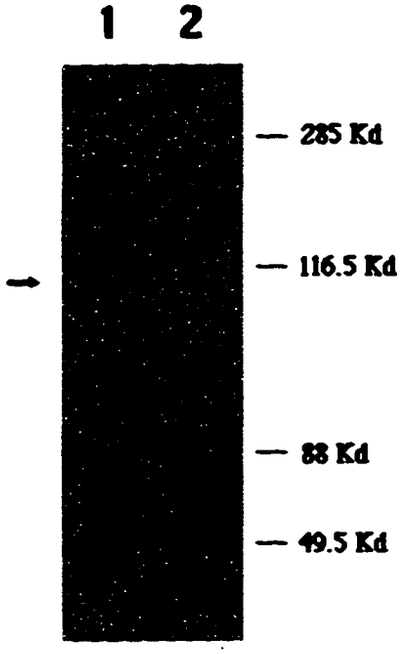


Figure 22. A 33 bp sequence contains a FTZ-F1α binding site adjacent to a FTZ binding site

Sequence of the 323 bp fPE is shown here. Five FTZ homeodomain binding sites are highlighted in black background. Three FTZ-F1α binding sites are highlighted in gray background. The overlapping FTZ-F1α and FTZ binding sites is underlined. This site (o-F1F) was used to test the cooperative interactions of the two proteins in gel retardation assays.

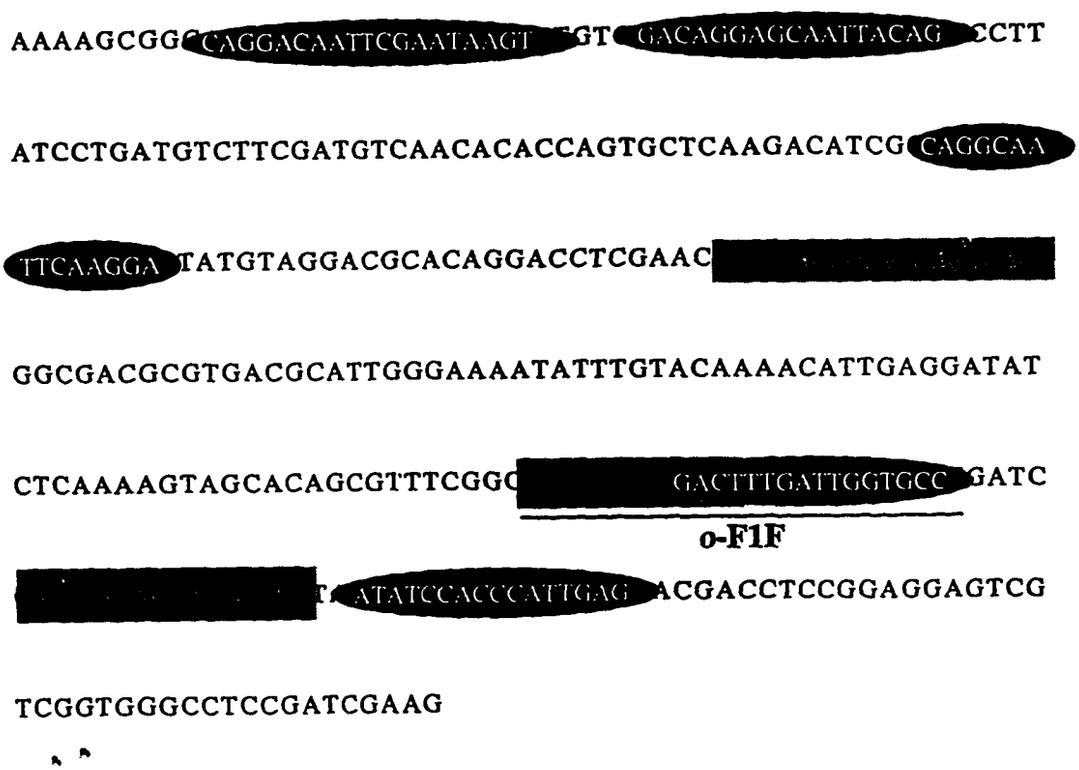


Figure 23. FTZ-F1α facilitates the binding of FTZ to its target DNA

(A) The binding of FTZ to DNA in the absence and presence of FTZ-F1α was compared. Cell extracts expressing different proteins were used in the binding reactions. 3 μg control protein (lane 1), 3 μg FTZ-F1α protein (lane 2), 3 μg FTZ protein (lane 3), both FTZ and FTZ-F1α proteins (lanes 4, 5, 6, 7, and 8), were used in the reactions. Anti-FTZ-F1α antibody (lane 5) but not preimmuserum (lane 6) supershifted both protein-DNA complexes. Binding was inhibited by specific competitor (50 fold molar excess of o-F1F) (lane 7) but not nonspecific competitor (50 fold molar excess of Slp1-site) (lane 8). **(B)** Anti-FTZ antibody abolished the formation of the triple complex. 3 μg of FTZ and FTZ-F1α were included in binding reactions for lanes 1-5. To deplete extracts of FTZ protein (lane 2), they were incubated with anti-FTZ antibody coated beads, and immunoprecipitated as described in methods. The upper complex was selectively lost demonstrating that it contains FTZ protein. Incubation with preimmuserum coated beads (lane 3), and uncoated beads (lane 4) had no effect on complex formation.

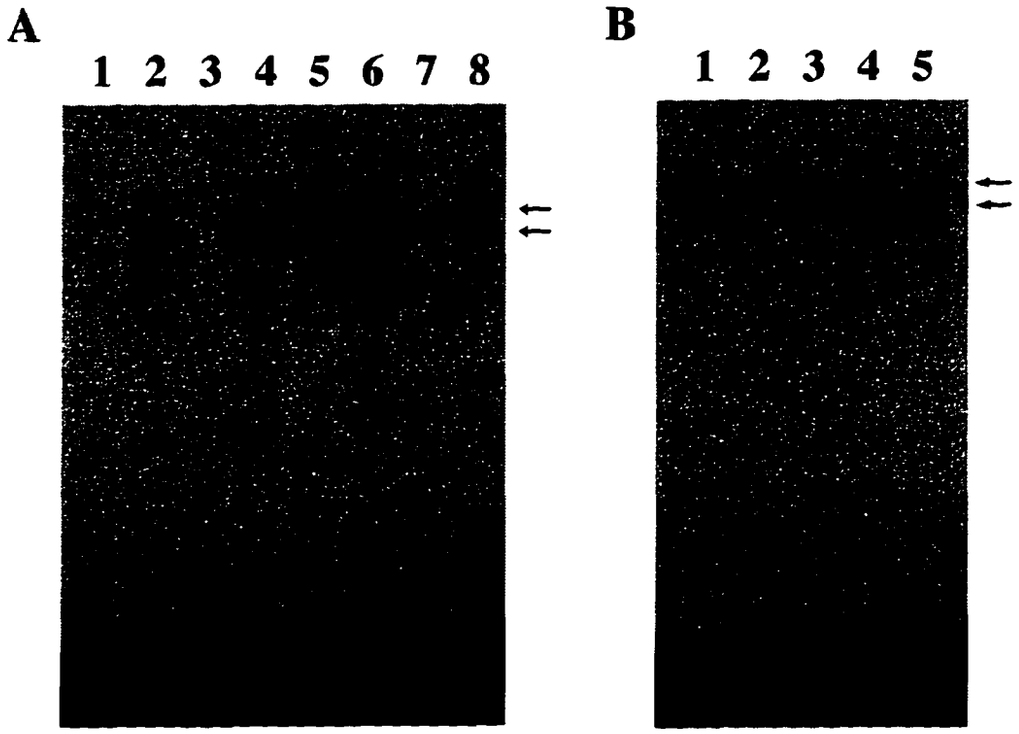


Figure 24. The triple interaction requires both FTZ binding and FTZ-F1α binding sites

Gel retardation assays using oligonucleotides carrying point mutations in the FTZ-F1α binding site (lanes 1-3) or FTZ binding site (lanes 4-6) were carried out. Mutation of the FTZ-F1α binding site abolished formation of the FTZ-F1α/DNA complex (lane 1) and of the FTZ/FTZ-F1α/DNA complex (lane 3). Mutation of the FTZ binding site had no effect on FTZ-F1α binding alone (lane 4) but abolished formation of the triple complex (lane 6). FTZ alone did not bind to wild type (see Fig. 23) or either mutant oligonucleotide (lanes 2 and 5). Binding of FTZ-F1α and the triple FTZ/FTZ-F1α/DNA complex with wild type oligonucleotides is shown in lane 7. 3 μg of either FTZ or FTZ-F1a protein extract was used in all binding reactions.

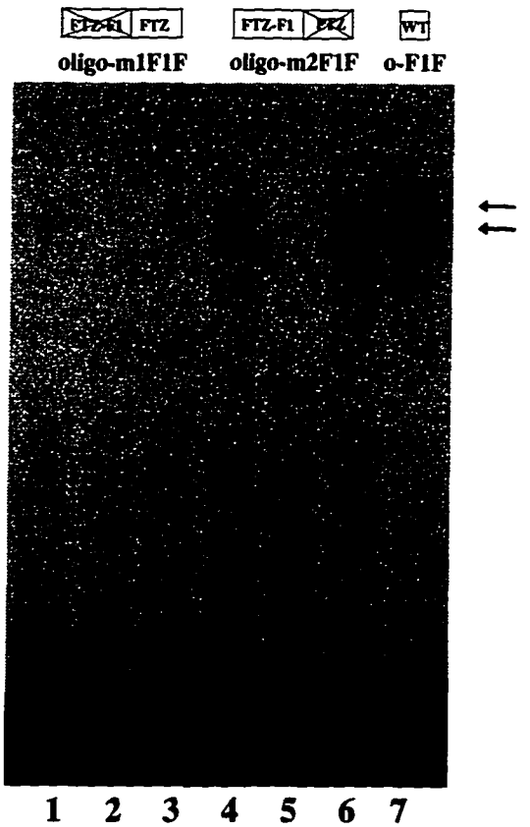


Figure 25. FTZ and FTZ-F1α bind to o-F1F DNA cooperatively

In gel retardation assays using wild type oligonucleotides (o-F1F), **(A)** the FTZ-F1α/DNA complex was competed off by the triple FTZ/FTZ-F1α/DNA complex at high concentrations of FTZ protein extract. In the presence of 3 μg FTZ-F1α protein extract, 1.5 μg FTZ protein extract resulted a strong FTZ-F1α/DNA complex and a weak ternary complex (lane 1); 7 μg FTZ protein extract almost abolished the FTZ-F1α/DNA complex (lane 2); 30 μg or 75 μg FTZ protein extract totally abolished the FTZ-F1α/DNA complex and produced a stronger triple complex (lane 3 or 4). 75 μg FTZ protein extract alone did not result any specific binding (lane 5). **(B)** At concentrations at which either FTZ or FTZ-F1α protein alone were unable to bind DNA, the two together could form a triple DNA-binding complex. 0.3 μg FTZ-F1α protein extract failed to bind the oligonucleotide (lane 1) but formed a triple FTZ/FTZ-F1α/DNA complex in the presence of 75 μg FTZ protein extract (lane 4). 0.75 μg or 1.5 μg FTZ-F1α protein extract interacted with the oligonucleotide as a FTZ-F1α/DNA complex in the absence of FTZ (lane 2 or 3), but formed a much stronger triple complex in the presence of 75 μg FTZ protein extract (lane 5 or 6).

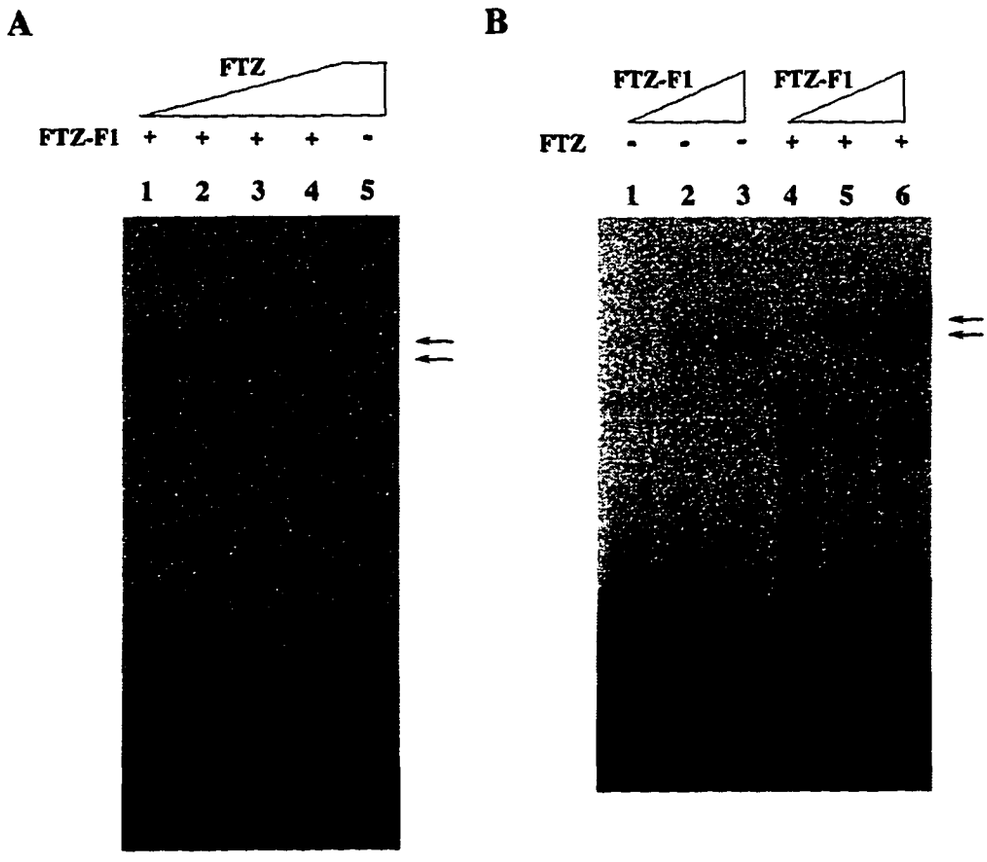
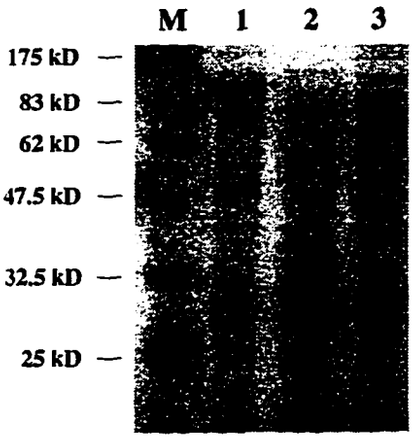


Figure 26. SDS-PAGE of FTZ and FTZ-F1α protein extracts used for the gel retardation assays

Protein extracts containing FTZ or FTZ-F1α protein were analyzed on an SDS-polyacrylamide gel stained with 0.1% Coomassie-Blue. **(A)** FTZ-F1α was expressed in *E.coli* as full length protein (lane 1, as indicated by an arrow); full length FTZ protein was not clearly detected by Coomassie staining at the position around 55kD (lane 2); control protein extract (lane 3). 15 μg protein was loaded in each lane. **(B)** Western blot detected a full length FTZ protein band at expected size (~ 55kD) in extracts expressing FTZ (lane 1, as indicated by an arrow), but not in the control extracts (lane 2). 7.5 μg protein was loaded in each lane. Molecular weights are indicated by protein markers (New England BioLabs).



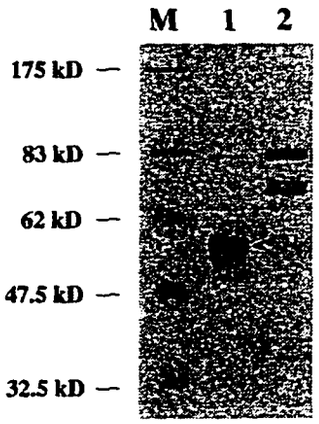


Figure 27. FTZ binds more strongly to its high-affinity site than to its medium-affinity site

The binding of FTZ to a high-affinity homeodomain binding site and a medium-affinity homeodomain binding site was compared. o-2, a synthetic oligonucleotides carrying an in vivo FTZ homeodomain-binding site, was used as a high-affinity binding site (lanes 1-4). o-F1F was used as a medium-affinity binding site(lanes 5-8). Gel retardation assays were carried out as described in Figure 23. Binding of FTZ to its high affinity site was readily detected using 6 µg of extract (lane 2). The broad complex formed was "supershifted" by anti-FTZ antibody (lane 3) but not preimmuserum (lane 4), demonstrating that FTZ is present in the complex. Five times more protein (lane 5, 30 µg) only resulted in non-specific binding also seen with control extract (data not shown). Anti-FTZ antibody (lane 6) and preimmuserum (lane 7) did not affect the non-specific binding suggesting that FTZ is not in this complex. Twenty five times more protein (lane 8, 150 µg) did not bind to o-F1F demonstrating that the binding of FTZ to its high affinity site is at least 25 times stronger than to its medium affinity site.

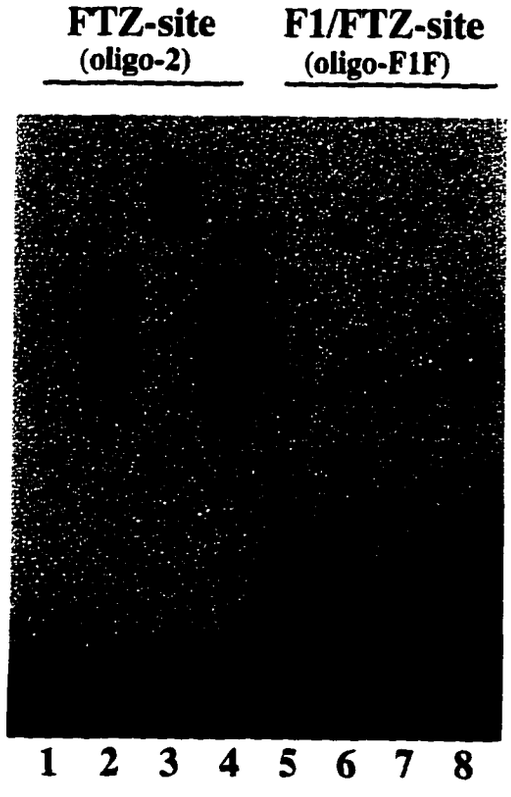
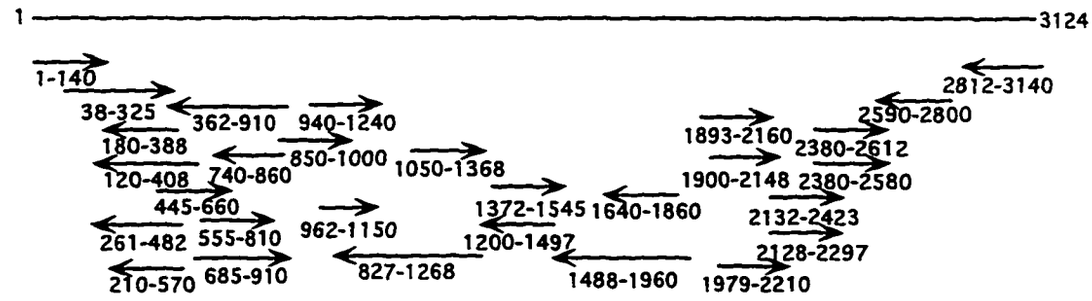


Figure 28. Sequencing of the *FIP2* gene

FIP2 cDNA was sequenced using ExoIII/Mung Bean nuclease system. A total sequence of 3124 bp was obtained. **(A)** End points for each deletion fragment that was sequenced are indicated. Fragments sequenced from 5'-3' are shown by arrows to the right; Fragments sequenced from 3'-5' are shown by arrows to the left. **(B)** Most regions were confirmed by sequencing from both strands (shown as double lines). Regions between position 120-326, 440-582, 740-1370. 1380-1550, and 1990-2550 were sequenced from both strands. Drawings are not to scale.

A



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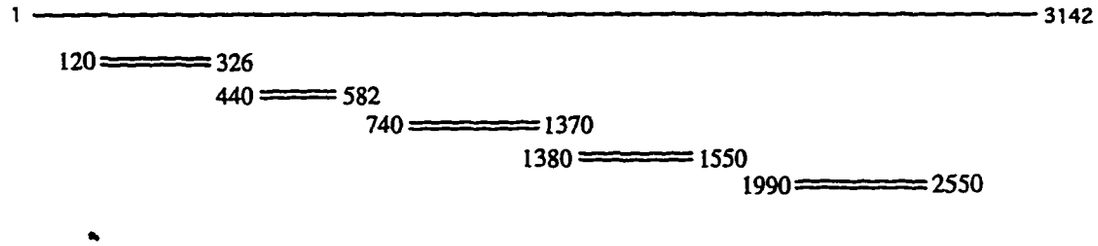

Figure 29. Partial cDNA sequence of the *FIP2* gene

Partial cDNA sequence of the FIP2 gene (~3124 bp) is shown. The first ATG start codon and a TAA stop codon are underlined. A poly (A) tail is at the end of cDNA. The cDNA encodes a putative polypeptide of ~70 KD (region shown as bold letters). There is still a fragment denoted as NNNNNNNNNN within the 3' untranslated region that needs to be sequenced.

GCCACGAAGGCCTCTAGATCGCGTCATAAACAATCCAAACTTTGTGACGATGAGCGGCTCGATATAAGCAGGG
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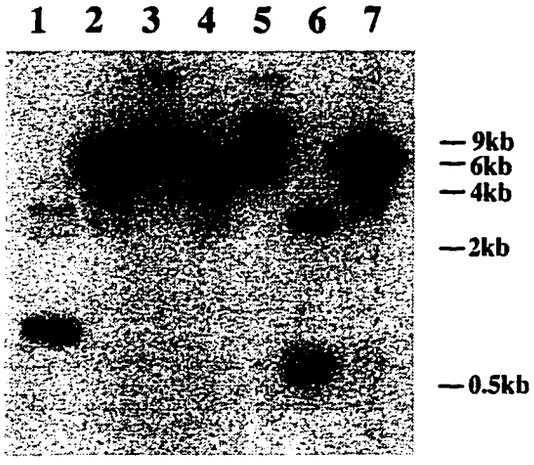
AAAAAAAAAAA

Figure 30 Southern and Northern analysis of the *FIP2* gene

(A) Southern blot of the *FIP2* gene. Genomic DNA isolated from *Drosophila* embryos was digested with BamHI (lane 1), EcoRI (lane 2), EcoRV (lane 3), HindIII (lane 4), NotI (lane 5), SacI (lane 6), and SalI (lane 7). DNA was separated through a 1% agarose gel and hybridized with a ^{32}P-labeled probe from 1156-2048 of the *FIP2* cDNA. An autoradiograph of the Southern blot is shown. One band was detected from each sample, except lane 6 in which two bands were detected, suggesting an internal SacI site within the *FIP2* gene. Thus, the *FIP2* appears to be a single copy gene in the *Drosophila* genome.

(B) Northern blot analysis. 3 μg of poly $(A)^+$ RNA isolated from 0-6 hour *Drosophila* embryos was run through a 1% agarose gel as described in methods. After transfer, the filter was hybridized with the FIP2 probe used in (A). One band was detected at about the same position as the 3638 bp size band of RNA markers (Promega).

A



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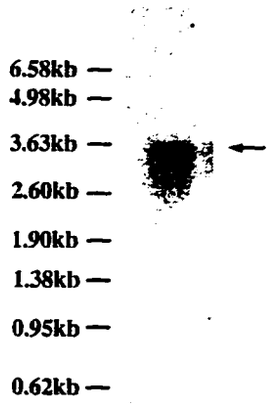


Figure 31. Expression pattern of the *FIP2* gene in the embryo

Digoxigenin labeled *FIP2* DNA probe was hybridized to 0-10 hour whole mount *Drosophila* embryos as described in the text. Photographs of the stained embryos are shown. Embryos at different stages of development are shown: **(A)** syncytial blastoderm, **(B)** cellular blastoderm, **(C, D, E)** germband extension, **(F)** germband retraction. All embryos are oriented anterior left and dorsal side up.

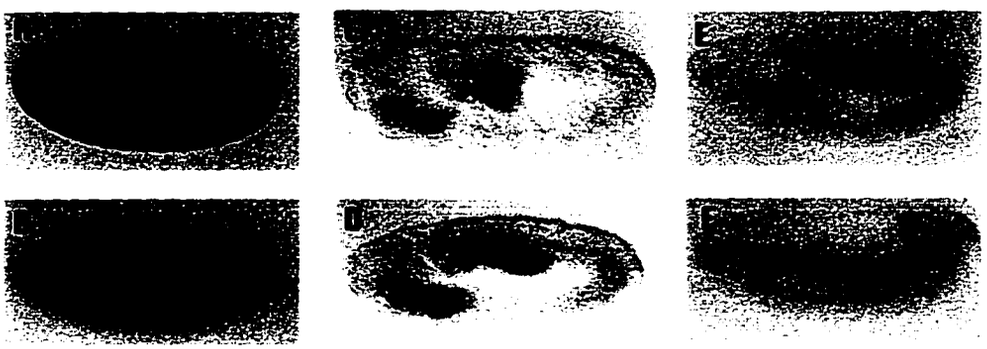


Figure 32. Expression of recombinant FIP2 protein

Recombinant FIP2 protein was expressed in a pET30b vector as a histidine- and S-tagged fusion protein as described in results. **(A)** Crude protein extracts were separated on a 10 % SDS-polyacrylamide gel. Lane 1, 5 µg of extract prepared from uninduced cells; lane 2 and 3, 3 µg and 7 µg of extract prepared from induced cells respectively; lane 4, protein markers. Three arrows indicate three forms of the fusion protein. The molecular weight of each marker is indicated. **(B)** An S-tagged Western blot described in results is shown. 2 µg of control extract was loaded in lane 1; 1 µg and 4 µg of FIP2 extract were loaded in lanes 2 and 3 respectively. Three arrows indicate three forms of the fusion protein. The molecular weight of each marker is indicated.

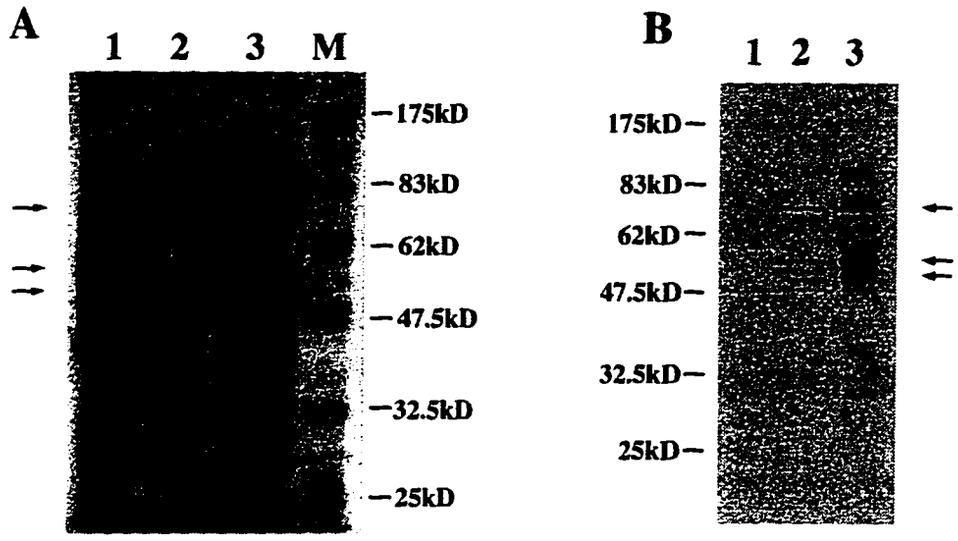


Figure 33. Purification of recombinant FIP2 protein

The purification of FIP2 protein with a Ni^{2+}-coupled sepharose column was monitored by a SDS-polyacrylamide gel. About 10 μg of total protein was loaded in each lane. Control protein was prepared from cells transformed with pET30b vector alone (lanes 1-3). The purification procedure is described in methods. For control extracts, fractions from the flow through (lane 1), wash (lane 2), and eluate (lane 3) did not contain bands of the correct size. FIP2 protein was purified from cells transformed with the pET30bFIP2 construct (lanes 4-7). Many bands were detected in the flow through fraction (lane 4), including the three fusion protein bands, probably because of column overloading; no fusion protein was detected in the wash (lane 5); two fractions of eluate (lanes 6, 7) contained three major bands corresponding the size of the fusion proteins (indicated by arrows). Protein markers (New England BioLabs) were loaded in lane M. Molecular weights are indicated.

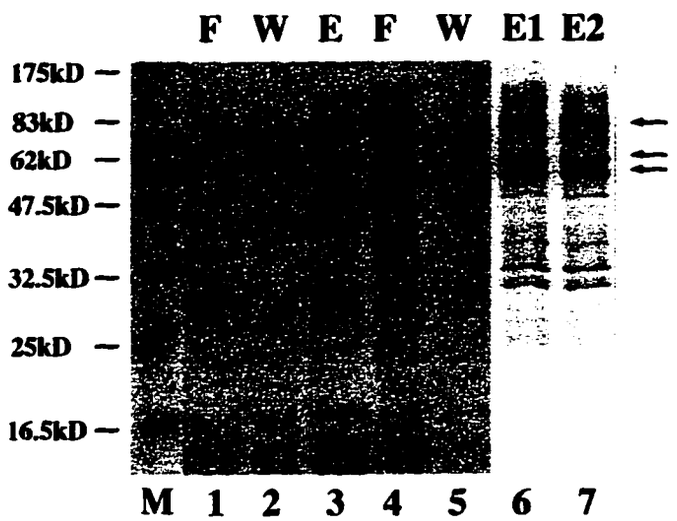


Figure 34. FIP2 selectively interacts with several homeodomain proteins

The ability of FIP2 to interact with other homeodomain proteins was tested in yeast cells carrying the 323 bp fPE-HIS3 reporter gene, and expressing different homeodomain proteins. The growth of cells in the presence of 3-AT with and without FIP2 was compared. Expression plasmids for the homeodomain proteins carried the URA3 markers. FIP2 expression plasmid carried the LEU2 marker. Growth was tested on SD-ura, his plates (for selecting plasmids expressing homeodomain proteins), or on SD-ura, leu, his plates (for selecting plasmids expressing homeodomain proteins and FIP2). Growth in the presence of 5 mM 3-AT was supported by Dfd, Ubx, and Abd-B (left panel); none of these cells grew in the presence of 25 mM 3-AT (middle panel). An interaction between FIP2 and Lab, FTZ, and Antp is indicated by growth in 25 mM 3-AT (right panel). Ubx and Abd-A appeared to interact more weakly with FIP2, since some growth was detected. Control cells, upper left corner in each panel, were transformed with empty expression vector.

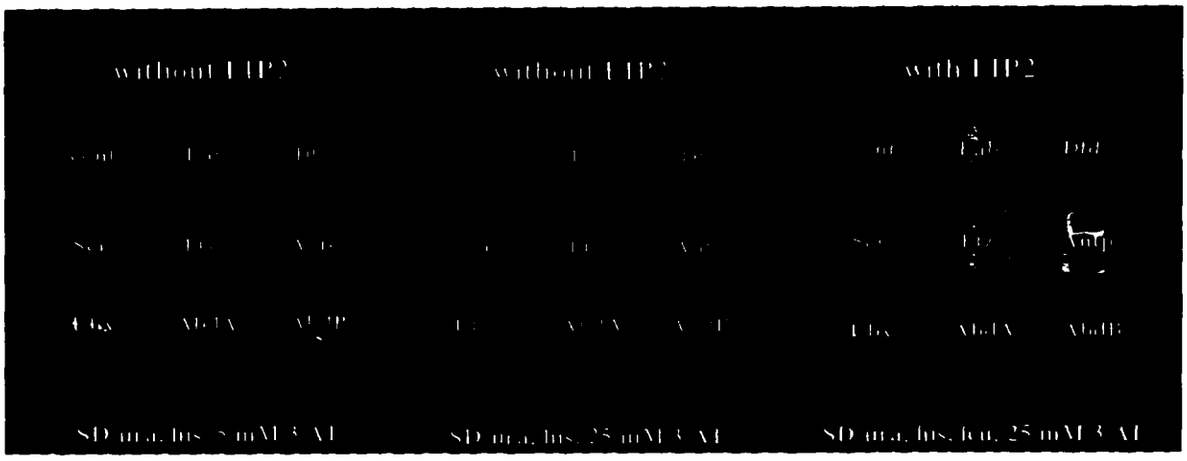


Figure 35. Homeotic genes are expressed in yeast cells

To confirm the proper expression of homeotic genes in yeast cells, expression plasmids for different homeotic genes were transformed into w/NP6His3 reporter cells. Each of the resulting transformant was able to grow on SD-plates containing 25 mM 3-AT, suggesting these genes were expressed in yeast cells and their gene products could activate transcription via the NP6 site. Control is w/NP6His3 cells transformed with an empty vector.

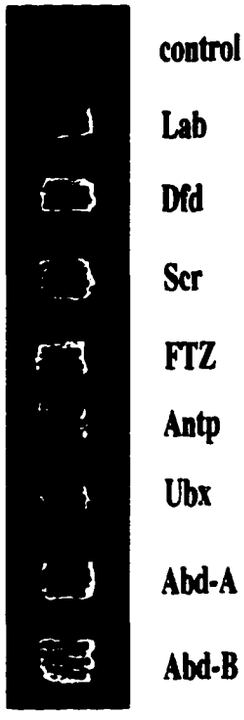
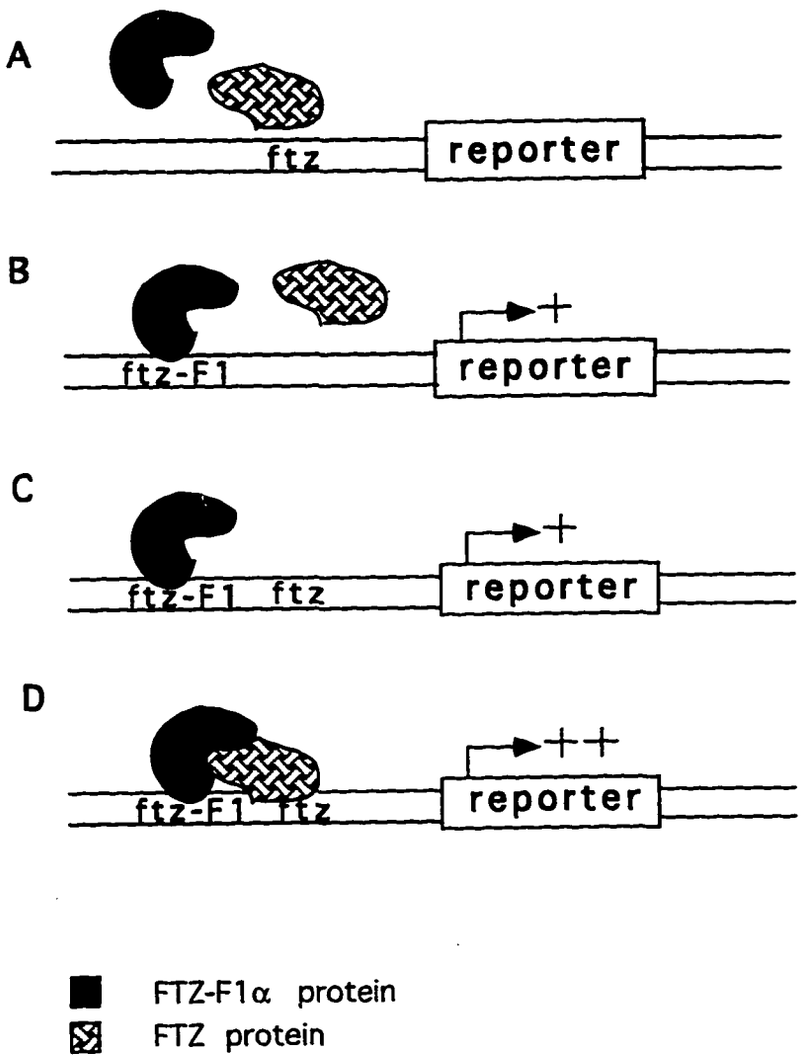


Figure 36. A model for FTZ and FTZ-F1αinteraction

We propose a model explaining the dual function of FTZ-F1α and its involvement in FTZ regulation. FTZ-F1α is capable of regulating the expression of the *ftz* gene and the activity of FTZ protein. The decision of which function it has is made by both the DNA target sequences and the availability of FTZ protein. Its cooperative interaction with FTZ happens only when both proteins can bind to a overlapping cognate sites **(D)** and not otherwise **(A, B, C)**. Binding sites for FTZ-F1α and FTZ are indicated.



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