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Potential *Drosophila melanogaster fushi tarazu (ftz)* regulators interacting with the *ftz* proximal enhancer.

by
Mary Frances Landrigan

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

Potential *Drosophila melanogaster fushi tarazu (ftz)* regulators interacting with the *ftz* proximal enhancer.

by

Mary Frances Landrigan

Advisor: Leslie Pick, Ph.D.

fushi tarazu (ftz) is a homeobox-containing gene of the pair-rule class. *ftz* is expressed in seven stripes in the cellular blastoderm, and later in the nervous system. Mutation of this gene is lethal, resulting in the loss of every other parasegment. Control of *ftz* expression is at the level of transcription. The 5' regulatory region of the gene has been subdivided into the zebra element, the neurogenic element, and the upstream element. The upstream element directs expression in stripes in the mesoderm and ectoderm in a distance- and orientation independent manner. The upstream element has been functionally partitioned into proximal and distal enhancer elements. The former directs reporter expression in seven mesodermal and ectodermal stripes. Nine protein binding sites have been identified in the proximal enhancer, and at least twelve separate proteins bind to these sites. Previously identified proteins which interact with these sites include Ftz protein, Ftz-F1, a Ftz cofactor, and Ttk, a putative *ftz* repressor. Two proteins which interact with site 2 are apparently unique to that site. Conditions which should allow the purification of these proteins were established but not fully implemented. A possible third protein which interacted with this site was identified. Adf-1 is another previously recognized protein which was found to interact with multiple sites in the proximal enhancer. The gene encoding this factor was mutated by P element insertion into its coding

region. Rescue by a P element containing the *adf-1* coding region and flanking sequence was successful. Mutants of *adf-1* fail to hatch, although the stage at which the mutation is lethal is not known. There is no effect of *adf-1* mutation on *ftz* expression. *ftz* stripes are expressed normally, and *ftz* nervous system expression is unaffected in *adf-1* mutants. *ftz-lac Z* reporter constructs are expressed normally. This suggests that the role of Adf-1 in the control of *ftz* is relatively minor.

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This thesis is dedicated to my grandfather, Thomas Magee, who died during its composition. I could not have asked for a more staunch supporter, and I only regret that he didn't live to celebrate its completion with me.

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Chapter 1. Introduction

A fundamental question in the study of development is how an elaborately formed multicellular organism develops from a fertilized egg. *Drosophila melanogaster* has long been studied as a paradigm, for invertebrates and vertebrates, of how the segmented pattern of the embryo is established. One of the genes involved in establishing the segmented body plan is *fushi tarazu (ftz)*. In this thesis I will describe work I have done to better understand some of the proteins which interact with the regulatory regions of *ftz*. The first experimental chapter describes work I did attempting to purify proteins binding to one particular binding site in the *ftz* proximal enhancer. The second concerns the production and characterization of a mutation in the *adf-1* gene, which encodes a protein that has been shown to bind to the proximal enhancer. All of these proteins are potential regulators of *ftz* transcription.

Following a discussion of the cascade of activation of the embryonic segmentation genes, I will review what is thus far known about the *ftz* cis-acting regulatory regions and the trans-acting factors which interact with these regions.

A. Genes Active in the Establishment of Embryonic Segments

Most of the genes active in specifying the body plan in *Drosophila* embryos were isolated in massive genetic screens (Gans et al., 1975; Nusslein-Volhard et al., 1987; Schupbach and Wieschaus, 1989; Schupbach and Wieschaus, 1986). Some screens produced female-sterile lines, among which were "maternal-effect" genes which when mutated produced females who laid eggs which died during development. Other screens identified recessive embryonic lethal mutations in zygotic genes which resulted in changes in the larval cuticle (Jurgens et al., 1984;

Nusslein-Volhard et al., 1984; Nusslein-Volhard, 1980; Read et al., 1990; Wieschaus et al., 1984). In the following pages I will discuss the basic outline of how each stage in the segmentation pathway is established and maintained, with particular emphasis on the effects on or by the gene *fushi tarazu*.

A. 1. Maternally Active Genes

The anterior-posterior and dorsal-ventral axes of the *Drosophila* embryo are established during oogenesis by maternal gene products acting on the developing egg. A limited number of embryonic phenotypes were observed in genetic screens to identify these genes (Gans et al., 1975; Nusslein-Volhard et al., 1987; Schupbach and Wieschaus, 1989; Schupbach and Wieschaus, 1986) (reviewed in St. Johnson (St. Johnston and Nusslein-Volhard, 1992)). In consequence, the genes involved in this process were assigned to one of four classes. As I will discuss below, the genes within each class have been shown to act in a common pathway.

Genes of the *anterior* class displayed a mutant phenotype which involved a reduction or loss of head and thorax structures. *Posterior* group mutations resulted in deletions of abdominal structures and, in some cases, of the precursors to the germ cells. The *terminal* class of mutations resulted in deletion of the acron and telson, the most anterior and posterior structures in the embryo. These three classes of maternal-effect genes determine the anterior-posterior pattern of the embryo (Nusslein-Volhard et al., 1987). The *dorsal-ventral* pattern is specified by a fourth group of genes. Mutations in this class of genes resulted in dorsalized or ventralized embryos (Anderson et al., 1985). Each class of genes relies on the asymmetric localization of a spatial signal. However, the method by

which this signal is localized varies greatly from system to system, illustrating the diverse ways in which the final action of a gene product can be regulated.

A. 1a. The Anterior Class of Maternal Effect Genes

Both the anterior and posterior groups of genes rely on the graded cytoplasmic distribution of a determining factor or factors. As I will discuss below, both groups rely on a transcriptional activator as well as the function of a translational repressor to ensure the proper spatial expression of their target genes. The first factor discovered for the anterior group was the product of the *bicoid (bcd)* gene. *bcd* RNA is synthesized in the nurse cells during oogenesis, and is localized in the anterior end of the embryo (Berleth et al., 1988; Frigerio et al., 1986). *bcd* RNA is translated following oogenesis to form an anterior-to-posterior protein gradient within the embryo (Berleth et al., 1988; Driever and Nusslein-Volhard, 1988; St. Johnson et al., 1989). That this concentration gradient is the key to determining the pattern of the anterior half of the embryo was shown by two lines of evidence. First, if the number of maternal copies of *bcd* was increased, more protein was produced, anterior structures were expanded, and the position of subsequently activated gap genes was shifted posteriorly (Driever and Nusslein-Volhard, 1988; Struhl et al., 1989). Secondly, if *bcd* RNA was directly injected into the embryo in an ectopic location, ectopic head and anterior structures would form from this point, showing that anterior structures formed at areas of high Bcd concentration, while posterior structures required lower Bcd concentration in order to form (Driever et al., 1990).

Sequencing of *bcd* showed that it encoded a homeodomain-containing protein (Berleth et al., 1988; Frigerio et al., 1986). Bcd protein has been found to play a variety of roles. The first is as a transcriptional activator for a number of

gap genes, including *hunchback (hb)*, whose expression in the anterior of the embryo is dependent on *bcd* concentration. The *hb* promoter has been shown to contain a number of *bcd* binding sites to which *bcd* binds via its homeodomain (Struhl et al., 1989) in a cooperative DNA-dependent manner (Ma et al., 1996) The region in which *hb* is activated depends on the concentration of Bcd, and will shift if that concentration profile shifts (Struhl et al., 1989). *bcd* has been found to activate the expression of some gap genes, and repress others, possibly using *hb* as an intermediate; this will be discussed below.

In addition to its action as a transcriptional activator, *bcd* has been found to repress aspects of the posterior patterning system (Frohnhofer et al., 1986). *caudal (cad)* is a homeodomain-containing protein which is expressed in a gradient from posterior to anterior in the embryo (Macdonald and Struhl, 1986), and which acts as a transcriptional activator of some gap genes (Rivera-Pomar et al., 1995). It has been found that the Bcd homeodomain binds to the 3' UTR of *caudal (cad)* RNA, repressing translation (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). Thus, in the anterior class of genes, *bcd* acts both as a transcriptional activator and as a translational repressor.

The other genes in the anterior class have been found to function in the proper localization of *bcd* RNA. *exuperantia* was shown to be necessary for localization of *bcd* RNA at the anterior pole (Frohnhofer and Nusslein-Volhard, 1987; Hazelrigg et al., 1990). *staufen* and *swallow* encode proteins which have RNA-binding motifs (Chao et al., 1991; Pokrywka and Stephenson, 1991). (Ferrandon et al., 1994; Ferrandon et al., 1997; St Johnson et al., 1991), and are thought to be necessary for the maintenance of *bcd* RNA localization by linking *bcd* RNA to the microtubules of the cytoskeleton (St. Johnson et al., 1989).

A. 1b. The Posterior Class of Maternal Effect Genes

All ten members of the posterior group show defects in abdominal segmentation, and in addition, eight genes in this class have defects in the formation of pole cells, which are the precursor to the germ cells (Gans et al., 1975; Lehmann and Nusslein-Volhard, 1991; Nusslein-Volhard et al., 1987; Schupbach and Wieschaus, 1989; Schupbach and Wieschaus, 1986). Cytoplasmic transplantation studies suggested a hierarchy in which the products of the pole cell-formation genes participated in either the production or the localization of the genes which specified abdominal structures (Lehmann and Nusslein-Volhard, 1991).

The nos gene was cloned and RNA injection experiments showed that it could rescue the abdominal phenotype of most of the other mutants in the posterior group (Wang and Lehmann, 1991), suggesting that this gene product acts as a posterior determinant. *nos* RNA was insufficient to rescue a true *pum* null mutant, whose phenotype appeared to be the same as a *nos* null, but had normally distributed Nos protein (Barker et al., 1992). *nos* and *pum* may therefore encode factors which act together.

The Nos/Pum posterior determinant acts to inhibit the translation of a transcriptional repressor. The repressor is the product of maternally supplied *hb* RNA which, unlike the later zygotic transcript, is uniformly distributed in the early embryo (Schroder et al., 1988). Pum and another unidentified factor (Murata and Wharton, 1995) have been shown to bind to *nos*-responsive elements in the *hb* 3′ UTR (Wharton and Struhl, 1991), and these proteins recruit Nos, which has no nucleic acid binding capability. This complex inhibits translation of *hb* RNA (Wharton and Struhl, 1991). It has been shown that in the absence of both maternal Hb and Nos, the posterior of the embryo will develop normally

(Hülscamp et al., 1989; Irish et al., 1989; Struhl, 1989). Therefore, Nos and Pum play a permissive rather than an instructive role in the determination of the posterior pattern of segments.

Another protein, not identified in a genetic screen, has been shown to play an instructive role in the establishment of the posterior segmentation pattern. *cad* was isolated on the basis of its homology to the homeobox motif, and was found to be expressed both during oogenesis and in the early embryo (Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987). Maternal *cad* transcript initially is distributed evenly in the embryo and by the time of cellularization has disappeared from the anterior of the embryo (Mlodzik and Gehring, 1987). Post-transcriptional regulation of *cad* RNA by Bcd, which was described above, is responsible for this graded distribution. *cad* has been shown to act as an activator not only of gap genes in the posterior of the embryo (Rivera-Pomar et al., 1995), but also of *ftz* (Dearolf et al., 1989).

Thus, in the anterior and the posterior of the embryo, similar strategies are utilized to establish the basic pattern of segmentation. Both systems rely on protein gradients of a transcriptional activator and a translational repressor. *bcd* plays both roles for the anterior system, while *nos/pum* and *cad* share the functions in the posterior system. These determinants direct proper expression of the gap genes, transcriptional activators which are discussed below, in narrow domains, which further subdivides the embryo along its anterior-posterior axis.

A. 1c. The Terminal and Dorso-ventral Classes of Maternal Effect Mutants

The terminal and dorsal-ventral classes share some superficial similarities. Both transmit a maternal signal across the embryonic cell membrane rather than relying on the localization of a maternally supplied RNA within the embryo.

However the intracellular consequences of the transduced signal are quite different in the two cases.

When mutated, all of the genes of the terminal class display a recessive phenotype which involves the loss of the acron, the eighth abdominal segment, and the telson (Nusslein-Volhard et al., 1987). One gene in this group, *torso (tor)*, also has a reciprocal dominant phenotype in which terminal structures are present, but thorax and abdominal pattern defects are evident (Strecker et al., 1989). Epistatic studies using the dominant gain-of-function *Tor* mutant have allowed the order of action of the genes in this group to be determined (Ambrosio et al., 1989; Stevens et al., 1990).

Many of the downstream targets of *tor* have been cloned and found to be highly homologous to previously known genes. *tor* itself is a receptor tyrosine kinase (Sprenger et al., 1989), while *l(1)polehole* is the Drosophila homologue of the vertebrate serine-threonine kinase *c-raf* (Ambrosio et al., 1989). *corkscrew (csw)* encodes a non-receptor tyrosine phosphatase which acts in concert with *D-raf* to transduce the *tor* signal (Perkins et al., 1992). The final action of this receptor tyrosine kinase cascade is the activation (Liaw and Langyei, 1993) of the gap genes *tailless (tll)* and *huckebein (hkb)*, which act at the poles of the embryo to repress the segmentation of these regions by suppressing the expression of "central" gap genes (Bronner and Jackle, 1991; Strecker et al., 1991; Weigel and Jackle, 1990).

The genes upstream of *tor* are not as well characterized. Tor protein was found to be widely distributed on the external cell membrane of the embryo, suggesting that its localized domain of action is due to some localized activating factor or factors (Casanova and Struhl, 1989; Sprenger et al., 1989). The candidate ligands are *torsolike*, which encodes a secreted protein which is transcribed only at the poles (Martin et al., 1994; Savant-Bhonsale and Montell, 1993), and *trunk*,

which is homologous to *spätzle*, the ligand for the dorsal-ventral system (Casanova and Struhl, 1993).

Therefore, in the terminal system, localized activation of a receptor tyrosine kinase allows the activation of a kinase cascade. This results in the de-repression of downstream transcription factors in highly restricted areas, which then act to further direct the development of the acron and telson.

The dorsal-ventral patterning system consists of 12 genes; 11 display a recessive mutant phenotype in which the embryo is completely dorsalized (Anderson and Nusslein-Volhard, 1984). Epistasis studies using a dominant negative form of Toll allowed the determination of the order of action of these 11 genes (Anderson et al., 1985; Anderson et al., 1985). The twelfth gene, *cactus*, has the opposite phenotype, so that when it was mutated the embryo is completely ventralized (Schupbach and Wieschaus, 1989). *dorsal* was found to act downstream of all other genes in the group. Later studies showed that while Dorsal protein was distributed over the whole embryo, it was localized to the nucleus in a ventral-to-dorsal gradient (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989) and directed the formation of ventral structures in a dose-dependent manner (Anderson et al., 1985). Mutants in any dorso-ventral gene except *cactus* block Dorsal nuclear localization, suggesting that the other genes' function is to establish this asymmetric nuclear localization. In *cactus* mutants, Dorsal is located in the nucleus of every cell, suggesting that its function is to block nuclear localization of Dorsal on the dorsal side of the embryo (Roth et al., 1991; Roth et al., 1989).

Sequencing of *dorsal* revealed that it is highly homologous to transcription factors of the vertebrate Rel family, such as NF-kB (Steward, 1987). *cactus* was identified as the Drosophila homologue of the NF-kB inhibitory protein, I-kB (Geisler et al., 1993). Like Dorsal, NF-kB is regulated by sequestration in the

cytoplasm until an external signal is transduced. In the case of *dl*, the signal is transduced via *Toll*, whose sequence shows that it is a transmembrane receptor protein (Hashimoto et al., 1988). *pelle*, which acts downstream of Toll, is a serine/threonine protease which is activated by *tube*, which encodes a protein with unknown biological activity (Grosshans et al., 1994; Hecht and Anderson, 1993). Upon activation, Pelle phosphorylates Cactus, which causes it to dissociate from Dorsal and degrade (Belvin and Anderson, 1996; Whalen and Steward, 1993). Freed Dorsal is also phosphorylated, and translocates to the nucleus (Gillespie and Wasserman, 1994).

Thus both the terminal and dorso-ventral systems rely on the localized maternal activation of a transmembrane signaling cascade in the embryo.

A. 2. *Zygotically Active Genes*

Like the maternal-effect genes, most zygotic genes affecting embryonic development were identified in genetic screens (Jurgens et al., 1984; Nusslein-Volhard et al., 1984; Nusslein-Volhard, 1980; Wieschaus et al., 1984). Initial analysis of the phenotypes caused by these mutations grouped the genes into categories based on the level of spatial disruption they caused. Mutations in genes of the "gap" class resulted in embryos which were missing a contiguous stretch of segments. "Pair-rule" genes, when mutated, caused the deletion of regions in every other segment. Mutants of the "segment polarity" class affected a portion of each segment (Nusslein-Volhard, 1980). By examining the expression of various genes in the context of mutations in others, it was possible to construct a hierarchy of gene action among the zygotically acting genes (Harding et al., 1986; Howard, 1986).

A. 2a. Gap Genes:

The gap genes are first in the hierarchy of zygotically induced segmentation genes. This can be shown by the fact that their expression is affected either by mutations of the maternal-effect class or of other gap genes, but not by mutations of pair-rule or segment polarity genes (Gaul and Jackle, 1987; Jackle, 1986; Kraut and Levine, 1991; Tautz, 1988). The expression domains of the gap genes, from anterior to posterior, are *tll/hkb, hb, giant (gt), Krüppel (Kr), knirps (kni), gt, tll/hkb.* In mutants of the gap genes, the segments corresponding to each gene's expression domain were found to be missing (Jurgens et al., 1984; Nusslein-Volhard et al., 1984; Nusslein-Volhard, 1980; Wieschaus et al., 1984). The gap genes are therefore thought to receive the basic positional information supplied by the maternally active genes and interpret it by subdividing the embryo into smaller units along the anterior-posterior axis.

For example, the protein gradient of Bcd is essential for directing the expression of at least three of the gap genes, *Kr, hb,* and the anterior domain of *gt* (Driever and Nusslein-Volhard, 1989; Driever et al., 1989; Schroder et al., 1988; Struhl et al., 1989; Tautz, 1988). For example, in *bcd* mutant embryos, the Kr domain was shifted anteriorly, indicating that Bcd acted to repress Kr expression at the anterior edge of its domain (Gaul and Jackle, 1987). Bcd binding sites have been identified in the Kr promoter, showing that Kr is a direct target of Bcd action (Hoch et al., 1991).

Once the gap genes are activated, a complex network of intra-group interactions is responsible for the maintenance of their expression pattern. Observation of the pattern of one gene when another was mutated led to the conclusion that most of these interactions involved repression of transcription, either mutual or unilateral. Mutually repressive interactions include Hb (at high

concentration) and Kr (Hülscamp et al., 1990), and Kr and Gt (Kraut and Levine, 1991). Other, non-reciprocal repression interactions are Hb repression of *kni* and *gt* (Hülscamp et al., 1990; Struhl et al., 1992), and Gt repression of *kni* (Capovilla et al., 1992).

Sequencing has revealed that all of the gap genes encode transcription factors. *hb* and *Kr* encode zinc-finger transcription factors (Redemann et al., 1988). gt encodes a transcription factor of the b-ZIP/leucine zipper class (Capovilla et al., 1992), while *kni* encodes a steroid receptor homologue (Nauber et al., 1988), the ligand of which is as yet unknown, making it an orphan receptor. Besides their action in refining the expression domain of other gap genes, the major action of the gap genes is to direct the expression pattern of the next genes in the zygotic hierarchy, the pair-rule genes.

A. 2b. Pair Rule Genes:

Establishment of the pair rule gene expression pattern:

Most of the pair-rule genes are characterized by a pattern of mRNA and protein expression in seven or eight stripes. These stripes peak at or around the time of cellular blastoderm formation (Gergen and Butler, 1988; Howard et al., 1988; Macdonald et al., 1986). An overlapping series of gap protein gradients directs the expression of many pair-rule genes, and mutants in different gap genes have been used to determine their effect on the expression of various pair-rule genes. For example, the *ftz* pattern was disrupted in *Kr*, *kni*, *hb* and *gt* mutant embryos (Carroll and Scott, 1986), as was the *evenskipped (eve)* pattern (Frasch, 1987). However, in the gap mutants examined, if Ftz was detected in a particular set of cells, Eve was not, and vice-versa (Frasch, 1987), indicating that these two pair rule genes responded to the same regulatory signals with opposite actions.

runt expression was repressed by *gt, hb, Kr, kni* and *tll*, although different stripes responded more or less well to repression by any one gap gene (Klingler and Gergen, 1993). Each pair rule gene is thus affected differently by the gap genes, establishing a pattern of expression for each pair rule gene which echoes but does not coincide with its neighbors' expression domain.

Two mechanisms by which gap genes establish the striped pattern of pair rule gene expression have been identified. For the *eve* and *hairy (h)* promoters, individual stripe-producing elements were identified by linking portions of the genes' regulatory regions to reporter genes (Goto et al., 1989; Harding et al., 1989; Klingler et al., 1996; Pankratz, 1990). These elements respond independently to gap proteins, and to some maternal effect proteins, to direct the expression reporter genes in individual stripes. The best example of this is an element directing *eve* stripe 2 expression. This element is activated by Bcd and Hb, and repressed by Kr and Gt (Small et al., 1992; Small et al., 1991; Stanojevic et al., 1989). Dissection of the *h* regulatory region also revealed a number of stripe-specific elements, each of which responded differently to the various gap genes (Howard et al., 1988; LaRosee et al., 1997; Pankratz, 1990). This differential response allows the anterior and posterior boundaries of each individual stripe to be established by the interaction of various gap and maternal-effect proteins with binding sites in the control elements for that particular stripe.

A point is raised by this data, which is that in the regulation of segmentation genes, arbitrary "hierarchical boundaries" can be breached. This is the case for the *eve* promoter, which contains binding sites for the maternal effect gene *bcd* in its regulatory region (Small et al., 1991). Classifying the segmentation genes as members of a hierarchy is a convenience only, and should not be considered to be a completely accurate picture of what is actually occurring in the embryo.

Not all pair-rule genes' regulatory regions contain elements which direct the expression of a single stripe. For example, *ftz* has a regulatory region which has been shown to direct the expression of seven stripes in a *ftz*-like pattern when it was placed upstream of a reporter gene (Hiromi et al., 1985). No element which directs the expression of a single stripe has been identified within this region, which suggests that all seven stripes are coordinately regulated in the case of *ftz*. This will be discussed below in detail. Therefore, the pair rule gene expression pattern can be built up from a combination of single stripes which are individually regulated, or all of the stripes which can be coordinately regulated.

Refinement and maintenance of the pair rule expression pattern:

Initially, the genes of the pair-rule class were subdivided into "primary" and "secondary" pair-rule genes. This was based on an apparent hierarchy of action within the group. For example, Ftz patterns were disrupted in *h* and *runt* mutant embryos, but the reverse was not true (Carroll and Scott, 1986; Frasch, 1987; Howard, 1986). H acted as a *ftz* repressor, so that Ftz stripes were expanded in *h* mutants and repressed in *h* overexpression (Ish-Horowicz and Pinchin, 1987). Runt acted as an activator of *ftz*. (Frasch, 1987). Based on these apparently epistatic relationships, *h*, *runt*, and *eve* were termed primary pair rule genes, while the rest including *ftz* were thought to be secondary. This led to a model that the primary pair rule genes responded to the gap genes and that they in turn influenced the expression of secondary pair-rule genes (Ingham, 1988).

However, it appears that the situation is more complex than this basic model would imply. While *h* is indeed necessary for fine-tuning of the *ftz* striped pattern, it is not in fact necessary for their initial expression. *ftz* stripes appeared at the same time as *h* stripes and their appearance was independent of mutations in any of the so-called primary pair rule genes (Ingham, 1988; Yu and Pick, 1995),

suggesting a role for the pair rule genes in maintenance of but not initiation of the *ftz* pattern. This model in which all pair rule genes respond directly to the gap genes' cues is supported by the fact, mentioned above, that *ftz* and *eve* responded to the same regulatory signals (Frasch, 1987).

Thus, like the gap genes, pair rule genes use positional cues provided by gene products expressed before them in the regulatory cascade to establish their expression domains in the embryo. Cross-regulation among members of the group refines and maintains the pattern. Again like the gap genes, sequencing of the pair rule genes has shown that they encode various types of transcription factors. *h* encodes a basic helix-loop-helix transcription factor (Rushlow et al., 1989), *eve* and *ftz* both encode proteins which contain homeodomains (Laughon and Scott, 1984; Macdonald et al., 1986). The pair rule genes, in their seven-striped expression pattern, thus influence each other's expression domains and define the parasegmental border by directing the expression of their targets, the segment polarity genes (Lawrence and Johnston, 1989; Macdonald et al., 1986).

A. 2c. Segment Polarity Genes:

The segment polarity class of zygotically active genes is responsible for directing the demarcation of the parasegmental border (Lawrence and Johnston, 1989) which functions to separate the posterior compartment of one segment from the anterior compartment of the next. Each segment is made up of one anterior and one posterior compartment, which are committed to different developmental routes (Lawrence & Morata 1976). Two of the major participants in the definition of both the parasegmental border and the fate of cells within a compartment are *engrailed (en)* and *wingless (wg)*. *en* was detected in a 14-stripe pattern only in cells of the posterior compartment (Kornberg et al., 1985) and *en*

mutants failed to form any structures associated with that compartment (Morata and Lawrence, 1975). *wg* appeared to play a complimentary role, since denticles, which are typically found only in the anterior compartment of each segment, in its absence were scattered all over the cuticle (Bejsovek and Martinez-Arias, 1991). Thus *wg* and *en* appeared to specify the development of their respective compartments.

The typical fourteen-striped pattern of *wg* and *en* is directly initiated by the pair-rule genes. The anterior edge of each parasegment was observed to coincide with the anterior edge of *ftz* or *eve* expression domains (Lawrence and Johnston, 1989). In an *eve* mutant, En stripes were missing from odd-numbered parasegments (Frasch, 1987; Macdonald et al., 1986). The same effect was seen in *paired (prd)* mutants (DiNardo and O'Farrell, 1987). In *ftz* or *odd-paired (opa)* mutants, the even-numbered En stripes were absent (DiNardo and O'Farrell, 1987; Laughon and Scott, 1984). These genes therefore acted as activators of *en*. On the other hand, *ftz* and *eve* were shown to be repressors of *wg*. In both *ftz* and *eve* mutants the number of Wg stripes was reduced by half and significantly broadened. This pattern was interpreted to be the result of ectopic expression in the Wg interstripes, due to a lack of repression by either *ftz* or *eve* (Ingham and Gergen, 1988). Thus one action of the pair-rule genes, particularly *ftz* and *eve*, is to specify the exact spatial pattern of segment polarity gene expression.

Sequence analysis has demonstrated the diversity of proteins encoded by this class of segmentation genes. This difference in types of gene products appears to relate to the relative timing with which the genes of the different classes are activated. Genes of the segment polarity class are activated at approximately the time of the cellular blastoderm. The free diffusion of nuclear factors that was possible previously is no longer a viable way to transmit information from nucleus to nucleus at this later stage (Slack, 1991).

Consequently, the genes of the segment polarity class encode the factors involved in cell-cell signaling.

en and *wg* were found to be necessary for the maintenance of each other's expression (DiNardo et al., 1988; Ingham et al., 1988). The signaling pathway by which *en*, a transcription factor (Macdonald et al., 1986; Poole et al., 1985), could upregulate the expression of *wg*, a secreted factor (Gonzalez et al., 1991; Rijsewijk et al., 1987), in a different cell has been the subject of intense scrutiny. One method of examination was epistatic analysis to determine which segment polarity genes affected which others. For example, the pattern of En expression in *armadillo (arm), disheveled (dsh)* and *porcupine (porc)* mutants was the same as in *wg*, indicating that these four gene products acted in the same pathway to transmit the Wg signal to the *eve*-expressing cell (van den Heuvel et al., 1993). Sequencing has also helped to establish the roles of many of the gene products in this system. Two serine/threonine protein kinases, *zeste-white 3 (zw3)* and *fused (fu)* have been identified (Preat et al., 1990), a zinc-finger transcription factor, *ciD* (Orenic et al., *1990*), a member of the β–catenin family of cell-adhesion molecules, *arm* (Peifer and Weischaus, 1990), one additional secreted factor, *hh* (Lee et al., 1992), and three multiple-pass transmembrane proteins which could be receptor molecules, *smoothened (smo), ptc*, and *porc* (Hooper and Scott, 1989; Kadowaki et al., 1996; van den Heuvel and Ingham, 1996).

Thus, a cascade of signaling on both sides of the parasegmental border is necessary to maintain the expression of genes initially activated by the pair-rule genes. The segment polarity genes then act to define the fate of cells as members of either the anterior or posterior compartment of a segment.

A. 2d. Homeotic Genes:

Until this point in the regulatory cascade, the various proteins act to sequentially subdivide the embryo into a pattern of repeated segmental units. The unique identities of each segment in the *Drosophila* embryo are then specified by the genes of the homeotic class (Lewis, 1978).

The homeotic genes are located in two clusters, the Antennapedia (ANT-C) and Bithorax (BX-C) clusters, on the third chromosome. All encode proteins of the homeodomain type of DNA-binding protein (Kaufman et al., 1990; Lewis, 1978). The homeobox was first identified as a region of cross-hybridization between the *Antennapedia (Antp)* and *fushi tarazu (ftz)* genes (Garber et al., 1983; Scott et al., 1983). This region was found to consist of 180 highly conserved bases, which were subsequently used as a probe to clone many genes which were found to be transcription factors, some of which were important in embryonic segmentation (McGinnis et al., 1984; McGinnis et al., 1984; Scott and Weiner, 1984). The domain encoded a 60 amino acid polypeptide, the homeodomain, which was predicted to form a structure of multiple α–helices, a structure which was very similar to previously-identified yeast and bacterial transcription factors (Laughon and Scott, 1984; Shepherd et al., 1984). NMR spectroscopy confirmed that the Antp homeobox formed a helix-turn-helix DNA binding motif (Otting et al., 1988; Qian et al., 1989). The helix-turn-helix motif is found in many different transcription factors, including yeast MATa1 and a2 factors, bacteriophage l cro protein, and the vertebrate Myb oncoprotein (Frampton et al., 1989; Harrison and Aggarwal, 1990; Laughon and Scott, 1984; Shepherd et al., 1984). Each of these proteins has a slightly different arrangement of helices, but in all of them, the so-called "recognition helix" makes contact with the DNA major groove at the site of the recognition sequence (Harrison and Aggarwal, 1990; Otting et al., 1988). In

the case of the homeodomain, the classic two-helix structure is supplemented by two additional helices, so that it is the third helix of the four which is responsible for DNA contact (Otting et al., 1988; Qian et al., 1989). The other two helices are postulated to maintain the DNA-binding helix in its proper conformation. It has been shown in many cases that the action and the specificity of DNA binding of homeodomain proteins depends on cofactors. One such cofactor is *extradenticle (exd)*, which has been shown to be necessary for co-activation of the homeotic gene Ubx's targets, such as *wingless, teashirt* and *decapentaplegic* in the midgut (Peifer and Wieschaus, 1990; Rauskolb and Wieschaus, 1994).

Mutations in these genes cause the classic "homeotic" transformations, in which the identity of one segment is replaced by a differing segment's identity. One example is *Antennapedia,* in which the antennae are replaced by second thoracic legs. The former is a over- or mis-expression phenotype; when Antp is missing, the second thoracic segment is transformed to a more anterior phenotype (Wakimoto and Kaufmann, 1981). Both of these phenotypes suggested that the major role of Antp is to specify structures in parasegment (PS) 4. However, there are more segments than there are homeotic genes, which has led to the proposal that each segment is not necessarily specified by the expression of a single homeotic gene, although this is apparently the case in some instances. In the remainder of the cases, a combination of homeotic genes is proposed to signify the identity of each segment (Lewis, 1978).

Activation of the homeotic genes depends on the action of the pair rule genes and the gap genes. For example, when *ftz* is mutated, *sex combs reduced (Scr), Antp,* and *Ubx* are all initiated incorrectly, so that no peak of activity is seen for any of the three genes in their specific segments (Ingham and Martinez-Arias, 1986).

In a theme which should by now be familiar, once the expression pattern of these genes is defined by extra-group influence, intra-group regulation refines and maintains the pattern. For example, in a phenomenon known as "posterior prevalence", genes which are expressed more posteriorly (i.e. *Ubx*) will repress the expression of more anteriorly expressed genes (i.e. *Antp*). This is necessary, since many of the homeotic genes are expressed in domains which extend far to the posterior of their apparent area of influence (Hafen et al., 1984; McGinnis and Krumlauf, 1992; Struhl, 1982).

The downstream targets of the homeotic genes are still largely unknown. That the proteins act as transcriptional activators has been established by tests of reporter gene activation in cell culture (Winslow et al., 1989). One target, the gene *Distalless (Dll)* has been found to be under the control of the Ubx protein. Dll is necessary for the formation of Keilin's organs, which are the vestiges of larval legs (Cohen et al., 1989). They are present on all three thoracic segments, and are suppressed in the abdomen by the action of Ubx (Mann, 1994). This is one example of a particular segmental structure which is regulated by the action of a homeotic gene product. Thus, once the segments are established by the action of the gap, pair-rule and segment polarity genes, the homeotic genes act to specify the structures of each individual segment.

B. *fushi tarazu*, A Segmentation Gene:

B. 1. *ftz* Expression Pattern:

ftz was originally identified as a gene necessary for establishing the proper segmental pattern in Drosophila embryos (Wakimoto and Kaufmann, 1981). It was subsequently classified as one of the pair-rule genes, since when it was

mutated the embryo lacked denticle bands for segments T2, A1, A3, A5, and A7, along with the adjacent naked cuticle making up those segments (Jurgens et al., 1984; Nusslein-Volhard, 1980). The gene is located within the *Antennapedia* complex (Wakimoto and Kaufmann, 1981) and was cloned based on its homology to the *Antp* homeobox sequence (Kuroiwa et al., 1984; Scott and Weiner, 1984). The gene encodes a single transcript of ~1.9 kb, which in turn encodes a protein of 413 amino acids (predicted MW of 45 kDa) (Laughon and Scott, 1984). The expression patterns of *ftz* RNA and protein have been extensively studied. As I will discuss below, these patterns are comparable, indicating that wherever *ftz* mRNA is transcribed, it is translated. Therefore, a fairly complete understanding of the regulation of *ftz* expression can be achieved by studying the control of its transcription.

B. 1. Ftz mRNA and protein patterns:

The Ftz protein pattern was first detected by immunofluorescent labeling (Carroll and Scott, 1985). No protein was detected until cellularization, at which time seven stripes of nuclear labeling, each approximately four nuclei wide, were visible. The stripes narrowed during gastrulation and finally disappeared by the end of germ band elongation. As the Ftz stripes faded, a second site of Ftz accumulation became visible in the nervous system. One cluster of cells in each segment was found to have Ftz protein in its nuclei. This protein persisted until the end of germ band retraction (Carroll and Scott, 1985). An even later region of Ftz expression has been identified in the primordia of the hindgut, where protein is detected from 12 to 15 hours of development (Krause et al., 1988).

In situ hybridization using a radioactive probe showed that *ftz* mRNA is expressed in seven stripes at the cellular blastoderm stage (Hafen et al., 1984; Weiner et al., 1984). Comparison of the site of transcript accumulation with the

fate map positions of segment primordia showed that the cells which expressed *ftz* transcript were the primordia of the segments missing in *ftz* mutant embryos (Hafen et al., 1984).

Later, a more sensitive *in situ* hybridization with digoxygenin-labeled probe revealed transitional stages in the establishment of stripe expression. A diffuse early stripe extending beyond the borders of stripe 1 was first detected, followed by an increase in transcript levels in the area of stripe 2. Stripe 5 then appeared, succeeded by stripe 3, stripes 6 and 7 (which were initially fused), and finally stripe 4 (Yu and Pick, 1995) (see Figure 1). This exactly corresponded to the pattern described in a more intensive investigation of the protein expression pattern (Karr and Kornberg, 1989), demonstrating that wherever *ftz* RNA was transcribed it was translated into protein. Thus, *ftz* is regulated solely at the level of transcription.

B. 2. *cis*-Regulatory Regions of *ftz*:

ftz regulatory regions were identified by ligating chromosomal fragments of various sizes into P element vectors and injecting these vectors into *Drosophila* embryos. Flies carrying these constructs were then bred to *ftz* mutant flies, and their offspring were scored for rescue of the *ftz* phenotype (Hiromi et al., 1985). In this way, a fragment containing approximately six kilobases 5' of the *ftz* transcription start site, the *ftz* coding region, and a ~2 kb 3' element was found to rescue *ftz* mutant embryos to adulthood. Smaller fragments had weak and variable rescue activity (Hiromi et al., 1985), indicating that this 10 kb fragment was probably the minimum necessary for rescue.

Through the use of fusion genes in which the *Escherichia coli lac Z* reporter gene was placed under the control of the *ftz* regulatory region, the 5' flanking

region of *ftz* was separated into three elements (see Figure 2A) (Hiromi et al., 1985). A "zebra" element, located within 0.62 kilobases of the transcription start site, directed reporter gene expression in seven mesodermal stripes (Hiromi and Gehring, 1987). A neurogenic element, located between -0.62 and -2.45 kb, was necessary for reporter expression in the developing nervous system, and was independent of the other two elements (Hiromi and Gehring, 1987). Finally, an upstream element located between -3.4 and -6.1 kb acted cooperatively with the zebra element in either orientation to direct stripes expression in the mesoderm and ectoderm (Hiromi and Gehring, 1987; Hiromi et al., 1985). While this pattern of stripes was indistinguishable from the normal *ftz* pattern once it was established, *in situ* hybridization using a *lac Z* probe has shown that the order in which they appeared was incorrect (Yu and Pick, 1995), indicating that additional stripe-control elements must be located elsewhere in the genome.

These *ftz-lac Z* constructs were expressed in various mutant fly lines. The zebra element was found to be sensitive to mutations in the gap and pair-rule genes, such as *h* and *runt*. The upstream element was responsive to mutations in the Ftz gene (Hiromi and Gehring, 1987), and thus autoregulatory. The *ftz* zebra element and the upstream element have been studied intensively, leading to a better understanding of many of the factors interacting with these elements. The control elements 3' of the *ftz* coding region have not been well studied. Deletions of this flanking sequence have obvious adverse effects on rescue activity (Hiromi et al., 1985). However, except for an element which drives reporter gene expression in a region corresponding to stripe 5 (L. Pick, unpublished), little is known about specific regulatory elements in this region.

B. 2a. The *ftz* zebra element:

DNAse I footprinting experiments using the zebra element revealed at least 12 specific sites of protein binding within 400 base pairs of the *ftz* transcription start site (Laughon and Scott, 1984). In order to study these sites, exonuclease III digestion of the zebra element from its 3' and 5' ends was used to create a series of sequential deletions. These fragments were placed upstream of a *lac Z* gene, and the expression of the reporter gene was analyzed in comparison to the expression of the construct preceding it in the series. Activator regions were identified by a drop in β–galactosidase expression following their deletion. Repressor regions, upon deletion, resulted in ectopic expression of the reporter gene outside the normal bounds of *ftz* expression. Thus at least five activator and four repressor elements were identified (Dearolf et al., 1989). None of the elements were found to be essential for expression of the reporter gene. However, removal of successive activator elements decreased the expression level to undetectable levels, while removing repression elements led to increasing expression in the interstripe regions. This suggests that these elements act additively to direct proper reporter gene expression.

Thus, the zebra element contains a number of semi-redundant activation and repression elements, which combine in an additive fashion to direct the striped pattern. These sub-elements could potentially interact with a number of *ftz* regulatory factors. However, except for Ftz-F1 and Ttk, none of the proteins implicated genetically in the control of *ftz* have been shown to interact directly with the zebra element, including the gap and pair rule genes.

B. 2b. The *ftz* upstream element:

B. 2b. *1.* Organization:

The *ftz* upstream element has many features in common with a classical enhancer. It acts to enhance expression of the seven stripes directed by the zebra element (Hiromi et al., 1985). This element was found to act on either the 5′ or 3′ side of the *ftz* coding region, in an orientation-independent fashion (Hiromi and Gehring, 1987). If this element was placed upstream of a heterologous hsp 70 basal promoter, it could independently direct expression of a reporter gene in seven *ftz*-like stripes. This effect was also independent of distance and orientation (Hiromi and Gehring, 1987).

Unlike the zebra element which acts as a unit to direct stripe expression, the upstream element can be subdivided into a number of independent seven-stripe-forming components. Exonuclease digestion of the upstream element generated a series of deletions which were tested for their ability to direct stripe expression when placed upstream of a heterologous promoter and the *lac Z* gene (Pick et al., 1990). It was shown that the upstream element could be separated into two independently-acting portions (see Figure 2B) which directed the expression of seven reporter gene stripes. The distal region directed expression in seven mesodermal stripes, while the proximal region directed the expression of stripes in both the mesoderm and ectoderm. Both elements could act in a distance- and orientation- independent manner (Pick et al., 1990). The two enhancers are probably not redundant, since deletion of the distal enhancer caused the intensity of reporter expression to decrease.

The proximal element was further subdivided into two germ layer-specific elements: Prox A and Prox B. Prox A was sufficient to direct expression in seven stripes in the mesoderm alone. Addition of Prox B to Prox A allowed expression

in the ectoderm and mesoderm. Prox B alone supported no reporter gene expression (Pick et al., 1990). The lack of expression by Prox B could be explained if the binding of a few specific proteins is sufficient to activate transcription in the mesoderm, while additional factors must be added to allow ectodermal expression. Hence, the upstream element consists of two independent enhancers, one of which contains a mesoderm-specific element and an accessory element necessary for ectodermal stripe expression.

B. 2b. *2.* Protein Binding Sites in the Upstream Element:

B. 2b. *2a.* Ftz binding sites:

Both proximal and distal enhancer elements require the presence of wild-type Ftz protein to direct reporter gene expression in seven stripes (Pick et al., 1990). DNAse I footprinting studies using the homeodomain of Ftz identified multiple binding sites of varying affinities within the two enhancers. Both enhancers contained one high-affinity Ftz homeodomain binding site and multiple medium- and low-affinity sites (Pick et al., 1990). Autoregulation from the enhancer could involve Ftz binding directly to these sites, or Ftz could be interacting with another protein or proteins which make direct contact with DNA.

To test whether Ftz protein bound directly to the proximal enhancer *in vivo*, a series of internal deletions within the enhancer were created and their effect on *lac Z* reporter gene expression was studied (Schier and Gehring, 1992). Each deletion removed the area surrounding one or more previously identified Ftz binding sites. The ablation of one site alone had little or no effect on *lac Z* expression. Deletion of two sites together resulted in a reduction of reporter gene expression to varying degrees. Deletion of all four sites completely abolished

expression. These results were interpreted as evidence that the Ftz binding sites in this enhancer were functionally redundant and that Ftz binding to the enhancer was vital to allow expression (Schier and Gehring, 1992). Unfortunately, later information (Han et al., 1993; Schier and Gehring, 1993) showed that these deletions removed the binding sites of many proteins besides Ftz, making it difficult to determine from this experiment whether Ftz actually interacted with the enhancer.

The strongest evidence to date that Ftz protein binds directly to the proximal enhancer *in vivo* was obtained by the study of compensatory second-site mutations. In these studies, two of the Ftz binding sites of the proximal enhancer, one of which was the high-affinity site, were mutated. The sequence of the Ftz binding site was changed to a sequence to which Bcd binds with higher affinity than Ftz (Driever and Nusslein-Volhard, 1989; Percival-Smith et al., 1990). When these mutated *ftz* binding sites were placed upstream of a reporter gene, the level of reporter gene expression dropped relative to wild type (Schier and Gehring, 1992). If this construct was expressed in an embryo whose *ftz* homeobox had been mutated at one base in the recognition helix so that it recognized the Bcd binding sequence (Treisman et al., 1989), reporter gene expression recovered to wild-type levels (Schier and Gehring, 1992). This suppression of the binding site mutation by a second mutation in Ftz strongly suggested that Ftz protein directly interacts with those sites of the proximal enhancer.

B. 2b. *2b*. Other protein binding sites in the upstream element:

Two studies in particular have investigated protein binding sites in the upstream element. One identified DNAse I protected sites in the proximal enhancer and the proteins which bound to these sites (Han et al., 1993). The other

created sequential small scale deletions in the same region and examined their effect on expression of a reporter gene (Schier and Gehring, 1993).

In the first study, the proximal enhancer was divided into seven approximately equal parts, and each part was used as probe in an electrophoretic mobility shift assay (Han et al., 1993). Three of the seven, all located in the 3' half of the enhancer, generated specific protein-DNA complexes in this assay. DNAse I footprinting of these three fragments revealed nine sites of protein-DNA interaction (see Figure 2C). Five of these sites (1, 2, 4, 8, and 9) overlapped or were adjacent to previously identified Ftz homeodomain binding sites (Pick et al., 1990). Site 2 overlapped the single high-affinity Ftz binding site. Methylation interference assays confirmed that there was direct protein-DNA contact at all nine sites (Han et al., 1993). By placing successively smaller DNA fragments of the proximal enhancer upstream of a reporter gene, it was found that 323 base pairs were sufficient to direct reporter expression in seven *ftz*-like stripes. This minimal proximal enhancer contained all of the nine identified protein binding sites in the proximal enhancer (Han et al., 1993). Thus, understanding how these sites interact with proteins to generate seven stripes will be a major step towards comprehending how the *ftz* pattern is generated.

In order to determine how many proteins interacted with the nine protein binding sites, oligonucleotides corresponding to their sequence (numbered 1-6 and 8-10) were synthesized and used in electrophoretic mobility shift assays (Han et al., 1993). Every site except for site 10 resulted in one or more protein-DNA complexes in the assay. This suggested that proteins which bind to site 10 did so in cooperation with proteins which interacted with other sites in the enhancer. Sites 1, 3 and 5 resulted in the formation of one complex each, sites 2 and 8 resulted in two, 6 and 8 resulted in three, and site 4 resulted in four discrete protein-DNA complexes. Seventeen complexes were detected in all.

Cross-competition mobility shift assays, in which unlabeled oligonucleotide corresponding to another site was added to the reaction, revealed that some of the sites interacted with the same proteins (Han et al., 1993). Reciprocal competition indicated that for sites 1, 5, 6, 8, and 9, one complex was identical for all sites, indicating that the same protein(s) interacted with these sites. Sites 6 and 9 were shown to generate the same three complexes, while site 8 formed with two of these three. Only sites 2, 3 and 4 formed protein complexes which were unique to a single site. Therefore, ten different protein complexes bound to these eight sites in various combinations (see Figure 3). The identity of some of these proteins has been discovered and will be discussed later

.

The importance of some of these sites for directing the seven-striped pattern was assessed. Point mutations of any one site which abolished protein binding in mobility shift assays had no effect on reporter gene expression, indicating a certain degree of redundancy among the sites (Han et al., 1998) Internal deletions of the minimal proximal enhancer were tested (Han et al., 1993) and showed that deletion of a region which included sites 8, 9 and 10 resulted in a reduction of reporter expression. Double and triple point mutations in sites 6, 8 and 9 were tested (Han et al., 1998). Double mutation of sites 6 and 9 greatly reduced reporter gene expression, and mutation of all three sites resulted in no detectable reporter gene expression, suggesting that some or all of these sites were necessary for proper gene expression.

Therefore, no one site in the proximal enhancer is essential for its expression. Sites 6, 8 and 9, while not individually necessary, did seem to be more important than the others, since point mutation of all three completely abolished expression by the enhancer. Site 2 is also of some interest since in addition to generating two unique complexes in EMSA, it overlaps the site of

highest-affinity Ftz homeodomain binding in the proximal enhancer (Pick et al., 1990).

The second study (Schier and Gehring, 1993) used fine-scale deletion analysis of the proximal enhancer, accompanied by quantification of expression, to reach much the same conclusions. Sequential deletions from the 5′ and 3′ ends of the proximal enhancer, removing approximately 20 base pairs at a time, were produced by PCR-mediated mutagenesis. Each deletion was used to direct *lac Z* reporter gene expression, which was quantified. Successive deletions resulted in progressive loss of reporter expression, until about half of the element was removed, after which no reporter expression was detected. None of the deletions resulted in expression of the reporter outside of the seven stripes, suggesting the lack of repressor elements in the proximal enhancer. However, since the enhancer's expression is Ftz-dependent, repressor elements cannot be ruled out by this result. In fact, the presence of some factor(s) which repress ectopic stripe expression was suggested, since in a background of ubiquitous Ftz expression, no interstripe expression was observed when the entire proximal enhancer construct was tested (Schier and Gehring, 1993). The effect on stripe expression of smaller fragments of the proximal enhancer in this background was not reported.

Even though no one portion of the enhancer was necessary for expression, some regions were more important than others. A region corresponding to sites 6, 8 and 9 (as identified in Han, 1993) was again identified as being of particular importance for expression. The three smallest constructs which supported any reporter expression all retained the area around site 6, along with either sites 4 and 5, or 8 and 9. The smallest of these was only 177 base pairs long, and included sites 4 through 9 as well as at least one Ftz binding site, and directed lac Z expression at 73% of the level measured for the full-length enhancer (Schier

and Gehring, 1993). Deletion of thirty base pairs around site 6 reduced expression to 75%. Removal of only bases corresponding to site 8 reduced expression to 64%, suggesting that these regions played a disproportionately large role in directing expression of this enhancer. Site 9 was not specifically removed, but since it binds the same factors as site 6, it can be assumed that its effect on expression is similar to that of site 6.

One unexpected result, in light of the protein binding sites previously identified in the enhancer (Han et al., 1993), is the fact that deletions within the 73 bp of "empty space" between sites 6 and 8 caused a significant decrease in reporter expression (Schier and Gehring, 1993). This region may be necessary for proper spacing of DNA binding proteins when they bind to adjacent sites. Possibly other factors bind there that were not identified by DNAse I protection. The latter explanation would seem to be more likely, since this sequence is conserved in both the *D. virilis ftz* regulatory region, and in the first intron of the *en* gene (Han et al., 1993).

In summary, these two studies showed that while no one protein binding site was necessary for expression directed by the proximal enhancer, sites 6, 8 and 9 appeared to be more important than the others. Overall, the enhancer seemed to act like the zebra element, in that the sites activated expression in an additive manner. Unlike the zebra element, no repressor elements have yet been identified.

B. 3. *trans*-Acting Factors Which Interact With The *ftz* Proximal Enhancer:

A number of proteins have been shown to interact with the *ftz* proximal enhancer. Besides Ftz, which was discussed above, Ttk, Ftz-F1 and alcohol dehydrogenase factor 1 (Adf-1) have all been found to interact with the proximal

enhancer. Many proteins which have been shown to bind to the enhancer by footprinting and mobility shift assays (Han et al., 1993) remain to be characterized. All of these proteins are potential regulators of *ftz* expression, although the part that any individual factor plays has yet to be fully determined. In the following section, I will discuss Ttk, Ftz-F1 and Adf-1 in the context of their effect on *ftz* expression.

B. 3a. Tramtrack:

ttk was originally isolated by screening a 0-16 hour *Drosophila* embryonic cDNA expression library with a protein binding site from the *ftz* upstream element (Harrison and Travers, 1990). This site, located at positions 1613-1644 of the upstream element (between the proximal and distal enhancers (Pick et al., 1990)), was chosen due to its differential binding of proteins in the course of embryonic development. The site was bound by proteins in extracts made from early and later-stage embryos, but the exact bases protected by each extract changed (Harrison and Travers, 1988). Analysis of one clone's predicted protein sequence showed that it contained two zinc finger DNA binding motifs, which was consistent with its observed requirement of Zn^{+} for DNA binding (Harrison and Travers, 1990)).

The expression pattern of *ttk* RNA was analyzed by *in situ* hybridization (Harrison and Travers, 1990). *ttk* RNA was detected in the earliest embryos tested, indicating that the transcript is maternally deposited. By the tenth nuclear division *ttk* RNA was almost undetectable; this was shown to be the time at which ftz RNA begins to accumulate (Hafen et al., 1984). *ttk* RNA was detected again in the anterior midgut during germ band extension, and persisted there (Harrison and Travers, 1990). In late germ band extension, after *ftz* RNA was no longer detected, *ttk* RNA was found in the mesoderm and ectoderm in a pattern

of 14 stripes which gradually condensed to form two longitudinal stripes on either side of the developing central nervous system (Hafen et al., 1984). This characteristic late expression was the basis for the gene's name.

The protein pattern of Ttk was examined by antibody staining (Read, 1992), which showed low uniform staining in 0-2 hour embryos (indicating maternal deposition) that decreased to background levels in embryos undergoing cellularization. Ttk was detected next in the midgut, corresponding to RNA localized to that area. In late germ band extension staining was detected in the epidermis, in rings surrounding each tracheal pit. This gradually expanded until the entire epidermis was expressing Ttk, a situation which persisted until the end of germ band retraction. The characteristic "tramtrack" staining was not observed (Read, 1992). Unless the antibody was nonspecific at this stage, I have no explanation for how such a restricted pattern of RNA could give rise to widespread distribution of a transcription factor.

Based on a number of findings, *ttk* has been implicated in the repression of *ftz* transcription. The effect of overexpressing *ttk* was examined by placing its coding region under the control of a heat shock promoter and inducing this construct in 2-4 hour old embryos. It was found that in those embryos both Ftz and Eve protein levels were drastically reduced (Read et al., 1990). All of the gap genes were expressed normally in these embryos, suggesting that the effect of overexpressed Ttk on *ftz* was direct (Read et al., 1990). Finally, it was shown that by increasing or decreasing the dose of Ttk supplied maternally, the appearance of *ftz* transcription would be delayed or advanced, respectively (Pritchard and Schubiger, 1996). This indicates that Ttk is a repressor of *ftz* transcription, and that the decay of maternally supplied Ttk is the trigger for the onset of *ftz* transcription.

More evidence that Ttk functions as a direct repressor of *ftz* expression came from a search for specific trans-acting factors in the zebra element which led to the identification of a 69 bp region (-131 to -200) which was protected from DNAse I digestion by some factor or factors in early (1.5 hours) embryonic extract (Brown et al., 1991). Two footprinted sites with similar sequences were identified within the region. One site was used to screen a cDNA expression library. The clone which was isolated was identical in sequence to *ttk*. Mutation of both binding sites or of the higher affinity site alone and insertion of this mutated zebra element upstream of a *lac Z* reporter led to reporter expression in the interstripes (Brown et al., 1991). In addition, in embryos carrying the doubly-mutated construct, low levels of reporter gene expression were detected by the third nuclear division, well before wild-type *ftz* is expressed. The two sites are located within regions of the zebra element previously identified as repressor elements (Dearolf et al., 1989). Thus, mutations in two of the three Ttk binding sites in the zebra element are sufficient to cause ectopic reporter gene expression, indicating *ttk's* role as a direct *ftz* repressor.

Ttk has also been shown to interact with the *ftz* proximal enhancer. fEBC 10, which interacted with sites 1, 5, 6, 8, and 9, had a consensus binding sequence of AGGA, the core of the Ttk binding sequence (Fairall et al., 1992). Anti-Ttk antibody could disrupt the formation of fEBC 10 using sites 1, 5, 6, 8, or 9 in mobility shift assays, indicating that this complex contained Ttk (Han et al., 1993). Additionally, affinity purification of factors binding to site 6 of the proximal enhancer resulted in the isolation of Ttk (Han, 1994). Thus, five sites of Ttk binding exist in the proximal enhancer, in addition to the zebra element sites.

A putative zygotic null mutation in the *ttk* gene has been isolated and found to be homozygous lethal during embryogenesis (Xiong and Montell, 1993). Examination of the cuticle of these mutants revealed that segmentation did

occur, but cuticular structures were severely perturbed (Xiong and Montell, 1993). These defects did not fall into any of the segmentation classes and thus may indicate that *ttk* has multiple functions in the early embryo, whose disruption leads to diffuse defects in embryogenesis. Therefore this factor, which has been shown to bind to a number of sites in the *ftz* regulatory region, is necessary for embryonic survival. Embryos derived from germline clone *ttk* mutants to this date have not been successfully generated.

B. 3b. Ftz-F1:

Ftz Factor 1 (Ftz-F1) was originally identified as an activity in 1.5-4 hour old embryo extract which interacted with the zebra element in mobility shift assays (Ueda et al., 1990). The protein responsible for the interaction was purified using the binding site immobilized on a column (Ueda et al., 1990). The zebra and neurogenic elements of the *ftz* regulatory region were screened for additional Ftz-F1 binding sites using the purified protein. Another zebra element site and two sites in the *ftz* coding region were found (Ueda et al., 1990). Comparison of these sites revealed consensus binding sequence, the core of which was CAAGG.

Ftz-F1 was cloned and sequenced and it was found that the gene sequence was homologous to nuclear hormone receptor proteins (Lavorgna et al., 1991). No *in vivo* ligand has yet been identified for Ftz-F1, making it an orphan nuclear receptor. However, it was recently shown that transcriptional activation by the mouse homologue of Ftz-F1 was enhanced by addition of 25-, 26-, or 27-hydroxycholesterol (Lala et al., 1997). It was unclear whether this sterol or one of its metabolites was the ligand and whether a similar molecule interacts with *Drosophila* Ftz-F1 has yet to be determined.

Northern blots showed that 0-2 and 2-4 hour embryos had a strong *Ftz-F1* RNA band, indicating that *Ftz-F1* was maternally supplied (Lavorgna et al., 1991). Ftz-F1 protein was likewise detected in early embryos and unfertilized eggs, peaking at cellularization (Yu et al., 1997). This expression coincided with Ftz expression, raising the possibility that Ftz-F1 acted as a positive regulator of *Ftz* expression. 4-14 hour embryos had no detectable Ftz-F1 RNA, but the RNA reappeared in 14-22 hour embryos. This suggested that Ftz-F1 had a later role which was not related to its role in Ftz regulation.

The segment of the zebra element (-273 to -293) with which Ftz-F1 originally interacted had been found to have dual activator/repressor capability (Dearolf et al., 1989; Topol et al., 1991). If one of two Ftz-F1 binding sites in the zebra element was mutated, and this fragment was used to drive reporter gene expression, overall levels of reporter gene expression were reduced (Ueda et al., 1990), which indicated that Ftz-F1 was acting as an activator of *ftz* expression.

Multiple Ftz-F1 sites in the proximal enhancer were identified based on the presence of the Ftz-F1 consensus binding sequence (Han et al., 1993). The consensus binding sequence for sites 6, 8 and 9, was similar to the derived consensus sequence for Ftz-F1 (Ueda et al., 1990). To test if fEBC 8 contained Ftz-F1, anti-Ftz-F1 antibody was added to a mobility shift assay using site 6 (Han et al., 1993). fEBC 8 was abolished by addition of this antibody, confirming that Ftz-F1 bound to sites 6, 8 and 9 of the proximal enhancer.

Ftz-F1 has more recently been shown to act as a cofactor for Ftz (Guichet et al., 1997; Yu et al., 1997). One study used a modified yeast two-hybrid system to screen a cDNA library for Ftz cofactors (Yu et al., 1997). Ftz-F1 was isolated in this screen as a factor which could bind alone to the target sequence, 323 base pairs which included all nine protein binding sites of the proximal enhancer. Alone, Ftz-F1 could activate low levels of reporter transcription, and together

with Ftz it could activate high levels of transcription. Ftz alone was unable to cause significant levels of transcriptional activation from this target sequence. Thus, the high level of activation caused by the two proteins together indicated cooperative action.

That the two bound DNA cooperatively was shown by mobility shift assays using a proximal enhancer element which contained a Ftz-F1 site adjacent to a Ftz medium-affinity binding site (Yu et al., 1997). This element corresponded to the area around site 8 of the proximal enhancer (Han et al., 1993). Ftz protein alone was unable to bind to this site, although it was able to bind to a high-affinity Ftz site under the same conditions, but Ftz could form a ternary complex with Ftz-F1 when both were used in the reaction (Yu et al., 1997). Co-immunoprecipitation experiments showed that anti-Ftz antibody could precipitate Ftz-F1, confirming that the two proteins were physically associated (Yu et al., 1997) Thus, Ftz-F1 increases the affinity of Ftz for medium-affinity binding sites.

ftz-F1 mutants have a phenotype which is very similar to that of *ftz*. Two mutations in the gene were independently isolated, and found to have a *ftz*-like pair rule cuticular phenotype (Guichet et al., 1997; Yu et al., 1997). This suggested that despite the widespread distribution of Ftz-F1 in the early embryo, its major function at that time was as a Ftz cofactor. In both mutants, Ftz-dependent *en* stripes were missing, an indication that Ftz action was disrupted. Interestingly, although mutations in Ftz-F1 binding sites in the zebra element implicated the factor as an activator of *ftz* expression (Ueda et al., 1990), the activation of Ftz striped expression was apparently normal (Yu et al., 1997). However, the Ftz stripes did decay more quickly than in wild type, suggesting that Ftz-F1 could have a role in autoregulatory Ftz stripe maintenance (K. Su and L. Pick,

unpublished). Thus, Ftz-F1 acts as a cofactor for Ftz binding and function, both on Ftz itself, and on Ftz downstream targets.

B. 3d. Adf-1:

Adf-1 was first identified as a factor in *Drosophila* embryo extracts which was necessary for *in vitro* transcription of the *alcohol dehydrogenase (adh)* gene. *adh* is transcribed from two distinct promoters, which result in two different transcripts (Benyajati et al., 1983) The two transcripts accumulate differently during development; the proximal transcript is more prevalent in late embryos and larvae, while the distal is expressed transiently in mid-stage embryos and strongly in adults (Savakis et al., 1986). Deletion analysis of the distal promoter identified one region in particular (-25 to -85) which was important for directing transcription from that promoter (Heberlein et al., 1985). Stage-specific nuclear extracts were used to DNAse I footprint the *adh* distal promoter. The activity which interacted with this site was strongly detected only in 8-12 hour extracts, suggesting that it might be temporally regulated (Heberlein and Tjian, 1988). Deletion of the distal promoter site was shown to cause a four-fold drop in *in vitro* transcription from the distal promoter (England et al., 1990), indicating the importance of Adf-1 in the activation of *adh* transcription.

Adf-1 was purified from Kc cell extract, using a combination of conventional chromatography and DNA affinity chromatography (England et al., 1990). A possible consensus binding site for Adf-1 was identified by comparing the sequence of the distal and proximal promoter Adf-1 binding sites in *D. melanogaster* and *D. orena* (Moses et al., 1990). The consensus sequence was made up of four to five repeats of the trinucleotide [G (C/T) (C/T)]. The only highly conserved bases in this sequence were the initial G's (England et al., 1990). Adf-1

binding sites were found in the *Antp* P1 promoter and the dopa decarboxylase promoters; binding was confirmed by footprinting. However, Adf-1 did not bind to the *Antp* P2 promoter, or to the *Ubx* promoter, indicating that this factor was not one which binds to all promoters to activate transcription.

The gene encoding Adf-1 was cloned by using sequences obtained from tryptic peptides to generate oligonucleotide probes which were used to screen a *Drosophila* genomic library (England et al., 1992). One genomic clone was used to probe a cDNA library, which resulted in the isolation of eight cDNAs for *adf-1*. Sequencing of the cDNA and computer searches of sequence databases revealed a domain in the N-terminal end of the protein which had homology to the Myb helix-turn-helix (H-T-H) DNA-binding motif (England et al., 1992). Deletion mutagenesis and alanine-scanning analysis of the protein has revealed that unlike some transcriptional activators, Adf-1 cannot be separated into distinct DNA binding and activation domains (Cutler et al., 1998). It was also shown that Adf-1 homodimerizes and interacts with TAFs to activate transcription through another Myb-like protein-protein interaction domain at the C terminal end of the protein. While monomeric Adf-1 could bind to DNA, its DNA affinity was lower than the dimer's. Thus, *adf-1* encodes a transcription factor which has a DNA binding domain similar to the Myb H-T-H, and a nonmodular transactivation domain which includes a novel Myb-like dimerization and TAF interaction domain.

An antibody raised against bacterially-produced Adf-1 protein was used to show that Adf-1 protein was detected in the cellular blastoderm and was found in the nuclei of all cells in the embryo except the pole cells (England et al., 1992). The levels of Adf-1 peaked by about 4-5 hours post fertilization and remained until the end of germ band retraction, after which the protein was no longer detected. The disparity between the constant protein expression of Adf-1

and its temporally restricted pattern of binding to the *adh* distal promoter could be explained by either the presence of a cofactor necessary for Adf-1 binding which is itself expressed for a limited time or by some time-limited modification of Adf-1. It further suggests that Adf-1 has roles in development beyond the activation of *adh* expression.

Adf-1 has been shown to bind to the *ftz* proximal enhancer. Sequencing of proteins purified from a binding site 6 affinity column showed that one peptide's sequence corresponded with the sequence of Adf-1 (Han et al., 1998). A mobility shift assay using bacterially produced Adf-1 showed that it interacted with site 6. Anti-Adf-1 antibody added to a mobility shift assay using nuclear extract and binding site 6 specifically abolished the formation of one of the complexes common to sites 6, 8 and 9. Interestingly, when anti-Adf-1 was added to assays using each of the sites in the proximal enhancer except 5 and 10, the antibody was found to abolish the formation of one complex in each reaction, suggesting that Adf-1 participates in the formation of complexes with each of these binding sites. This is not consistent with the EMSA competition experiment results, which suggested that sites 2, 3, and 4 interacted with factors that did not recognize sites 6, 8 or 9 (Han et al., 1993). It is possible that Adf-1 interacts with different proteins at these sites, and that these proteins change its DNA affinity, but until the proteins binding to these sites have been unambiguously identified, this issue remains unresolved.

The consensus binding sequence for Adf-1 (England et al., 1990) is not present in sites 6, 8 or 9. The site derived from the proximal enhancer, [(C/T) C N (A/G) (G/A) G (A/G) N (G/A)], differs markedly from the consensus repeats of [G (C/T) (C/T)], particularly in its lack of conservation of the initial G's. Titration of Adf-1 binding to either site 6 or to the binding site in the *Adh* distal promoter using mobility shift assays demonstrated that Adf-1 bound with 10-fold lower

affinity to site 6 (Han et al., 1998). Thus, while Adf-1 can bind to non-consensus sites in the proximal enhancer, it does so with reduced affinity. This suggests that *ftz* may not be the primary target of Adf-1 in the embryo.

Using a yeast system, Adf-1 was shown to act as an activator of transcription from the 323 bp ftz minimal proximal enhancer (Han et al., 1998). Three copies of binding site six were placed upstream of a *lac Z* reporter and cotransfected into yeast cells with Adf-1. Adf-1 was able to activate transcription of the reporter at about one-quarter the level at which Ftz-F1 could activate the same reporter. The 323 bp element was placed upstream of a histidine reporter gene and also tested for Adf-1 transcriptional activation. Adf-1 was able to activate expression of this reporter gene, although approximately ten times less well than Ftz-F1 (Han et al., 1998; Yu et al., 1997). Thus Adf-1 can interact with the *ftz* proximal enhancer and activate some level of transcription.

Summary and Future Directions:

The proximal enhancer of *ftz* performs a fundamental role in the expression and regulation of this essential segmental patterning gene. Without the *ftz* upstream element, which includes the proximal enhancer, rescue of *ftz* mutant embryos to adulthood was impossible (Hiromi et al., 1985). The proximal enhancer was necessary to direct expression of a reporter gene in stripes in the ectoderm, a function which is performed by no other known *ftz* regulatory element (Pick et al., 1990). Thus, in seeking to understand *ftz* regulation, it is essential to determine the ways in which this regulatory element is controlled.

While some of the proteins which bind to the *ftz* proximal enhancer have been identified, many questions remain to be answered. The identity of many of these proteins has not been established. The identity of the proteins interacting

with binding site 2 are of particular interest, because the two proteins are apparently unique among the proximal enhancer binding proteins (Han et al., 1993), and because site 2 has been identified as the only high-affinity Ftz homeodomain binding site in the proximal enhancer (Pick et al., 1990).

The exact action of many of the proximal enhancer binding proteins is also unclear. Of the known proteins which interact with the proximal enhancer, only Ftz-F1 has been mutated and tested for its effect on *ftz* expression (Yu et al., 1997). The modest effect on *ftz* expression which is seen in *ftz-f1* mutants is possibly due to redundancy among the factors which bind to the same sites which it recognizes. Thus, lack of one factor could be compensated for by the presence of others which bind to the same site (see Figure 4). Other factors which bind to the same sites as Ftz-F1 include Ttk and Adf-1, neither of which has a mutant whose action has been tested in this system. Thus, the possible effect on *ftz* of mutating more than one of these factors simultaneously has to be determined.

It is possible that the mutation of more than one of these factors at once would have a drastic effect on *ftz* proximal enhancer expression. It is known that deletion of the sites to which these proteins all bind, sites 6, 8, and 9, causes a dramatic decrease in reporter gene expression. Consequently, it seems likely that mutation of more than one of the proteins which bind to these sites could cause a similar decrease. This can only be confirmed by testing *in vivo*.

Therefore, in order to gain a clearer understanding of how expression from the *ftz* proximal enhancer is regulated, the proteins which interact with the enhancer must be identified and the contribution which each of these proteins makes must be assessed, both individually and in concert with other binding proteins.

Chapter 2. Materials and Methods

I. Materials:

1. Fly Strains

P[sced]/CyO flies (Sullivan et al., 1993) were a generous gift by Dr. W. Theurkauf, SUNY Stonybrook. *adf*^{le60}/CyO and ve 48 flies were provided by Dr. J. Dezazzo and Dr. T. Tully, Cold Spring Harbor Laboratory. The following lines were provided by Dr. N. Perrimon, Harvard University: P1598, FRT^{3L}/ TM3, w, FLP^{22}; TM3/CXD, and w; *ovoD1*, FRT^{3L}/ TM3 (Chou and Perrimon, 1996; Perrimon et al., 1996). Ore R wild type flies were obtained from Dr. M. Biggin, Yale University. The following strains were obtained from the Mid-America Drosophila Stock Center: *ry506* Sb^{1} P[Δ2-3](99B) / TM6, and Df(2R)42, en^{1} / In(2L)Cy. The following stocks were generated in our laboratory: 1) w; CyO/Gla was created by mating P[sced]/CyO flies to CyO/In(2LR)Gla flies (obtained from MidAmerica) which had lost the Cy marker, and selecting Gla, CyO offspring for maintenance. 2) w; Sp/CyO; P1598, FRT^{3L}/ TM3 flies were generated by mating laboratory stock w; Sp/CyO; TM3/Sb to P1598, FRT^{3L}/ TM3 and mating Sp, Sb offspring to CyO, Sb offspring for maintenance. 3) w; *adf*^{le60}/CyO; TM3/Sb were created by mating laboratory stock w; Sp/CyO; TM3/Sb to *adf*^{le60}/CyO flies and mating CyO, Sb to CyO, Ser offspring for maintenance. All strains were maintained at 25°C.

2. Antibodies

Monoclonal anti-Ftz antibody was provided by Dr. I. Duncan (Kellerman et al., 1990). Polyclonal rabbit anti-Ftz was prepared by Yan Yu of our laboratory. Polyclonal rat anti-Ftz was provided by D. Kosman of Dr. Reinitz's laboratory at

Mt. Sinai (Kosman and Reinitz, 1998). Rabbit anti-β–galactosidase was purchased from Cappell. Biotinylated anti-rabbit antibody was purchased from Boehringer Mannheim. Biotinylated anti-rat antibody was provided by Dr. R. Hardy at Mt. Sinai.

II. Methods:

1. Preparation of Embryo Nuclear Extract

This procedure was done essentially as described by Han, *et al.* (Han et al., 1993), with modifications by Kai Su of this laboratory. Embryos of the appropriate age were harvested from mass population cages of *Drosophila melanogaster* (Oregon R). The embryos were dechorionated by immersion in 3% sodium hypochlorite (Clorox) for 90 seconds, followed by extensive washing with water. All of the following steps were performed at 4°C. 30 ml of homogenization buffer was added for every 10 grams of embryos. Homogenization buffer was made up of 10 mM HEPES, pH 7.6, 25 mM KCl, 0.15 mM spermine-HCl, 0.5 mM spermidine HCl, 1 mM EDTA, and 0.35 M sucrose. 1 mM DTT and a mixture of protease inhibitors: 50 μg/ml soybean trypsin inhibitor, 1 mM benzamidine-HCl, 1 μg/ml aprotinin, 1 μg/ml antipain and 1 μg/ml bacitracin (final concentrations), were freshly added. Embryos were homogenized by passing them through a Yamato Teflon-glass motor-driven homogenizer set at 100 rpm. Pre-wetting of the homogenizer greatly increased the efficiency of this step. The homogenate was spun at 12,000 rpm for 10 minutes, and the supernatant was discarded. The whitish portion of the pellet, which represented the nuclei separated from the yellowish yolk material, was resuspended in 10 ml homogenate buffer for every 10 grams of embryos. The solution was passed again through the homogenizer and re-spun at 12,000 rpm

for 10 minutes. The whitish portion of the pellet was resuspended in 5-10 ml of lysis buffer, depending on its size. Lysis buffer was composed of 10 mM HEPES, pH 7.6, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA and 10% glycerol. 1 mM DTT and the protease inhibitors were freshly added. A 1/10 volume of 4 M ammonium sulfate, pH 7.9, was added to the resuspended pellet and the tubes were rotated end over end for 30 minutes. The tubes were spun for one hour at 36,000 rpm. 0.3 grams of solid ammonium sulfate were added per ml of supernatant from this spin, and this was mixed for 15 minutes. 1 µl of 1 N NaOH per gram of AS was added to the solution, and it was mixed for 15 minutes. The solution was centrifuged for one hour at 36,000 rpm. The pellet was dried and resuspended in 50 µl no-salt HEMG (25 mM HEPES, pH 7.6, 0.1 M EDTA, 12.5 mM $MgCl_2$, and 10% glycerol) per gram of starting material. 1 mM DTT and the protease inhibitors were freshly added. The protein concentration was measured by the Bradford method (Bradford, 1976), using a BioRad protein assay solution. Nuclear extract was stored at -80°C.

2. <u>Electrophoretic Mobility Shift Assay</u>

This procedure was performed as described by Han, *et al.* (Han et al., 1993), after the manner of Carthew (Carthew et al., 1985). DNA fragments with 5'-protruding ends were labeled with α-^{32}P-dCTP and the Klenow fragment of DNA polymerase. Protein of varying amounts (see figure legends) was incubated with 10 fmol of labeled probe in a total reaction volume of 25 µl. The reaction contained 0.1 M KCl, 25 mM HEPES, pH 7.9, 0.5 mM DTT, 10% glycerol, and 0.5 mM EDTA, pH 8.0. Nonspecific competitors, 0.1 µg poly (dI-dC) (Pharmacia) and 0.1 µg random single-stranded oligonucleotide, were included in each reaction. Antibody or pre-immune serum, if applicable, was added to the reaction at this stage. Samples were incubated on ice for one hour and analyzed

by electrophoresis through a 4% native polyacrylamide gel, using 0.5 X TBE as running buffer. The gels were run at 12 mA for approximately 1/2 hour in the cold room. Dried gels were either exposed to film (-70°C with an intensifying screen for 4 hours, or overnight at room temperature) or to a PhosphorImager screen (Molecular Dynamics), in which case the data collected from the screen was analyzed using ImageQuant software (Molecular Dynamics).

Oligonucleotide 2, which was used as the probe in the assays described in this study, corresponds to the sequence of binding site 2 of the *ftz* proximal enhancer. The upper strand sequence is $^{5'}$ AGCTTGACAGGAGCAATTAA $^{3'}$. The lower strand sequence is $^{5'}$ ACTGTCCTCGTTAATTTCGA $^{3'}$. M-O-1, M-O-2 and M-O-9 sequences are given in Figure 15. Oligonucleotides were annealed by mixing equimolar amounts of each oligonucleotide with 10 mM Tris, pH 7.9 and 5 mM $MgCl_2$, and incubating them for 2 minutes at 88°C, 10 minutes at 65°C, 10 minutes at 37°C, and 5 minutes at room temperature.

3. Silver-staining of SDS-polyacrylamide gels

This procedure was modified from that described in Ausubel (Ausubel et al., 1992). Proteins were separated by SDS-polyacrylamide gel electrophoresis, using sample loading buffer consisting of 0.32 M Tris, pH 6.8, 0.1% SDS, 0.01% DTT, 0.008% bromophenol blue, and 50% glycerol. Other sample buffer recipes were found to result in nonspecific brown background in the gel, particularly 3X SDS Loading Buffer supplied by New England Biolabs. The gel was washed in the following solutions, all of which were made up in sterile dH_2O, and used in extremely clean containers. It was helpful to use a separate container for the color formation step, so that residue from these reagents did not increase background in later stainings. The gel was washed for 45 minutes in Solution I (50% methanol, 12% trichloroacetic acid, and 2% $CuCl_2$). The gel was next washed in

Solution II for 15 minutes, until the gel was transparent. Solution II was composed of 10% ethanol and 5% acetic acid. This solution was removed and the gel was washed with 0.01% $KMnO_4$ for 10 minutes, and then with Solution II for 10 minutes. The gel was washed with 10% ethanol for 15 minutes, and then with sterile dH_2O for 15 minutes. The gel was washed with 0.1% $AgNO_3$ for 10 minutes. This solution was made up fresh each time, and kept in the dark until it was used. The gel was then washed for 10 seconds in sterile dH2O, and then with 10% $K_2C O_3$ for 1 minute. The color solution consisted of 0.01% formaldehyde and 2% K_2CO_3, and was prepared each time from stock. The gel was incubated in this solution until the color had developed, and then was immediately removed to Solution II for 10 minutes, and then held in sterile dH2O. The gels were scanned into the computer immediately, as the colors faded very quickly. To increase contrast, the gel was briefly immersed in Coomassie gel staining solution (0.1% Coomassie brilliant blue, 50% methanol, 10 acetic acid) and equally quickly in destaining solution (50% methanol, 12% acetic acid).

4. <u>Ultraviolet DNA-Protein Crosslinking</u>

This procedure was modified from that described in Ausubel (Ausubel et al., 1992). Oligonucleotide with 5'-protruding ends was labeled using the Klenow fragment of DNA polymerase and 0.5 mM bromodeoxyuridine-substituted uridine (BrdU) in place of deoxythymidine. 1-5 µg of protein were incubated with 200 fmol probe in a final reaction volume of 20 µl. The reaction contained 25 mM HEPES, pH 7.6, 40 mM KCl (including the salt concentration of the protein solution), 0.1 mM EDTA, 1 mM DTT and 0.1 µg poly (dI-dC). The reactions was left on ice for 10 minutes, after which 10 µl were removed for separation on a 4% native polyacrylamide gel. The remainder was placed on a chilled Parafilm-coated heating block and crosslinked at 2.5 cm distance using a Stratagene

Stratalinker 1800. The solution was placed in a fresh tube on ice, containing 1 µl of 100 mM $CaCl_2$ and 1 µl of 1:10 diluted DNAse I. The solution was incubated for 30 minutes at room temperature. 10 µl of SDS sample loading buffer was added, and the sample was run on an SDS 8% polyacrylamide gel, with a 4% stacking gel. The dried gel was exposed to film overnight at -70°C.

5. **Southwestern Blotting**

This protocol was developed by modifying the protocols of Towbin, LeLong, and Bowen (Bowen, 1980; LeLong, 1993; Towbin et al., 1979). 25 to 50 µg of protein were separated on a 10% SDS-polyacrylamide gel with 6% stacking gel. The proteins in the gel were renatured by shaking the gel in three changes of incubation buffer for a total of 90 minutes. "Renaturation" buffer was composed of 4 M urea, 50 mM NaCl, 2 mM EDTA and 10 mM Tris, pH 7.5. 0.1 mM DTT and 0.1 mM benzamidine were freshly added. The purpose of this step was to remove SDS from the gel, which facilitated the subsequent renaturation of the proteins. Proteins were transferred to nylon membrane by use of a Hoefer electroblotting apparatus, for 2 hours at 80 V or overnight at 25 V, using a transfer buffer composed of 25 mM Tris, pH 8.3, and 192 mM glycine. The membrane was blocked overnight at room temperature with binding buffer containing 10 µg/ml poly (dI-dC). Binding buffer was composed of 100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.2, 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% BSA. The blocking step should be at least overnight for best results. The membrane was washed three times for 10 minutes each with binding buffer. Approximately 2 pmol of oligonucleotide probe, which had been radiolabeled on its 5'-overhanging ends by the Klenow fragment of DNA polymerase, was hybridized to the membrane for four hours in binding buffer containing 1-2 µg/ml poly (dI-dC). The membrane was washed for one

hour in at least 4 changes of binding buffer, and the dried membrane was exposed to film at -70°C for at least 12 hours.

6. Ammonium sulfate precipitation

This is based on the protocol outlined by Harris (Harris and Angal, 1989). Five aliquots of protein, each consisting of 4.3 mg of crude nuclear extract with an initial ammonium sulfate concentration of 1% (determined by conductivity measurement), were diluted in 3 ml no-salt HEMG. Ammonium sulfate was added with stirring over the course of 15', to a final concentration of either 20%, 30%, 40%, 50% or 60% saturation. 1 µl per gram of ammonium sulfate of 1 N NaOH was added to each solution, and stirred for 15 minutes. Following the addition of NaOH, the solutions were centrifuged in a refrigerated microcentrifuge at 10,000 rpm for 40 minutes. The pellets were dried and allowed to resuspend in 300 µl no-salt HEMG. The resuspended pellets were tested by EMSA for purification of O2 DNA binding activities.

7. Fly population cages

Fly populations cages were kept at >55% humidity, 25°C in a controlled-environment room. Flies were fed with plates made up of 4% agar and 16% molasses, with 0.01% p-hydroxybenzoic acid methyl ester (Nipagin) added to retard fungal growth. The recipe was communicated to us by Dr. M. Biggin, Yale University. Plates were coated with ~3-5 ml of bakers' yeast diluted in water to a paste-like consistency. Plates were changed at least every 24 hours, or more frequently depending on the desired age of embryos being harvested. Cages were typically started from hatching embryo boxes on a Monday, and new embryos boxes were begun that Friday. Flies in the cage were allowed to lay until the following Wednesday or Thursday, after which they were killed by

placing the cage overnight in the cold room. The cages were then cleaned with Lysol and re-started.

Embryos were propagated in 2.8 L Rubbermaid food storage boxes which had a hole cut in the lid which was covered with fine mesh. Boxes were cleaned with Lysol between uses, rinsed thoroughly, and autoclaved. The box was lined with non sterile cotton (Carolina), with the cotton cut large enough that it went up the sides of the box about 2 inches. The boxes were then filled with about 330 ml yeast mixture. Yeast mixture was made by adding, in order, 228 ml sterile dH_2O, 32 g sucrose, 0.16 ml 100% propionic acid, 0.9 ml 85% phosphoric acid, 3 ml 10% Nipagin, 11 mg of tetracycline or kanamycin (this alternated between generations) and 98 g live bakers' yeast. The yeast mixture was allowed to sit in the boxes for approximately 3 hours at room temperature, until the production of carbon dioxide by the yeast had subsided enough that the mixture would not rise and smear the embryos against the lid of the box. Approximately 1 gram of embryos harvested from population cages were washed thoroughly in water, immersed for 30 seconds in 70% ethanol, and rinsed several times with dH_2O. Embryos were distributed with the aid of minimal 70% ethanol and a small brush on two discs of Whatman #1 paper which had been placed on top of the yeast mixture. Boxes were placed in the fly room, and sprayed with dH_2O daily to prevent over-drying.

8. <u>Analysis of embryo hatching rates</u>

This was based on the procedure outlined by Littleton (Littleton and Bellen, 1994). 0-9 hour embryos were collected and rinsed several times with dH_2O to remove any yeast mixture. Embryos were lined up on molasses agar plates and allowed to age for approximately 36 hours. The number of embryos

left on the plate was counted under a dissecting stereomicroscope. Covering embryos with halocarbon oil to reduce over-drying was found to be dispensable.

9. Embryo antibody staining

This protocol was modified from that used in Dr. Frasch's laboratory at Mt. Sinai (Yin et al., 1997). Embryo fixation was performed after the protocol of Dr. Small's laboratory at New York University (Kosman and Small, 1997). Embryos were collected and dechorionated by immersion for 90 seconds in 3% sodium hypochlorite (Clorox). The bleach was rinsed away extensively with water and then with 0.4% NaCl, 0.25% Triton X-100. Embryos were transferred to Fixation Buffer (4% formaldehyde, 1X PBS, 50 mM EGTA mixed 1:1 with heptane). Embryos were fixed by rotation in this buffer for 25 minutes. The lower, aqueous, phase was removed and 500 µl methanol was added. The embryos were vortexed for 10 seconds to devitellinize them, and the phases were allowed to separate. The upper phase was removed and 500 µl methanol was added. The embryos were washed 2 times more with methanol and 3 times with ethanol. At this stage the embryos were either used or stored at -20°C.

Embryos were rehydrated by washing them 3 times for 10 minutes each in filtered PBST (phosphate buffered saline with 0.1% Tween 20). Antibody, which had been previously diluted and preabsorbed by incubation for 2 hours at room temperature with fixed devitellinized embryos, was added and allowed to incubate with rotation overnight at 4°C or for 4 hours at room temperature. If antibody staining was to be followed by *in situ* hybridization, 5 units of RNAsin was added to antibody solutions and to the ABC reagent. Antibody was removed and embryos were washed 3 times 10 minutes with PBST. Antibody was re-used up to three times. Secondary antibody, appropriately diluted, was added and allowed to incubate for 2 hours at room temperature. Antibody was

removed and embryos were washed 3 times 10 minutes with PBST. During these washes, ABC reagent was prepared by mixing 10 µl reagent A and 10 µl reagent B (ABC kit, Vector Laboratories) with 1 ml PBST and allowing it to sit at room temperature for 30 minutes. ABC reagent was then added to embryos and incubated for one hour. ABC was removed and embryos were washed 3 times 10 minutes with PBST. Color was developed by adding color solution (10 µl 30% H_2O_2, 10 µl Tris, pH 8.0, and 10-50 µl 10 mg/ml dH_2O freshly prepared diaminobenzidine (DAB) in 1 ml total volume PBST). This solution resulted in a brown color. 5µl $NiCl_2$ was added to produce a dark blue. The color reaction was monitored using a stereomicroscope, and was stopped by removing embryos to 600 µl PBST. Embryos were washed 3 times 10 minutes with PBST and then allowed to settle in 50% glycerol, and then in 90% glycerol prior to mounting on slides. If embryos were to be subsequently *in situ* hybridized, they were not placed in glycerol.

10. Whole mount embryo *in situ* hybridization

This protocol was communicated to us by Dr. S. Small, New York University (Kosman and Small, 1997). Embryos were dechorionated, fixed and devitellinized as described in the antibody staining protocol. All incubations were done at room temperature with rotation unless specified. Embryos were removed from the freezer and warmed to room temperature and washed with ethanol three times, and two times with methanol. 600 µl methanol and 600 µl PBST/formaldehyde (6:1) were added and incubated 5 minutes. If embryos had already been antibody stained, the previous steps were omitted. The tube was then filled with PBST/formaldehyde and incubated 25 minutes. Embryos were then washed 3 times 5 minutes with PBST. Proteinase K freshly diluted 1:2500 in PBST was added and incubated for 5 minutes, and the embryos were washed

three times with PBST. Embryos were fixed again for 25 minutes with PBST/formaldehyde. They were then washed 6 times 5 minutes with PBST. The tube was then filled 1:1 with PBST and Hybridization Solution (50% formamide, 5X SSC, 100 µg/ml salmon sperm DNA, 50 µg/ml heparin, and 0.1% Tween 20) and inverted several times. The tube was filled with fresh Hybridization Solution and placed in a 55°C water bath for 30 minutes. This was repeated, and as much of the solution as possible was removed. To this small volume, DGG-labeled RNA probe (which had been boiled for 5 minutes and kept on ice) was added, and the embryos were placed overnight in a 55°C water bath. Following hybridization, the tubes were filled with Hybridization Solution and inverted several times. The solution was replaced with fresh Hybridization Solution and the tube was placed in a 55°C water bath for 30 minutes, with inversion every 10 minutes. The solution was replaced with fresh Hybridization Solution and the 30 minute incubation with inversion was repeated. Hybridization Solution was removed and the tube was filled 1:1 with Hybridization Solution and PBST and incubated 10 minutes. Embryos were then washed 3 times 10 minutes with PBST, and then anti-DGG antibody (diluted 1:2000) was added and incubated for one hour with rotation. Antibody was removed and embryos were washed 3 times 10 minutes. Embryos were rinsed with Staining Buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris, pH 9.5, and 0.1% Tween 20). Embryos were then washed for 5 minutes in Staining Buffer. Color was developed by adding 1 ml of Staining Buffer to which had been added 4.5 µl of 100 mg/ml nitro blue tetrazolium (NBT) and 3.5 µl of 50 mg/ml 5-bromo-4-chloro-indolyl phosphate (BCIP). Color formation was monitored under the stereomicroscope and stopped by removing embryos to 600 µl PBST. Embryos were washed 2 times 5 minutes with PBST and fixed for 25 minutes in PBST/formaldehyde. Embryos were washed 3 times 5 minutes with PBST and then for 5 minutes in 1:1 PBST/methanol. Embryos were

then rinsed 2 times with methanol, once with ethanol, and twice with methanol. The tube was then filled 1:1 with PBST and methanol and rocked for 5 minutes. Embryos were then washed twice with PBST and allowed to settle in 50% and then 90% glycerol and mounted on a slide.

RNA probe was prepared using the Boehringer Genius kit. 1 µg of linearized plasmid DNA containing the template sequence of interest was labeled by incubating it for 2 hours at 37°C in a 20 µl reaction containing 2 µl NTP Labeling Mix, 2 µl 10X transcription buffer, 1 µl RNAse inhibitor and 40 units of SP6 or T7 RNA polymerase. The reaction was stopped by the addition of 2 µl 0.2 M EDTA, pH 8.0 and 2.5 µl LiCl. 75 µl of ethanol were added to precipitate nucleic acids, and the solution was spun down following a 30 minute incubation at -70°C. The pellet was resuspended in 100 µl dH_2O. 0.2 to 0.5 µl of probe were used per reaction, the amount used was determined empirically.

11. <u>Fly genomic DNA preparation</u>

This procedure is adapted from Bingham's protocol (Bingham et al., 1981). 100-200 flies were homogenized in a ground-glass tissue grinder with 3 ml homogenization buffer (100 mM Tris, pH 8.0, 60 mM NaCl, 10 mM EDTA, 0.15 mM spermidine, and 0.5% Triton X-100). The homogenate was decanted through fine gauze into a 15 ml glass centrifuge tube. An additional one ml of buffer was used to rinse out the tissue grinder and added to the centrifuge tube. This was centrifuged at 4°C, 7 minutes at 7000 rpm. The supernatant was discarded and the pellet was resuspended in 3 ml homogenization buffer, and recentrifuged. The pellet was resuspended in 1.8 ml homogenization buffer, and 0.2 ml 20% Sarcosyl was added and gently mixed by inversion. 25 µl of proteinase K (10 mg/ml dH_2O) was added and the solution was incubated at 55°C for 2-16 hours. 200 µl of 3M sodium acetate, pH 6.3 was added and the solution was extracted

twice with phenol/chloroform and once with chloroform, and 3 volumes of ethanol were added to precipitate the DNA. Following 15 minutes at -20°C, the solution was centrifuged for 10 minutes at 14,000 rpm at 4°C. 2 ml of TE was added to the pellet, which had the ethanol pipetted off, but was not extensively vacuum-dried. The pellet was allowed to resuspend, and 25 µl of 10 mg/ml dH_2O proteinase K was added and the solution was incubated at 55°C for 2 hours. This second digestion was found to be helpful for preparations in which the DNA did not readily digest. The DNA was again phenol/chloroform and chloroform extracted and ethanol precipitated. The pellet was finally resuspended in about 1 µl TE per fly.

12. Southern Blotting

This protocol was modified from that described by Ausubel (Ausubel et al., 1992). 25 ng DNA probe was radiolabeled to 10^8 cpm/ng using the NE Blot random priming kit (New England Biolabs). DNA and dH2O up to 33 µl total volume was boiled for 5 minutes and placed on ice. To this mixture, 5 µl of 10X Labeling Mixture, 2 µl each of 0.5 mM dATP, dTTP, and dGTP, 5 µl of α-^{32}P-dCTP (300 Ci/mmol), and 1 µl of Klenow fragment of DNA polymerase were added. The solution was incubated at 37°C for one hour, and the reaction was terminated by addition of 5 µl 0.2 M EDTA, pH 8.0. Labeled probe was separated from unincorporated nucleotides by passing it through a spin column. Incorporation was determined by placing one drop of resuspended DNA on a Whatman DE-81, and rinsing the filter with 0.5 M Na_2HPO_4. The filter was counted in a scintillation counter, and the number of counts remaining on the filter was multiplied by the total volume of the DNA solution, and divided by the approximate concentration of DNA as determined by agarose gel electrophoresis with known standards.

5-10 µg of genomic DNA was digested overnight using 3-4 units of enzyme per µg DNA at 37°C. Digested genomic DNA was separated on a 0.8% agarose gel, using 1X TBE as running buffer. The gel was washed for 2 times 15 minutes in 0.25 N HCl, for 30-60 minutes in denaturing solution (0.5 N NaOH, 1.5 M NaCl) and for 30-60 minutes in neutralization solution (1 M Tris, pH 8.0, 1.5 M NaCl). DNA was transferred overnight to a nylon membrane by capillary action, using 10X SSC as transfer buffer. The membrane was washed for 1 minute in 5X SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0) and blotted dry. The membrane was UV crosslinked using the Stratagene Stratalinker "Autocrosslink" setting. The membrane was wet in 6X SSC, placed in a hybridization tube with 1 ml hybridization solution (5X SSC, 5X Denhardt's solution, and 1% SDS, to which 100 µg/ml salmon sperm DNA was added before use) per 10 cm^2 of membrane, and incubated with rotation for 15 minutes at 68°C in a Hybaid Maxi Oven. Fresh hybridization solution to which all of the labeled probe (which had been denatured by 10 minutes of boiling) had been added was poured into the tube and incubated overnight at 68°C. The hybridization solution was removed, and 2 volumes of 2X SSC/0.1% SDS was added, and incubated with rotation for 5 minutes. This was repeated. An equal volume of 0.2X SSC/0.1% SDS was added and incubated 2 times 5 minutes. The membrane was next washed 2 times 15 minutes at 42°C in a Hybaid oven with 0.2X SSC/0.1% SDS. The membrane was washed 2 times 15 minutes at 68°C, with 0.1X SSC/ 0.1% SDS. The membrane was rinsed with 2X SSC, blotted, wrapped in Saran Wrap, and exposed to film overnight at -70°C with an intensifying screen.

13. <u>Single-embryo PCR</u>

Single stained embryos (in which $NiCl_2$ had not been used in the color solution) were added to 2.5 µl digestion solution (50 mM KCl, 10 mM Tris, pH

8.0, 2.5 mM $MgCl_2$, 0.45% Tween 20, and 100 ng/µl dH_2O Proteinase K (S. Wang, Ph.D. thesis, Columbia University, 1997) in a 0.5 ml Eppendorf tube. Embryos were placed directly into the droplet, to prevent desiccation. It was essential that the proteinase be fresh, or frozen at most one time. Embryos were briefly spun down, vortexed and re-spun, and incubated at 50°C for one hour. The tubes were spun down and placed immediately at -80°C for at least one hour. Prior to PCR, the tubes were boiled for 10 minutes and placed on ice. PCR reactions were performed in a total volume of 100 µl. They contained the embryo in its 2.5 µl of digestion buffer, and a mix consisting of 100 ng of each primer, 1.8 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP and dGTP, and 1X Promega Mg-Free Buffer. 2.5 units of Promega Taq DNA polymerase was added to the tubes during the "hot-start", after the block temperature had reached 75°C. Reaction conditions were as follows: a 5 minute "hot-start" at 95°C was followed by 10 cycles in which 1 minute at 95°C was succeeded by a "touchdown" annealing step, in which the temperature decreased from 75°C to 50°C by 1 degree every 5 seconds (for a total of 125 seconds), and an extension step of 2 minutes at 72°C. This was followed by 20 cycles of one minute each at 95°C, 50°C, and 72°C. A ten minute incubation at 72°C completed the reaction, to allow any unfinished fragments to be extended. The reactions were held at 4°C until they were analyzed on a 2% agarose gel.

Chapter 3. Results I: Purification of Proteins Interacting with Proximal Enhancer Binding Site 2

Introduction:

Previous work from this laboratory identified nine protein binding sites in the proximal enhancer of *ftz* (Han et al., 1993). These are indicated in Figure 2, and were discussed in the introductory section. Oligonucleotides corresponding to the sequence of each of these sites were synthesized and used in electrophoretic mobility shift assays (EMSA). These oligonucleotides each resulted in the formation of various protein complexes in mobility shift assays using nuclear extract prepared from 0-12 hour *Drosophila* embryos. Competition by unlabeled oligonucleotide corresponding to other binding sites to the mobility shift reaction showed that certain proteins interacted with more than one site. Other proteins were shown to be resistant to competition by any other oligonucleotide binding site, indicating that they were able to interact with a unique site in the proximal enhancer. These results are summarized in Figure 3.

In particular, it was found that an oligonucleotide corresponding to site 2 (O2) resulted in the formation of two apparently unique complexes in EMSA. The protein components of these complexes are referred to as *ftz* enhancer binding proteins (fEBP) 1 and 2. These could be either single proteins or a protein complex; it is impossible to make this distinction using the mobility shift assay. The interaction of these proteins with the oligonucleotide probe was unaffected by competition by any other unlabeled proximal enhancer protein binding site, suggesting that these proteins bound only to this site in the proximal enhancer. Oligonucleotides constructed with point mutations at the deduced site of protein-DNA interaction (derived from methylation interference studies) could

not interact with fEBP 1 or 2, indicating that their interaction with site 2 was sequence-specific (Han et al., 1993). Thus, site 2 specifically interacts with two proteins or protein complexes which are unique among proximal enhancer binding proteins.

Site 2 was previously identified by DNAse I footprinting to be the single high-affinity Ftz homeodomain binding site in the proximal enhancer (Pick et al., 1990). These results were discussed in more detail in the introductory chapter. This raises the possibility that either fEBP 1 or 2 could contain Ftz. Alternatively, fEBP 1 and 2 could contain Ftz-interacting factors, factors which compete with Ftz binding, or factors which repress Ftz interaction with site 2. Isolation and identification of these factors would allow us to determine the action of these proteins and distinguish between these possibilities.

Determination of the stage at which embryos should be harvested:

The goal of these experiments was to establish the time of development at which fEBP 1 and 2 were most strongly expressed, and the storage conditions under which these proteins were most stable prior to processing. Additionally, we hoped to establish a method for storing embryos prior to nuclear extract preparation so that a few large-scale nuclear extract preparations could be substituted for numerous smaller ones.

Ore R wild type *Drosophila melanogaster* embryos of various ages (0-2.5 hours, 0-6/7 hours, 0-9/10 hours) were collected from fly mass population cages. These collection times were chosen to reflect the stage before Ftz expression peaks, during and just after the peak of Ftz expression at cellular blastoderm (Carroll and Scott, 1985; Karr and Kornberg, 1989), and embryos past the peak of Ftz expression. After harvesting, the embryos were either immediately subjected

to the nuclear extract preparation procedure, or dechorionated and frozen at -20°C in 50% glycerol (Han, 1994). For embryos in the latter group, nuclear extract was prepared from a pool of embryos collected over several days. In every case, nuclear extract was stored at -80°C following preparation.

Figure 5A illustrates the results of electrophoretic mobility shift assays (EMSA) using extracts prepared from embryos of various ages and oligonucleotide 2 (O2). The extracts were either prepared fresh or frozen prior to preparation. Lanes 1 and 2 show the result of reactions using extract prepared from 0-7 hour embryos, fresh (lane 1) or frozen (lane 2). Lanes 3 and 4 show the same for extract prepared from 0-10 hour embryos. Comparison of 0-7 hour with 0-10 hour embryos revealed that fEBP 1 and 2 are more strongly expressed in older embryos. However, expression does not continue at such a high level, since 0-16 hour embryos (lane 5) showed a decrease in the levels of both complexes. Comparison of lanes 2 and 4 showed that the detrimental effect of freezing on the DNA binding activity of the proteins was more pronounced on younger embryos, and on fEBP 1 rather than fEBP 2. Evaluation of lane 4 versus lane 6 showed that the extract in lane 6 had significantly less activity than that in lane 4. The lane 6 extract was prepared from embryos of the same approximate stage as those used for the extract in lane 4, but the lane 6 embryos were held at -20° C for over one year, after which they were prepared using the same protocol as those in lane 4. Embryos used to make extract used in the reaction shown in lane 4 were held at -20°C for less than two months. This effect was observed with a number of extracts made from embryos held at -20° for over a year, and appears to be a result of long-term storage. Therefore, both fEBP 1 and 2 were degraded by the freezing of embryos prior to nuclear extract preparation.

To confirm that fEBP 1 and 2 were expressed primarily in older embryos, nuclear extract from 0-2.5 hour embryos was compared to extract from 0-6 and 0-

9 hour embryos. In Figure 5B, the results of this mobility shift assay are shown. Lane 3 shows the result of an assay using 0-9 hour embryos, lane 2 shows the same for 0-6 hour embryos, and lane 1 shows an assay using 0-2.5 hour embryos. Comparison of lane 1 with lanes 2 and 3 shows that young embryos had low levels of fEBP 2, and negligible amounts of fEBP 1. No additional DNA binding complexes were detected in 0-2.5 hour embryos, indicating that there is apparently no change over time in the proteins which bind to this particular site, and that fEBP 1 and 2 are not strongly co-expressed with *ftz*.

The third, most quickly migrating complex seen in all three lanes of panel B (indicated by an open arrow) is apparently non-specific. Examination of panel A shows that the complex was not present in every extract prepared. It was not originally identified as a protein which specifically bound to site 2 (Han et al., 1993). Since this shifted complex presumably did not contain a specific binding protein for this site, it was not considered in future evaluations.

Thus, fEBP 1 and 2 were expressed most strongly in embryos of 0-9 hours. Freezing of embryos prior to processing had a deleterious effect on fEBP 1 and 2 DNA binding ability, which was more pronounced in 0-6 hour embryos. Consequently, since both proteins seemed to be more stable and more abundant in 0-9 hour embryos, we decided to harvest embryos of this age and process them immediately into nuclear extract in order to maximize fEBP 1 and 2 recovery.

Examination of the possible presence of Ftz protein in fEBP 1 or 2:

Protein binding site 2 was previously determined to be the highest-affinity Ftz homeodomain binding site in the *ftz* proximal enhancer (Pick et al., 1990). We wished to determine if we could detect native Ftz protein interacting with this

site. Accordingly, the ability of an anti-Ftz antibody to interfere with the interaction of fEBP 1 or 2 with oligonucleotide 2 in EMSA, either by abolishing the complex formation or by causing a "supershift" of the complex was investigated. Either result would indicate that the antibody recognized some component of the fEBP 1- or fEBP 2-DNA complex.

Affinity-purified anti-Ftz polyclonal rabbit antibody was prepared in our laboratory by Yan Yu, and shown by her to supershift bacterially produced Ftz in EMSA (data not shown). Antibody or pre-immune serum was incubated in an oligonucleotide 2 mobility shift assay with nuclear extract prepared from 0-10 hour embryos. Figure 6 shows the results of this experiment. Lane 1 shows nuclear extract alone, lane 2 shows the same extract incubated with 1 ml of antibody. Lane 3 shows extract incubated with 1 ml of pre-immune serum, as a control for antibody specificity. Comparison of these three lanes showed that neither binding factor's interaction with the oligonucleotide was abolished, nor was either complex "supershifted" by the addition of antibody. The increase in band intensity upon addition of antibody is a non-specific effect of the serum, since the same effect was seen in the lane where pre-immune serum is added. This experiment was repeated on extract prepared from embryos of 0-2 hours and 0-6 hours in age, and on fresh and frozen embryos (data not shown). In no case was any change in the pattern of bands detected upon addition of anti-Ftz antibody. The most likely explanation for these results is that neither fEBP 1 or 2 contain Ftz protein. It is formally possible that Ftz protein is present and is somehow not accessible to the antibody. This cannot be ruled out without purification of the two factors.

Tests of various methods of protein purification:

To isolate the protein(s) interacting with oligonucleotide 2, an approach that had proved successful in the past (Briggs et al., 1986; England et al., 1990; Han et al., 1998) was planned. In this scheme, Drosophila embryo nuclear extract was first subjected to two or three steps of "conventional" purification, and then to several rounds of DNA affinity purification (Kadonaga and Tjian, 1986). Purified protein(s) recovered from affinity chromatography would be sent to the Mount Sinai Protein Core for microsequencing.

A number of potential purification steps were tested in order to develop a scheme for large-scale purification of the proteins making up fEBP 1 and 2. The results of these tests are summarized in Table 1. In each case, the fractions were assayed by mobility shift assay for the presence of DNA binding activity which recognized oligonucleotide 2. These assays were quantified using an PhosphorImager screen (Molecular Dynamics) and ImageQuant software, and were not usually exposed to film. As a result, only the quantified DNA-binding data was available for most of these tests. All column resins were obtained from Pharmacia, unless otherwise noted.

1) DEAE Sephacel:

148 mg of 0-9 hour embryo nuclear extract were applied to a 10 ml DEAE Sephacel anion exchange column which had been previously equilibrated with 0.1 M KCl/HEMG. The column was washed through with 0.1 M KCl/HEMG, and was eluted with 0.25 M KCl/HEMG and then with 0.5 M KCl/HEMG. The protein peaks from each step were pooled and assayed by EMSA for the presence of oligonucleotide 2 DNA-binding activity. fEBP 1 and 2 activities were found only in the flowthrough pool, indicating that DEAE did not bind either protein.

fEBP 1 specific activity went from 393 U/mg in the embryo nuclear extract to 413 U/mg in the flowthrough pool. fEBP 2 specific activity went from 446 U/mg to 256 U/mg. The unit yield for fEBP 1 was 53%, and was 29% for fEBP 2, and 86% of the protein was recovered in the flowthrough. Thus, no increase in specific activity of either protein was observed in the pooled flowthrough fraction. Consequently, DEAE Sephacel was rejected as a potential purification step.

2) P11 Phosphocellulose:

Flowthrough from the previously described column was divided and loaded onto a number of columns before the quantification data had been analyzed. Since the relative concentration of fEBP 1 and 2 was approximately the same in the DEAE flowthrough as in embryo nuclear extract, I proceeded to use this pool for a preliminary test of various columns. 11.4 mg of DEAE flowthrough were loaded onto a P11 phosphocellulose column (Whatman), a weak cation exchange column. This was washed with 0.1 M KCl/HEMG and eluted with a KCl gradient which increased from 80 mM to 1 M. Every other fraction from the elution, and the pooled protein peak from the flowthrough, was assayed by EMSA. fEBP 1 and 2 were detected in fractions 13-17 which were shown by measurement of their conductivity to correspond to a KCl concentration of 250 mM. Quantification of these pooled fractions' EMSA by PhosphorImager showed a 3.5-fold purification for fEBP 1 with 4.7% unit yield, and 3.5-fold purification for fEBP 2, with 4.8% unit yield. 1.4% of the protein was recovered in these fractions. Thus, while P11 phosphocellulose resulted in purification of fEBP 1 and 2, due to its very low unit yield it would not be very useful in large-scale purification of these factors.

3) CM Sepharose:

DEAE flowthrough was also loaded onto CM Sepharose, a weak cation exchange matrix which lacks the phosphate side groups of P11 phosphocellulose. Following the loading of 12.5 mg, the column was washed with 80 mM KCl/HEMG and eluted stepwise with 0.25 M, 0.5 M, and 1 M KCl/HEMG. EMSA of the protein peaks from the flowthrough and the 1 M eluate revealed O2 binding activity only in the flowthrough pool, indicating that neither protein interacted with this column. The 0.25 and 0.5 M washes did not result in the elution of an appreciable protein peak, and were thus not assayed. Figure 7 shows the autoradiograph of this assay. Lane 1 shows a sample of the load, lane 2 shows the pooled flowthrough peak, and lane 3 shows the pooled 1 M KCl peak (adjusted to 0.1 M KCl before assaying). Since neither protein was bound by this column, it was dropped from consideration.

4) SP Sepharose:

SP Sepharose, a strong cation exchanger, was also tested for its ability to purify fEBC 1 and 2. 15 mg of DEAE flowthrough were loaded onto the column, which was then washed with 0.1 M KCl/HEMG. The column was sequentially eluted with 0.25 M and 0.5 M KCl/HEMG, and protein peaks from each step were pooled and assayed. fEBP 1 and 2 were detected in the 0.5 M pooled eluate. Quantification of this pool's DNA binding activity revealed a 8-fold purification for fEBP 1 with a 22% unit yield, and an 16-fold purification for fEBP 2 with a 42% unit yield. 2.9% of the protein was recovered in this pool. Since this result was better than that seen with P11 phosphocellulose, SP Sepharose displaced it as the ion-exchange column of choice for the final scheme.

5) Phenyl Sepharose:

Phenyl Sepharose, which contains a hydrophobic moiety that makes it particularly useful for purifying proteins with large hydrophobic domains, was also evaluated. The column was loaded with 15 mg of DEAE flowthrough and washed in 0.1 M KCl/HEMG, and eluted with successive steps of 0.25 M and 0.5 M KCl/HEMG. No DNA binding activity was detected in any pooled protein peaks. This could be due to dissociation of proteins from each other, if fEBP 1 and 2 are multi-protein complexes, or the dissociation of some necessary cofactor from the protein(s). In either case, loss of DNA-binding activity following this column made it unacceptable as a purification step. However, its future potential for dissecting the functions of fEBP 1 and 2 should be considered.

6) Single-stranded DNA Cellulose:

Single-stranded DNA cellulose was loaded with 15 mg of DEAE flowthrough, washed with 0.1 M KCl/HEMG and eluted with 0.25 M and 0.5 M KCl/HEMG. EMSA of the pooled protein peaks showed that fEBP 1 and fEBP 2 were both in the 0.5 M HEMG/KCl pool. fEBP 1 had an increase in specific activity of 3.4-fold, and an unit yield of 39%. fEBP 2 was purified 4.3-fold and had a unit yield of 50%. 27% of the protein was recovered in this pool. These results showed that the column could bind and moderately purify both fEBPs. However, it was discarded from consideration when later tests of the column revealed that this result was not reproducible.

7) Sephacryl S-200:

A 38 ml Sephacryl S-200 size-separation column, was loaded with 19 mg of nuclear extract and washed with 0.1 M KCl/HEMG, with a flow rate of 9 ml/hour. Every other fraction of the protein peak of this column was assayed by EMSA and quantified using the PhosphorImager. Pooling of the fractions

containing fEBP 1 and 2 showed that fEBP 1 had been purified 11 fold, with a 141% unit yield. fEBP 2 had been purified 33 fold, with a 430% unit yield. 12.5% of the protein had been recovered in this pool. This column was marked as a possible first step in the purification scheme, since it resulted in the purification of both factors, with excellent unit retention.

The apparent increase in units recovered from the S-200 column was postulated to be due to the loss of some inhibitory factors(s) which normally masked fEBP 1 and 2 activity in nuclear extract. To test this possibility, a "combinatory" EMSA was performed, in which pooled fEBP 1 or 2 fractions from an S-200 column were combined with various fractions from the rest of the S-200 protein peak (see Figure 8). Quantification of this assay is showed that the fEBP 1 pool alone had an activity of 32,050 units. Combination of this pool with itself resulted in 67,283 units, which was approximately what was expected. The combination of the fEBP 1 pool with fractions 50, 54, 68-78, or 82 had no effect on fEBP binding activity, as in these cases the activity of fEBP was unchanged. The combination of the fEBP 1 pool with fractions at the beginning of the protein peak up to fraction 46, resulted in a decrease in fEBP 1 binding activity. In these assays, fEBP 1 activity was reduced from ~30,000 units to less than 20,000. The effect was most marked in fraction 34, where fEBP 1 activity was cut in half. This suggests that there was some fEBP 1 inhibitory factor in nuclear extract which was separated from it by the action of the S-200 column.

A similar result was found for fEBP 2. Pooled fEBP 2 fractions alone gave an activity of 125,162 units. Combination of the fEBP 2 pool with itself resulted in 281,366 units. Addition of fraction 82 had no effect on fEBP 2 binding activity. However, addition of any fractions which eluted earlier than the fEBP 2 pool resulted in a decrease of fEBP 2 binding activity. The most conspicuous fEBP 2 inhibition was seen with the addition of either fractions 50 or 54, where binding

activity was reduced to ~35,000 units, a three-fold drop in activity. The large spread of this inhibitory effect over the protein peak could in theory represent more than one factor, or one factor which is spread over a wide range of the column eluate. Thus, this experiment confirmed the existence of fEBP 2 inhibitory factors, although the exact number of factors remains to be determined.

In summary, DEAE Sephacel, P11 phosphocellulose, CM Sepharose, phenyl Sepharose, and single-stranded DNA cellulose were eliminated from consideration based on their inability or limited ability to purify fEBP 1 or 2. SP Sepharose and Sephacryl S-200 were selected as candidates for the final purification scheme, based on their ability to purify fEBP 1 and 2 with fair retention of total activity.

A possible third protein binding to oligonucleotide 2:

One additional column was tested for its utility in purifying proteins which bound to oligonucleotide 2, with surprising results. Cibacron Blue Sepharose (Sigma), which has a blue dye molecule attached to the column matrix, was loaded with 12.5 mg of DEAE flowthrough, washed with 0.1 M KCl/HEMG, and eluted with 0.25 M and 0.5 M KCl/HEMG steps. EMSA of the pooled protein peaks showed that fEBC 1 and 2 were not recovered from this column (see Figure 9, panel A), possibly for the same reasons advanced in the case of phenyl Sepharose, and possibly because they bound so tightly to the column that they could not be eluted. To test this latter possibility, the column was eluted with 1 M KCl/HEMG. EMSA of this pool, adjusted to 0.1 M KCl,

showed neither binding activity (Figure 9A, lane 5), leaving this question unanswered.

However, the 0.5 M KCl pool contained a protein which generated a novel complex in EMSA with oligonucleotide 2 (Figure 9A, lane 4, indicated by arrow). This complex had a mobility which was intermediate to fEBP 1 and 2. This complex could represent a breakdown product of fEBP 1. Alternatively it could be a novel complex whose interaction with oligonucleotide 2 is normally inhibited by the presence of fEBP 1 and 2. To determine if this complex contained Ftz protein, anti-Ftz polyclonal rabbit antibody was included in a mobility shift assay of this pool. Figure 9, panel B shows that while the complex was apparently abolished by the addition of antibody (lane 2), preimmune serum had the same effect (lane 3), indicating that this was a non-specific result. Further testing is necessary to determine if this complex contains Ftz protein, a breakdown product of fEBP 1, or a novel DNA binding protein.

Attempted large-scale purification of fEBP 1 and 2:

A scheme for the purification of fEBP 1 and 2 was established, based on the results of the previously detailed tests of various columns, and on schemes employed to purify other transcription factors (Briggs et al., 1986; Han et al., 1998) (Figure 10). Nuclear extract would be initially separated on the basis of the size of its components by passing the extract over an Sepharose S-200 size separation column. Those fractions which contained fEBP 1 or 2 would then be pooled and loaded onto an SP Sepharose cation exchange column. The 0.5 M KCl eluate from this column would be adjusted to 0.1 M KCl, and loaded onto the first of two DNA affinity columns. After two rounds of DNA affinity chromatography, the resulting proteins would be separated by electrophoresis

on an SDS-polyacrylamide gel. The bands corresponding to either fEBP 1 or 2 would be cut out and sent to the Protein Core at Mt. Sinai Medical Center for microsequencing. The identification of the band which contained fEBP 1 and 2 would be made on the basis of its size, as determined from UV crosslinking or Southwestern blotting.

500 mg of nuclear extract was loaded onto a 500 ml S-200 column and washed with 0.1 M KCl/HEMG, at a flow rate of 90 ml/hour. EMSA of every other fraction of the protein peaks was performed, and the fractions containing oligonucleotide 2 binding proteins were pooled and held on ice at 4°C with added protease inhibitors for a maximum of 3 days while the rest of the fractions were collected. Figure 11 shows the result of EMSA on one of the four S-200 columns' fractions. fEBP 1 was detected in fractions 48 to 52. fEBP 2 was found between fractions 46 and 58. Fractions 48-58 were pooled for loading onto the next column.

All four fEBP peaks were pooled, for a total of 98.7 mg, a 19% protein yield. This was loaded onto a 3 ml SP Sepharose and washed with 0.1 M KCl/HEMG. The SP Sepharose column was eluted with sequential steps of 0.25 M and 0.5 M KCl/HEMG. The 0.5 M KCl peak, which contained 12.9 mg, was adjusted to 0.1 M KCl and loaded onto an 1 ml oligonucleotide 2 DNA affinity column. The O2 column was washed with 0.1 M KCl/HEMG, and eluted with 0.25 M and 0.5 M KCl/HEMG. EMSA of the protein peaks showed that fEBP 1 and 2 were in the 0.5 M eluate, therefore this pool was adjusted to 0.1 M KCl and loaded a second time onto the O2 column. This column was washed and eluted as before.

EMSA was performed on samples which were reserved from each step of the procedure and stored on ice at 4°C. This assay was quantified using a PhosphorImager, and was then exposed to film (Figure 12). Lane 1 shows nuclear

extract, lane 2, a sample from the pooled S-200 fractions, lane 3, the SP Sepharose 0.5 M KCl pool. Lanes 4 and 5 show the two rounds of DNA affinity chromatography. fEBP 1, the more fragile of the two complexes, did not survive the procedure, and degraded even in the nuclear extract which was held at 4°C (lane 1). fEBP 1 was originally present in the nuclear extract, as Figure 11, lane 1 shows it before it had degraded. fEBP 2 appeared to increase in intensity until the SP Sepharose step (lane 3), and then decrease with each round of DNA affinity chromatography. However, examination of the amounts of proteins used in each assay (see Figure 12 legend) revealed that the EMSA for the two rounds of DNA affinity columns used 2.5 and 8 times less protein, respectively, than the amount used to assay the SP eluate. Thus, it appeared that fEBP 2 had been purified at each step of the procedure.

PhosphorImager quantification of the EMSA shown in Figure 12 showed that fEBP 2 had been purified in most, but not all, stages of the process (Table 2). The S-200 column resulted in a 54-fold purification, as measured by the increase in specific activity, and a recovery of 1070% units. This apparent increase in units was probably due to the loss of some inhibitory factor, and is consistent with the findings reported in Figure 6. SP Sepharose resulted in a further 7-fold increase in specific activity, with a 93% retention of units. The first round of DNA affinity chromatography resulted in a slight decrease in specific activity, accompanied by a loss of 90% of the units of activity. The second pass over this column gave a 6-fold increase in specific activity, and retention of 20% of the units. Thus, the overall purification of fEBP 2 was approximately 1800-fold.

Samples from each step of the purification were separated on a 10% SDS polyacrylamide gel, and the gel was silver-stained. Following silver-staining, the gel was briefly immersed in Coomassie Blue to increase the contrast of the bands (Figure 13). Lane 1 shows nuclear extract, lane 2 shows the S-200 pool, lane 3

shows SP Sepharose 0.5 M pool, and lane 4 shows the 0.5 M eluate from the second DNA affinity column. At least 6 discrete bands remained after the second round of DNA affinity chromatography (lane 4). One of these bands, at about 55 kDa, was particularly abundant. Unfortunately, this band was also visible throughout the procedure. This was also the case for the bands at 40 and 32 kDa. Given that transcription factors are in very low abundance in the cell, if a protein band could be discerned in nuclear extract, it was unlikely to be a transcription factor. Thus, the most visible bands in the DNA affinity column eluate may represent non-specific proteins. This made it difficult to immediately determine which, if any, bands should be isolated and sent for sequencing. Alternate methods to determine the sizes of fEBP 1 and 2 were therefore pursued.

Determination of the sizes of fEBP 1 and 2:

UV Crosslinking:

Nuclear extract and the eluate of both rounds of affinity chromatography from the large-scale preparation of fEBP 1 and 2 were incubated with bromodeoxyuridine-substituted oligonucleotide 2, UV crosslinked, separated on an SDS gel, and exposed to film. Figure 14, lane 1, shows nuclear extract, lane 2 shows the eluate of the first DNA-affinity column, and lane 3 shows the eluate of the second affinity column. The major crosslinked species, which increased in specific activity as the purification proceeded, had a mobility of approximately 55 kDa. The size of this band corresponded to the size of the predominant band observed in the silver-stained SDS gel shown in Figure 13. However, the possibility that these bands were non-specific still existed. Thus, Southwestern blotting using mutant forms of the oligonucleotide binding site was undertaken to determine if the proteins binding to oligonucleotide 2 were specific.

Southwestern Blotting:

A Southwestern blot was performed to confirm the specificity of binding of the 55 kDa protein. By probing membrane-immobilized proteins with native oligonucleotide probe and with mutated probe which did not interact with fEBP 1 or 2, it was expected that we could distinguish between specific and non-specific proteins interacting with oligonucleotide 2. Two different mutant versions of oligonucleotide 2 (prepared by W. Han (Han et al., 1993), shown in Figure 15A) were tested by EMSA for their ability to interact with fEBP 1 and 2 and Ftz protein (Figure 15B). Lanes 1 and 2 show EMSA using wild-type oligonucleotide 2 and either nuclear extract or bacterially produced Ftz protein. Lanes 3 and 4 show EMSA using mutant oligonucleotide 1 (M-O-1), which was designed to disrupt the interaction between Ftz protein and its binding site (Pick et al., 1990). M-O-1 did not bind to bacterially produced Ftz protein (lane 2), nor did it bind fEBP 1, although its interaction with fEBP 2 was apparently unaffected (lane 1). Mutant oligonucleotide 9 (M-O-9) was designed to disrupt the Ftz binding site and the site at which fEBP 1 and 2 were thought to contact DNA (Han et al., 1993). EMSA using this oligonucleotide is shown in lanes 5 and 6. This oligonucleotide did not interact with bacterially produced Ftz protein (lane 6) or with fEBP 1 or 2 (lane 5). It did, however cause the formation of a new set of protein-DNA complexes (lane 5, indicated by open arrows), possibly because a new protein binding site was inadvertently created in this oligonucleotide. Thus, the mutant oligonucleotides resulted in a different spectrum of specific protein binding.

M-O-1 and M-O-9 were tested in comparison to wild-type O2 in a Southwestern blotting experiment. The Southwestern blot on nuclear extract and the 0.5 M elution from an SP Sepharose column was performed by running each

sample on an SDS gel and transferring the proteins to a membrane. The renatured proteins immobilized on the membrane were hybridized to either radioactively labeled oligonucleotide 2 or a mutant oligonucleotide (Figure 16). Lanes 1 and 2 show the result using native oligonucleotide 2 as a probe. Three major bands hybridized to the probe in the nuclear extract and in the SP eluate, one at about 60 kDa, one at 51 kDa, and one at about 35 kDa. The smallest band appeared to be more intense in the SP eluate, suggesting that it had been enriched by the action of this column. The size of the 51 kDa appeared to agree with the size of the protein identified by UV crosslinking, shown in Figure 14. However, as lanes 3 and 4 show for M-O-1 and lanes 5 and 6 show for M-O-9, the same bands hybridized to these mutant oligonucleotides, indicating that they were not specifically binding to O2. One additional band at about 27 kDa was seen in lane 6 (indicated by open arrow), which could correspond to one of the newly-created complexes formed with this oligonucleotide in EMSA (see Figure 15, lane 5).

These results strongly suggested that the most prominent bands seen in the silver-stained gel shown in Figure 13 were non-specific DNA binding proteins which were similar enough in their physical characteristics to be co-purified with fEBP 1 and 2. Based on the apparent abundance of non-specific proteins seen in the silver stained gel, along with the fact that neither Southwestern blots nor UV crosslinking were able to identify any specific bands which interacted with the oligonucleotide 2 probe, it was determined that this preparation was unsuitable for recovering proteins for microsequencing.

Revising the purification procedure:

In the course of re-examining and optimizing the purification process (see Discussion for a detailed treatment of this topic), a few additional experiments were performed.

In order to increase the resolution of fEBP 1 and 2 during size separation chromatography, a fast-pressure liquid chromatography (FPLC) column was tested as a possible replacement for traditional low-pressure columns. A 15 ml Superose-12 FPLC column was loaded with 0.78 mg of ammonium sulfate-precipitated eluate from an SP Sepharose column, and washed with 0.1 M KCl/HEMG. Figure 17 shows the result of EMSA of the column's fractions. fEBP 1 eluted off the column from fraction 19 to fraction 31. fEBP 2 eluted from 23-37, although it was strongest in 25-31. The other complexes seen in this gel are apparently nonspecific, as they were not observed in other gel retardation assays. Fractions 19-31 were pooled, assayed by EMSA, and quantified using a PhosphorImager. Specific activity of fEBP 2 increased from 1856 to 5165 units, a 2.8-fold purification, with a unit yield of 77%. Thus, this column did not perform with the same efficiency as its low-pressure counterpart. The apparent increase in units observed following low-pressure size separation columns was not seen in the case of the FPLC column. However, Table 3 shows that the SP Sepharose column used prior to the FPLC column resulted in an unprecedented 32-fold purification of fEBP 2, with 100% retention of units. This suggests that the loss of inhibitory factors which was previously noted when size filtration chromatography was used as the first step in the purification of crude nuclear extract was now occurring on the ion-exchange column.

In order to increase the unit yield from the ammonium sulfate precipitation step shown in Table 3, various concentrations of the salt were tested

for their efficiency in precipitating fEBP 1 and 2. Table 4 shows the result of quantification of protein precipitation by ammonium sulfate. Ammonium sulfate was added to five aliquots of nuclear extract in amounts corresponding to 20%, 30%, 40%, 50%, or 60% saturation. The precipitate was resuspended in no-salt HEMG, assayed by EMSA and quantified by analysis of PhosphorImager data. The greatest recovery of units of fEBP 1 and fEBP 2 was found in the 20% AS precipitate. 55% of fEBP units were recovered with a 4.6 fold purification, and 60% of fEBP 2 units, with a 5 fold purification. No other concentration of AS resulted in more than a 25% fEBP 1 unit recovery, and a one-fold purification. Similarly, no other concentration allowed more than a 40% recovery of fEBP 2 units, with a one-fold purification. Thus, low concentrations of ammonium sulfate resulted in higher unit recovery of oligonucleotide 2 binding activity, as well as moderate purification.

A detailed description of proposed changes to the purification protocol is included in the Discussion.

Chapter 4. Results II: Mutagenesis of *Drosophila adf-1*

Background:

As I discussed in the introduction, Adf-1 was identified as one of the proteins which interacts with protein binding sites of the *ftz* proximal enhancer (Han et al., 1993). The sites with which Adf-1 interacted were also those which interacted with a number of other potential *ftz* regulators, including Ftz-F1 and Ttk (see Figure 3). A germline clone of *ftz-f1* mutant has been shown to have a subtle effect on the maintenance of *ftz* expression (K. Su and L. Pick, unpublished). It is thought that redundancy among the proteins interacting at the sites where Ftz-F1 binds could account for the modest degree of change in *ftz* expression in these mutants. Consequently, in order to determine the contribution of Adf-1 in regulating *ftz*, mutagenesis of the *adf-1* gene was carried out.

***adf-1* mutagenesis outline:**

A line of flies carrying a P element insertion on the second chromosome ($P^{[sced]}$/CyO) was given to us by W. Theurkauf, at the State University of New York at Stonybrook (Sullivan et al., 1993). The insertion was characterized by his laboratory to be within 3 kilobases of the *adf-1* start site. Given that *adf-1* does not appear to be expressed maternally (Engels, 1992), we initially designed a screen for mutations at the *adf-1* locus based on zygotic lethality. Mutations in many zygotically expressed embryonic patterning genes were found to be homozygous lethal (Nusslein-Volhard, 1980).

The strategy for the mutagenesis of the *adf-1* locus is as follows (see Figure 18): flies carrying the P element insertion were mated to flies carrying an

immobilized copy of the P element transposase (Robertson et al., 1988). The male progeny of this cross, carrying both the transposase and the P element, were then mated to flies carrying two balancer second chromosomes. This cross removed the transposase and at the same time, by virtue of the balancer chromosome, prevented any further recombination at the site from which the P element was mobilized. The rationale for selecting male progeny whenever possible is that mitotic recombination in the germ cells is suppressed in male *Drosophila* (Greenspan, 1997). The progeny of the second cross were next mated to flies carrying a deficiency in the chromosomal region of the *adf-1* locus. This deficiency, Df(2R)42, extends from band 42 C2-C8 to 42 D2-D3, which includes the location of the *adf-1* locus (42 C2-C7), as determined by *in situ* hybridization (England et al., 1992). Their offspring were assessed for lethal disruptions in the region "uncovered" by the deficiency. This is described below. The $P^{[sced]}$ insertion were found to be viable over this deficiency.

A detailed scheme of the cross, indicating the phenotypic markers used at each stage is shown in Figure 19A. The P element insertion line, $P^{[sced]}$, was characterized by the presence of the *miniwhite* gene included in the P element vector as a marker (Bier et al., 1989). This marker gives an orange eye color when it is expressed in a *white* background. The insertion was maintained over a balancer chromosome, CyO, which is marked by curly wings. The P element transposase $P^{[\Delta 2\text{-}3]}$ was on a chromosome which carried the Stubble marker (Sb), a bristle phenotype. Male offspring from cross 1 were chosen if they carried both the orange eyes characteristic of the P element, and the short bristles indicating the presence of the transposase.

The second cross (F1), to female flies which were doubly balanced on the second chromosome, had several possible outcomes. The P element could either excise exactly or inexactly, in which case the *miniwhite* gene would be lost and

the eyes of the progeny would be white. Inexact excision could remove part of the *adf-1* coding or regulatory regions. It is important to note that white-eyed flies which had not experienced P element excision would result from this cross. These flies would have inherited one balancer second chromosome and one second chromosome which had never had a P element insertion, resulting in white eyes. These would be phenotypically indistinguishable from flies which had experienced P element excision. Alternatively, the P element could relocate within the genome ("hop"), in which case the eyes would be orange. It has been shown that P elements will tend to reinsert near the site of their former insertion (Tower et al., 1993). Thus, some of the flies which had experienced a P element hop were likely to have disrupted the nearby *adf-1* gene.

The offspring of the test cross of P element-rearrangement flies to flies carrying the deficiency were assessed on the basis of their wing phenotypes (see Figure 19B). A lethal interaction between the deficiency and the P element hop/excision was inferred on the basis of wing and eye phenotype. If the altered second chromosome, identified by normal eyes, was inherited along with the chromosome carrying the deficiency, the fly's wings would be straight. This fly would be viable only if the P element hop or excision did not adversely affect an essential gene which was likewise deleted as part of the deficiency on the other chromosome. Thus the lethal effect of the P element hop or excision could be assessed by the presence or absence of normal-eyed, straight-winged offspring from this cross.

Mutagenesis Results:

The first cross was established with 60 P element-bearing males and 30 females carrying the transposase. 163 male offspring of the first cross were mated

to 121 Gla/CyO females. Of 3070 flies obtained from this cross, 140 of the offspring had white eyes and no transposase, while 192 had orange eyes and no transposase. Some of the white-eyed flies represented those which had never contained a P element, as was discussed above. In addition, a certain number of the orange-eyed offspring of this cross could represent flies in which the P element had not moved. However, the majority of flies with orange eyes had eyes of a shade that was different from that of the original P insertion line, or had variegated eyes. These differences in color indicate that the P element had probably moved, since the *miniwhite* gene is quite variable in its expression depending on its site of insertion (Pirrotta, 1988). Since it was impossible to distinguish these two classes of flies from those which had actually experienced a P element excision or hop, all white-eyed or orange-eyed F2 offspring were mated individually to females carrying Df(2R)42, since this test cross would allow the unambiguous identification of lines which had relevant P element mobilizations. 266 individual crosses were established, 137 excisions and 129 hops. A few lines were lost in the extra cross necessary to replace the CyO with the Gla chromosome in half the F2 offspring (see Figure 19). From these crosses, two excisions and eight hops were found to be lethal over the deficiency.

Non-complementation by an *adf-1* null allele:

During the course of this study, a strain of flies carrying a putative deletion of most of the *adf-1* coding region was generated in the laboratory of T. Tully, at Cold Spring Harbor. A P element insertion into the *adf-1* gene was identified in a screen for flies with impairments in learning and memory. This P element was then excised to create a deletion of 1.6 kb, which included most of the coding region of *adf-1*. Figure 21 shows a diagram of the *adf-1* coding region,

the P element insertion identified in their screen, and the deletion, adf^{de60}, which was generated. They agreed to enter into a collaboration with us to determine the embryonic implications of this *adf-1* null mutation.

The eight lethal P element hops and two lethal P element excisions generated in our lab were crossed to adf^{de60} flies in order to determine if any of them could complement that allele (Figure 22). Failure to complement could indicate a lesion in the same gene affected in adf^{de60}. One excision and three hops (excision E2 and hops H2, H3, H4) failed to complement adf^{de60}, indicating that these four lines had lethal disruptions of *adf-1*. In addition, two hops (H5, H8) showed a partial ability to complement the adf^{de60} allele. This was identified by the presence of straight wings in the cross of adf^{de60}/CyO x Hop/CyO at frequencies lower than the 33% expected of full rescue. H5 and H8 each had only 17% straight wings among the progeny of this cross. This could indicate that these two hops resulted in hypomorphic alleles of the gene, which produced a partially active *adf-1* gene product. In sum, of the ten lines which were identified in our genetic screen to be lethal over a deficiency in the region of (2R)42, four of them proved to be *adf-1* null mutants, and two more were candidate hypomorphic alleles.

Molecular analysis of mutations in the *adf-1* locus:

A Southern blot was performed to verify that the *adf-1* gene was disrupted in lines which were identified genetically as *adf-1* mutants. Approximately 15 mg of genomic DNA from either wild type flies or *adf-1* mutant lines E2, H2, or H4 was digested with Eco RI, Bam HI or Hind III. The digested DNA was separated on a 0.8% agarose gel and transferred to a nylon membrane. The membrane was hybridized overnight with radioactively labeled

cDNA corresponding to the complete *adf-1* coding region, washed with increasing stringency and exposed to film (Figure 23).

Wild type DNA (lanes 1-3) showed only one major band hybridizing to the probe, confirming that a single copy of the *adf-1* gene exists in the genome. A previous blot confirmed that the $P^{[sced]}$ chromosome and the CyO chromosome had banding patterns identical to wild type (data not shown). *adf-1* mutant line E2 (lanes 4-6) showed a slight reduction in the apparent mobility of the Eco RI band. This could correspond to a significant change in the size of this band, but due to its large size it was not resolvable on this gel. The 6 kb Bam HI and 9 kb Hind III bands were unchanged. *adf-1* mutant line H2 (lanes 7-9) exhibited changes in the hybridization pattern which were consistent with an insertion into the coding region of *adf-1*. The Eco RI band was unaffected; however, the Bam HI lane showed two bands hybridizing to the probe, a ~6 kb band which corresponded to the unaffected CyO chromosome and a larger band of undetermined size. Similarly, the Hind III digested DNA had two hybridized bands, of which the smaller 9 kb band corresponded to the unaffected CyO chromosome. This altered hybridization pattern confirmed that the P element with its additional restriction enzyme recognition sites had inserted into the *adf-1* coding region. The H4 (lanes 10-12) hybridization pattern was seemingly unchanged from wild type. This could indicate that the insertion in this line took place outside of the *adf-1* coding region, in some essential regulatory sequences for the gene. In sum, of the four *adf-1* null alleles tested, two had definite insertions into the *adf-1* coding region, and one had an excision of unknown size removing part of the *adf-1* coding region.

To pinpoint the exact site of insertion in H2, I attempted to sequence DNA around this location. Plasmid rescue was performed on genomic DNA from H2 flies, a procedure which takes advantage of the P element having been

engineered to include a bacterial origin of replication and ampicillin resistance gene. DNA was digested with an enzyme which cuts only once in the P element, the digestion was then ligated, and the ligation reaction was transformed into bacteria. Miniprep DNA from the resulting colonies was used for sequencing. Unfortunately, although primers within the P element generated readable sequence, no attempt to read outside the P element was successful. The reasons for this are unclear at this time.

Reversion of the H2 P element insertion:

To confirm that lethality in the H2 line was the result of P element insertion, H2 flies were subjected to a series of crosses to cause the P element to precisely excise. It was predicted that this would cause the *adf-1* gene to regain its wild type function and thus be able to complement the adf^{le60} *adf-1* null allele. The strategy used for this was similar to that which was employed for the *adf-1* mutagenesis was accomplished (see Figure 24). The one major difference was that in order to test for reversion to wild type gene function, $P^{excision}$/Gla males were mated to adf^{le60}/CyO females and scored for complementation of adf^{le60} (Table 5). Of 75 putative revertants mated to adf^{le60} flies, 41 (54%) complemented adf^{le60}, indicating a reversion to wild-type gene function. Given that not all P element excisions are precise, this is strong evidence that removal of the P element restores the gene to its original state. 23 flies (30%) did not regain the ability to complement adf^{le60}, and probably represent imprecise excisions. 11 (14%) exhibited a partial ability to complement adf^{le60}, as demonstrated by the fact that they had less than 50% straight-winged flies in the progeny of the test cross (see Figure 24B). As with the two lines isolated in the original mutagenesis, these probably produce a partially active protein. These two latter groups are

particularly interesting. Those lines which did not revert probably include some imprecise excisions which have removed most of the gene, creating a true null mutant. The hypomorphs are equally interesting, since examination of the site of their gene disruption may give us some insight into gene function or regulation. Thus, in H2 it seems that lethality is the result of P element insertion into the *adf-1* coding region.

Rescue of putative *adf-1* mutants:

A fragment containing the *adf-1* coding region along with 11 kb of upstream and downstream sequence was ligated into the Carnegie-20 P element vector by members of Dr. Tully's laboratory. This vector was injected into embryos by Dr. Pick, and individual lines carrying this vector inserted into various loci in the genome were established. adf^{le60} flies and H2 flies were each mated to two lines, 27-1 and 76-2, both of which carried the P element insertion on the X chromosome. Figure 25 shows a diagram of the rescue crosses. The P element insertion lines were bred to a second chromosome balancer line, so that they carried the Gla marker. These P element; Gla flies were mated to either adf^{le60} or H2 flies, and their offspring which carried both the P element and one copy of the *adf-1* mutation balanced over Gla, were mated to each other. Since *adf-1* homozygotes were lethal, it was predicted that normal eyes, indicating two copies of the *adf-1* mutant chromosome, would appear in the offspring only if the P element insertion rescued the *adf-1* mutation. As Table 6 shows, both P element insertion lines were able to rescue the *adf-1* mutants adf^{le60} and H2. In crosses to the P insertion line 27-1, adf^{le60} and H2 resulted in 22% and 26% normal eye offspring. For line 76-2, adf^{le60} resulted in 12% normal eyes, and H2 had 14%. The predicted percentage of normal eyes which would be seen with full rescue was

27% (Figure 25). Since the rescue construct was not homozygous in the first cross, some *adf-1/adf-1* homozygotes in the third cross did not have a copy of the construct, thereby increasing the fraction of embryos which would be obligate lethals. Thus, P insertion line 27-1 was fully able to rescue adf^{le60} and H2. The slightly lower percent of normal eyes in the 76-2 line could be due to the effect of its different site of insertion.

Phenotypic Analysis of *adf-1* Mutants:

The mutations in adf^{le60}, E2, H2, 3, and 4 are homozygous lethal. In maintaining these lines over a CyO balancer chromosome, no straight-winged adult flies, indicating the presence of *adf-1* mutant homozygotes, are ever seen. To determine if any obvious defects were apparent in the adf^{le60} embryos, cuticles were prepared from this line of flies. Examination revealed only normal wild-type cuticles (data not shown). This could be explained in a few ways. First, the mutant embryos could develop normally and die as larvae. Secondly, they could die before hatching, but the fatal defect could be in an internal system, allowing the cuticle to appear normal. Thirdly, the mutant embryos could be dying before they secrete cuticle, meaning that the normal cuticles seen from this line represented those embryos which were not homozygous mutants.

By investigating the percent of embryos which hatched from the adf^{le60} line, we hoped to distinguish between the first and the other two explanations. Individual embryos were placed on agar plates and allowed to age for 36 hours. The number of embryos which had not hatched were counted, and these percentages were compared to control lines (Figure 26). Wild type flies had a baseline non-hatching rate of 5.2%. ve 48/CyO flies, a revertant line generated from the same P element insertion line which gave rise to the adf^{le60} deletion, was

included to control for the effects of that particular parental strain, and to account for lethality due to the CyO chromosome. These embryos had a non-hatching rate of 24.3%. This was probably solely due to the balancer, which is homozygous lethal in late embryogenesis (Ward, 1923). adf^{de60}/CyO embryos had a non-hatching rate of 44.9%. While half of this was due to CyO/CyO embryos, the rest was attributed to the lethal effect of adf^{de60}/ adf^{de60}. Therefore, adf^{de60} homozygous embryos die during embryogenesis, although whether or not they are able secrete cuticle was not distinguishable by this experiment.

The effect of *adf-1* mutation on *fushi tarazu* expression:

The expression of *ftz* in *adf-1* homozygote null mutants was examined, to judge if the lack of this potential *ftz* regulator resulted in any disruption of the *ftz* expression pattern. So that *adf-1* homozygotes could be unambiguously identified, adf^{de60} was balanced over a CyO chromosome carrying an insertion of the *lac Z* gene under the control of the *hunchback* promoter. Embryos carrying this chromosome express *lac Z* in the anterior of the embryo from very early stages until the end of germ band extension (Figure 27, panel A). Thus, *lac Z in situ* or anti-β–galactosidase antibody staining allows one to distinguish by process of elimination adf^{de60}/ adf^{de60} from adf^{de60}/CyO-*hb lacZ* embryos. Figure 27 shows the results of a *ftz*, *lac Z* double *in situ* staining performed on these embryos. *ftz* expression in stripes was apparently normal in adf^{de60} homozygotes, with the stripes appearing in the proper order (panels B - F), and decaying during germ band extension (panels G, H).

Expression of *ftz* in the nervous system of *adf-1* mutant embryos was given particular attention, since *adf-1* mutants were identified by Dr. Tully's lab due to learning and memory defects. In order to identify adf^{de60} homozygotes in these

embryos, a CyO balancer containing an insertion of *lac Z* under the control of the actin promoter was used. β–galactosidase in embryos carrying this balancer is expressed from the beginning of germ band extension through the end of embryogenesis (see Figure 28, panel I for an example). Figure 28 shows embryos from this population which were stained with polyclonal rat anti-Ftz antibody and *lac Z in situ* hybridized. The expression pattern of Ftz in the nervous system begins during late germ band extension, approximately stage 11 (Campos-Ortega and Hartenstein, 1985). At this stage only a few cells per segment expressed Ftz (panel A), but as germ band extension continued, the number of cells expressing Ftz increased in each segment to peak at approximately 22 cells per segment (panel B). Cells were counted in embryos which had been dissected flat and mounted ventrally (not shown). As the germ band began to retract, the number of cells expressing Ftz began to decrease from anterior to posterior (panel C). Ftz expression was detected until stage 13, the end of germ band retraction (panel D). No change in the pattern of Ftz expression in *adf-1* mutants was detected at any stage of expression in the nervous system (panels E-H). Nor was any change in the number of cells expressing Ftz detected (not shown). Thus, adf-1 mutation has no effect on the expression of ftz in the nervous system.

Development of reliable single-embryo PCR:

A number of reporter constructs containing the *lac Z* gene under the control of portions of the *ftz* regulatory region have been generated in the course of dissecting the *ftz* promoter (Han et al., 1993; Hiromi et al., 1985; Pick et al., 1990). We wished to test the expression of some of these constructs in *adf-1* mutant embryos. We were unable to identify homozygous mutants in these crosses of *adf-1* mutant flies to flies carrying the constructs by the simple method

described above of using balancer chromosomes with an expressible marker. Therefore, in order to identify homozygous *adf-1* null mutants in a mixed population of embryos, single-embryo PCR was developed.

In short, a population of embryos would be harvested, dechorionated, fixed and devitellinized, and stained with anti-β–galactosidase antibody. Following the staining reaction, embryos which had stained (indicating expression of the reporter construct) and those which had not would be individually genotyped, and the presence or absence of *adf-1* mutants among the population of staining embryos would be assessed. In order for this strategy to be successful, reliable PCR amplification of individual embryos' DNA was essential.

The primer pairs used in these PCR reactions were designed to hybridize to various regions in the *adf-1* locus. Figure 21D shows a diagram of the *adf-1* gene, as well as the PCR primers used in these experiments. The primer pair Adf-fwd/ Adf-rev, which was used in the reactions whose DNA products are shown in Figure 29A, was predicted to produce a fragment of 495 bases. The primer pair M3/M-rev (provided by J. Dezazzo and T. Tully) was used to distinguish a adf^{de60} chromosome from a normal one, based on the presence or absence of the 38 base pairs left at the original site of P element insertion in the parental line. The normal chromosome would result in a PCR product of 520 bases, while the adf^{de60} chromosome would generate a product of 558 bases (Figure 29B).

Initial preparation conditions were established using fixed, devitellinized, unstained embryos. Fixed embryos were placed in individual 0.5 ml Eppendorf tubes and incubated with a Tween/proteinase K solution for two hours (see Methods for details), in order to permeabilize the embryos. The embryos were then placed in a boiling water bath for 10 minutes to inactivate the proteinase.

Following inactivation of the proteinase, embryos were either stored at -20° C, or used immediately in a PCR reaction.

Approximately 50% of embryos pretreated in this manner resulted in PCR product using a pair of test primers (Figure 29A, lanes 2-5), as long as the proteinase was freshly prepared or frozen no more than once (data not shown). A number of variations on the proteinase K pretreatment were tested, and their success was measured by the number of embryos which could generate PCR product. In one test, embryos were manually broken, using a 21-gauge needle, prior to proteinase treatment (lanes 6-9). In another, the embryo was pipetted up and down in the proteinase solution every 15 minutes during the two hour incubation (lanes 10-13). In a third variation, the embryos were frozen at -80° C for one hour following the two hour proteinase treatment, and then boiled (Panel B, lanes 2-7). Neither manual breakage nor pipetting showed any increase in the number of PCR-positive embryos over 50%. However, freezing prior to heat-inactivation of the proteinase increased the number of embryos which gave PCR product to greater than 80%. It is assumed that the freezing and thawing of the embryo further permeabilized it, making the DNA more accessible to the PCR reagents. Thus, the most successful pretreatment of embryos was proteinase K/Tween digestion, freezing at -80°C, and then boiling.

A variety of reaction conditions were also tested. To overcome the problem of determining the ideal annealing temperature, "touchdown" PCR was used in which the temperature during the annealing step of each cycle dropped from 75°C to 50° C by 1 degree C every 5 seconds. The effect of varying the concentration of $MgCl_2$ was tested (Figure 30A). Lane 1 shows DNA from a reaction using control adf^{le60}/CyO DNA with the M3/M-rev primer pair. The two bands of 520 and 558 bp expected from this heterozygous DNA are visible, although indistinct. This separation problem was solved later by using 2%

agarose instead of the 1.5% used here. Lanes 2-6 show the results of reactions on single embryos, using [$MgCl_2$] of 1.5 mM, 1.625, 1.75, 1.875, and 2 mM, respectively. Lane 4 shows a single band of 558 bp, indicating that the starting material was a homozygous adf^{de60} / adf^{de60} embryo. Lane 5 shows a single band of 520 bp, from a homozygous wild type embryo. Lane 6 shows both bands, indicating that the template was a heterozygote. The bands in lanes 4 and 5 are the sharpest, indicating that a magnesium concentration of 1.75 to 1.875 mM would be ideal. Accordingly, a concentration of 1.8 mM was used for future reactions. Other reagents were used according to the suggestions offered by the manufacturer of the Taq polymerase, and are detailed in the Methods.

It was unknown prior to these studies if it was possible to perform PCR on embryos which had been stained by the horseradish peroxidase method (see Methods), or whether some component of the color-forming reaction would inhibit PCR. As the color reaction could result in either a brown or blue precipitate, embryos were stained by both methods and assessed for their ability to produce PCR product (Figure 30 B and C). Panel B shows DNA from PCR reactions on embryos stained to result in a blue precipitate, a staining method which involves $NiCl_2$. No PCR product was observed in any embryos stained by this method, possibly because the $NiCl_2$ competed with $MgCl_2$ in binding to the Taq polymerase. Panel C shows the results of PCR reactions using embryos stained brown. The brown color reaction uses only diaminobenzidine and hydrogen peroxide. In this case, the percent of embryos which successfully generated PCR product was over 80%. Thus, stained embryos can be used in PCR reactions, as long as $NiCl_2$ is not included in the color-forming reaction.

In summary, the most important factors for successful PCR using single stained embryos are as follows: $NiCl_2$ should be excluded from the antibody staining reaction which precedes the PCR. Pretreatment of embryos for PCR

must include freezing at -80°C for at least one hour following proteinase digestion. Proteinase should be freshly made, or frozen no more than once prior to use. Finally, "touchdown" annealing is used instead of establishing a single annealing temperature for each primer set. A detailed description of the entire protocol is given in the Methods.

The expression of *ftz-lac Z* reporter constructs in *adf-1* null mutants:

The expression of four *lac Z* reporter constructs in *adf-1* null mutants was assessed. Flies carrying reporter constructs which contained either the full-length 5' *ftz* regulatory region, the zebra element, the upstream element, or the 323 base-pair proximal enhancer element (Han et al., 1993; Hiromi et al., 1985; Pick et al., 1990) (Figure 31A) controlling the expression of *lac Z* were mated to adf^{le60}/CyO flies. Their offspring were mated to each other to generate *adf-1* null homozygote embryos which also carried the reporter construct. Embryos from this cross were harvested and stained with anti-β–galactosidase antibody. Individual embryos were sorted based on their expression of the reporter construct and genotyped by PCR using the M3/M-rev primer pair which is diagnostic of the adf^{le60} chromosome. It was expected that if a construct could not be expressed in the context of the *adf-1* null mutation then adf^{le60} homozygotes would be found only among the non-staining members of the population. If, however, the absence of the *adf-1* gene product had no effect on reporter construct expression, then adf^{le60} homozygotes should make up 25% of the stained population, CyO homozygotes 25%, and heterozygotes 50%.

Representative embryos expressing the reporter constructs are shown in Figure 31. Panel B shows the expression of the *ftz*-lac C full-length construct in its typical seven mesodermal and ectodermal stripes. The seven stripes generated

by the *ftz*-lac A zebra element construct are restricted to the mesoderm, as panel C shows. A distinct posterior-to-anterior gradient of decreasing intensity is characteristic of this construct's expression. A variant in which the gradient of expression was not observed is shown in panel D. The upstream element controls *lac Z* expression in seven mesodermal and ectodermal stripes, shown in panel E. The 323 base-pair element of the proximal enhancer also directs the expression of seven reporter gene stripes, which are weaker than those directed by the upstream element (panel F). With the exception of the variant of the zebra element-driven expression, these expression patterns agreed with the reported expression of these constructs.

PCR was performed on stained embryos from each group described above, using the M3/M-rev primer pair. Figure 31G shows the results of reactions performed on embryos expressing the *ftz-lac A* zebra element reporter construct. Lanes 1 and 5 show the single 520 bp band diagnostic of CyO homozygotes. Lane 2 shows DNA from a heterozygote. Lanes 3 and 6 have a single band of 558 bp, indicating that the embryos were $adf\text{-}1^{le60}$ homozygotes. The CyO balancer was shown to produce the wild-type band using this primer pair (data not shown). The lower band visible in all but lane 4, indicated by an open arrow, was from a set of primers included as an internal reaction control. Table 7 lists the results of PCR reactions performed on embryos expressing the various *lac Z* reporter constructs. Of 33 embryos expressing the full length *ftz*-lac C construct, 36% were heterozygotes, 39% were CyO homozygotes, and 24% were adf^{le60} homozygotes. Embryos expressing the zebra element *ftz*-lac A construct were separated into those which displayed a posterior-to-anterior expression gradient, and those which did not. The former group was not depleted of adf^{le60} homozygotes (27.5% of 40 embryos). The lack of expression gradient did not correlate with any genotype, indicating that this was probably a

staining artifact. 21 323-*lac Z* expressing embryos were divided into 33% adf^{dc60} homozygotes, 28.5% CyO homozygotes and 38% heterozygotes. Thus, the expression of these three reporter constructs appeared to be unaffected by mutation in *adf-1*.

Of the four constructs tested, only the UPS-*lac Z* showed any appreciable deviation from the 25%/50%/25% ratio. 10% of the 50 embryos tested were adf^{dc60} homozygotes. 48% were heterozygotes and 42% were CyO homozygotes. It is difficult to attribute this to anything other than sampling error, since the expression of the 323 base-pair element, which is a truncated form of the upstream element, was apparently unaffected. Another possible explanation would have to invoke a strong Adf-1-responsive element in the upstream element which is not present in the 323 base-pair element.

Therefore, all four *lac Z* reporter constructs are expressed in *adf-1* null mutants, although it is possible that the UPS-*lac Z* construct is less robustly expressed than the others. It is quite different from the results obtained in our laboratory when these same reporter constructs were expressed in *ftz-f1* mutants. In that mutant, the 323-*lac Z* reporter was not expressed, and the UPS-*lac Z* reporter was expressed at negligible levels, confirming the importance of *ftz-f1* for proper *ftz* expression (K. Su and L. Pick, unpublished). This result suggests that Adf-1 has little effect by itself on the regulation of *ftz* expression.

Chapter 5. Discussion

Two avenues, one biochemical and one genetic, were taken in our endeavor to reach a more complete understanding of how proteins which interact with the *ftz* regulatory region contribute to the control of its expression during development. In the first, we attempted to purify factors which bound specifically to protein binding site 2 of the *ftz* proximal enhancer. In the other, we mutated the gene encoding Adf-1, a factor which was known to interact with sites 6, 8, and 9 in the *ftz* upstream element, to evaluate the effect of this mutation on *ftz* expression.

I. Attempted purification of fEBP 1 and 2:

In purifying the proteins which specifically interacted with protein binding site 2 of the *ftz* proximal enhancer (Han et al., 1993), we planned to follow a scheme by which crude nuclear extract would be subjected to a few steps of conventional chromatography, followed by DNA-affinity chromatography. fEBP 1 and 2 were found to be most strongly expressed in 0-9 hour embryos. Less active protein was recovered from embryos which had been frozen prior to extract preparation. Nuclear extract was therefore prepared from fresh embryos of 0-9 hours.

A number of potential purification steps were evaluated for their ability to effect an increase in fEBP 1 or 2 specific activity without excessively depleting the units recovered. Of the column resins tested, DEAE Sephacel, CM Sepharose, P11 phosphocellulose, single-stranded DNA cellulose, phenyl Sepharose, and Cibachron Blue Sepharose were judged to be unsuitable. Neither DEAE Sephacel nor CM Sepharose bound or purified either protein. P11 phosphocellulose bound

and purified both proteins, but was rejected in favor of SP Sepharose, which has a similar mode of action. Single stranded DNA cellulose also interacted with both proteins and purified them fairly well. However, it was eliminated from consideration due to instability of the resin. fEBP 1 and 2 were not recovered from Phenyl Sepharose or Cibachron Blue Sepharose. This could be due to the loss of some cofactor, the dissociation of a complex of proteins which were necessary for DNA binding, or irreversible binding of the proteins to the matrix. The binding hypothesis is unlikely in the case of Phenyl Sepharose, since this column matrix interacts with large hydrophobic regions, such as membrane-spanning domains. Irreversible binding could account for the disappearance of fEBP 1 and 2 in the case of Cibachron Blue Sepharose. Cibachron Blue Sepharose generated a possible third DNA-binding protein, which is discussed below. SP Sepharose and S-200 Sephacryl HR were found to result in good purification and unit yields of fEBP 1 and 2, and were therefore selected for use in the purification of these factors.

A procedure for purifying fEBP 1 and 2 was designed which employed S-200 Sephacryl and SP Sepharose, followed by two rounds DNA affinity chromatography. 500 mg of protein from crude nuclear extract were subjected to this procedure. The purification of fEBP 1 and 2 was assessed by the increase of specific activity in EMSA using oligonucleotide corresponding to site 2. Quantification of EMSA performed on samples from each step of the procedure showed that fEBP 1 had apparently degraded. However, fEBP 2 had been purified 1800-fold.

SDS polyacrylamide gel electrophoresis was used to separate the proteins from the final stage of the purification. At least 6 discrete bands were identified in this manner, the most abundant being at about 55 kDa. UV crosslinking had identified an oligonucleotide 2 binding protein with an approximate molecular

weight of 55 kDa, suggesting that this band in the SDS gel was fEBP 2. However, Southwestern blotting using either wild type or mutated oligonucleotide 2 showed that a DNA binding protein at about 51 kDa would interact with mutant and wild type probe, indicating that it was a nonspecific DNA binding protein. Thus, the presence of nonspecific DNA binding proteins which had been purified along with fEBP 2 made it impossible to identify a band for microsequencing from this preparation.

A possible third binding protein interacting with site 2:

Elution of a Cibachron Blue Sepharose column with 0.5 M KCl/HEMG resulted in the appearance of a third oligonucleotide 2 complex in EMSA. The apparent mobility of this complex was intermediate to fEBP 1 and 2. This complex could represent a breakdown product of fEBP 1, the more slowly migrating of the two previously identified complexes. Alternatively, it could be a novel complex, possibly one which contains Ftz protein. Our attempts to determine whether Ftz protein was present by using anti-Ftz antibody in EMSA were inconclusive, since the antibody and the pre-immune serum each abolished the interaction of the protein with oligonucleotide probe, indicating that the disruption was non-specific. Thus, the question of the presence of Ftz in this protein-DNA complex has not been answered.

It is possible that fEBP 1 and/or 2 antagonizes the binding of this third complex to site 2, which is why it was never identified in crude nuclear extract. We attempted to verify if such an antagonism existed by mixing the third complex with crude nuclear extract in EMSA, but the results were inconclusive. If the third complex does contain Ftz protein, this could imply that fEBP 1 and 2 function as repressors of Ftz binding to its highest-affinity site in the proximal

enhancer (Pick et al., 1990). The timing of fEBP 1 and 2 expression would appear to support this possibility, since they are more strongly expressed in 0-9 hour embryos, after Ftz expression in stripes has begun to taper off (Carroll and Scott, 1985; Karr and Kornberg, 1989). Alternatively, the third complex could encode a Ftz cofactor, whose interaction with the proximal enhancer was repressed by the action of fEBP 1 or 2. This third complex is another obvious candidate for future purification and identification.

Analysis and revision of the purification strategy:

While the attempt described in these results was not successful, these findings do confirm that this general approach for the purification of factors binding to the *ftz* enhancer is feasible. A number of DNA binding proteins have been purified by a combination of conventional and DNA-affinity chromatography (Briggs et al., 1986; England et al., 1990; Han et al., 1998; Kadonaga and Tjian, 1986), and the specific activity of fEBP 2 did in fact increase appreciably during the procedure we performed. Thus, it seems likely that fEBP 1 and 2 could be purified, if the purification strategy were modified so that these factors could be better separated from non-specific DNA binding proteins

A number of ways in which the purification strategy could be improved for future attempts have been identified. One hindrance to efficient purification was the order in which the columns were employed. In our experience, the size fractionation column tended to become clogged with debris from crude nuclear extract. In addition to causing loss of protein due to poor loading, it would tend to promote inefficient flow through the column, resulting in suboptimal protein separation. While this column has been used by others as the first stage of a purification scheme (Briggs et al., 1986), in this case it may not have been ideal.

Column elution with salt concentration "steps" was identified to be another probable source of non-optimal purification. Accordingly, it was determined that gradients of increasing salt concentration should be used exclusively for column elution. This would result in more precise separation of proteins from contaminants with similar but not identical column matrix affinities.

Another major problem which was identified was with the DNA affinity columns. The performance of this column was less efficient than previous reports had led us to expect. Previous studies showed a unit retention of 50-100%, with a purification of 80-500X for the first round of affinity chromatography. The second round resulted in 40-50% unit retention and 2.5-13 X purification (Biggin and Tjian, 1988; Briggs et al., 1986; Perkins, 1988). Table 2 shows that fEBP 2 had only 10% unit retention with essentially no purification on the first round, and 22% retention with 5X purification on the second. We theorized that this could be due to the presence of fewer available binding sites on the column than had been the case in other studies. The protocol for preparation of this column matrix called for ligation of monomer oligonucleotide binding sites to an average length of 1 kb prior to linking them to CNBr Sepharose (Han, 1994; Kadonaga and Tjian, 1986). In our experience, the average length of the ligated oligonucleotides rarely exceeded 500-600 bases. To counteract this problem, a few approaches have been considered for the future. One is to concatamerize the oligonucleotide binding site prior to ligation. A five-mer of oligonucleotide 2 was produced in our laboratory, and it is predicted that this 100 base fragment would more readily concatamerize into longer fragments. The other approach is to consider more efficient methods of linking oligonucleotides to the column matrix so that we could increase the density of available binding sites. Linking DNA to CNBr Sepharose relies on the production of a covalent bond between the oligonucleotide and the cyanide moiety. This method is estimated to bind

approximately 0.6 nanomoles of DNA per milliliter of column resin (Kadonaga and Tjian, 1986). Another method of attaching DNA to the column matrix involves biotinylating one end of an oligonucleotide and attaching it to streptavidin-agarose (Chodosh et al., 1986; LeBlond-Francillard et al., 1987). This method has a binding capacity of approximately 44 nanomoles of DNA per milliliter of column resin. Thus it is possible that using this type of resin could increase number of attached oligonucleotides and thus increase the binding affinity of the column for specific DNA-binding proteins.

A revised purification scheme is shown in Figure 32. Nuclear extract would first be separated by passing it over an SP Sepharose column, and eluting the column with an increasing gradient of KCl. Following concentration of the pooled oligonucleotide 2 binding activity by ammonium sulfate precipitation, it would be passed over a size separation column. The activity from this column would be loaded onto an improved DNA affinity column, which would also be eluted by a gradient. At least two rounds of DNA affinity chromatography would probably be necessary. Due to a number of factors, the most significant being the increasing demands of the *adf-1* mutagenesis, these revisions were not utilized in another purification attempt.

The proximity of site 2 to the highest affinity Ftz homeodomain binding site in the proximal enhancer makes the identity of proteins which interact with this site particularly interesting. The possibility arises that proteins which bind to site 2 could act as Ftz cofactors, as is the case for binding site 8. Site 8 of the proximal enhancer is adjacent to a medium affinity Ftz binding site, and one of the proteins which binds there, Ftz- F1, was shown to be a Ftz cofactor (Yu et al., 1997). Alternatively, proteins interacting with this site could act as transcriptional activators or repressors, with no direct interaction with Ftz protein. Despite the current failure to identify the factors, two known and one newly identified,

which bind to this site, their identity remains an important and ultimately solvable riddle.

II. Adf-1 mutagenesis:

The generation of mutations in *Drosophila* is a somewhat more complex process than in mouse, since homologous recombination is not a possibility. The most common ways to generate a mutation involve disrupting the genome in an undirected way, and then screening the offspring of this treatment for disruptions in the gene of interest. In the case of the *adf-1* locus, we used P element-mediated mutagenesis, primarily because of the fortunate chance that a suitable parent stock became available.

A P element within 3 kb of the *adf-1* start site was mobilized, resulting in the production of two excisions and eight P element local hops which were unable to complement a lethal deletion in the region of the *adf-1* locus. As the result of a collaboration with Dr. T. Tully at Cold Spring Harbor Laboratory, we obtained a lethal allele of *adf-1*, adf^{de60}, in which a 1.6 kb deletion removed most of the Adf-1 protein coding region. Of the 10 lethal events generated in our crosses, one excision (E1) and three hops (H2, H3, H4), were unable to complement adf^{de60}, indicating that they were also lethal alleles of *adf-1*. Two additional hops (H5, H8) were able to partially complement adf^{de60}, suggesting that they encoded a partially active form of Adf-1. Southern blots on genomic DNA confirmed that E1, H2, H3 and H4 had experienced disruptions of the *adf-1* locus.

The P element in the H2 line of flies was remobilized. 55% of the *white*$^-$ progeny from this procedure had regained the ability to complement adf^{de60}, confirming that the lethal event in H2 was due to P element insertion. 30 % of the offspring still did not complement adf^{de60}, suggesting that they had experienced

an imprecise excision of the P element. While some of these imprecise excisions would simply leave some of the P element behind, some could have all or part of the *adf-1* coding region excised. The remainder of offspring from this cross were able to partially complement adf^{le60}, indicating the production of a partially active protein. These could be useful for future studies of the structure and function of the Adf-1 protein. H2 and adf^{le60} were both rescued by addition of a P element carrying a fragment of genomic DNA containing the *adf-1* coding region along with approximately 11 kb of flanking sequence. Two separate P element insertions onto the X chromosome were bred into adf^{le60} or H2 lines. These transgenes were able to rescue the lethal homozygous condition. Thus, H2 and adf^{le60} represent true *adf-1* null alleles.

Investigation of the rates of hatching showed that adf^{le60} was lethal during embryogenesis, with 44.9% of adf^{le60}/CyO embryos failing to hatch. Workers in Dr. Tully's laboratory have confirmed this embryonic lethality, but have noticed that a few percent of *adf-1* homozygotes can survive to the first larval instar before dying (J. Dezazzo, personal communication). Thus far, neither our group nor Dr. Tully's group have been successful in determining when in embryogenesis death occurs. No disruptions of cuticle have been observed in these lines, which could suggest that the homozygotes die either before they secrete cuticle, or that they are able to deposit cuticle normally and die due to defects in some internal organ system. The latter seems more likely, given the presence of some larval *adf-1* homozygotes. If that is the case, an obvious candidate system would be the nervous system, since Dr. Tully's laboratory identified an *adf-1* hypomorphic allele as being deficient in memory and learning (J. Dezazzo, personal communication). However, no obvious disruptions of the nervous system have been observed to date.

Determination of the exact time at which death occurs in the homozygous embryos remains a difficult proposition. It is possible that by collecting staged embryos at one hour intervals, PCR could be performed on them to observe when homozygous mutants are no longer detected in the population. However, it is quite possible that dead embryos would still yield PCR product, resulting in false positives until their DNA had sufficiently degraded. Sectioning and examining microscopically a population of staged embryos would be possible. This would require the use of a balancer which could be identified histochemically. However, determining microscopically the time at which the embryos had died would be limited to looking for the onset of necrosis and concluding that death must have happened at some stage prior to the stage at which widespread degradation was observed. One other possible approach would be to videotape a population of live embryos under mineral oil (Ede and Counce, 1956; Valdes-Perez and Minden, 1995). It may be possible to deduce the stage of lethality based on the observation of gastrulation movements and when they cease in mutant embryos. Use of a newly-generated CyO-green fluorescent protein balancer (T. Hazelrigg, personal communication) could be helpful in identifying non-mutant embryos in that situation. In summary, while *adf-1* mutation is lethal during embryogenesis, the stage at which lethality occurs and the organ systems affected remain to be determined.

Development of single-embryo PCR:

In order to carry out the studies described below, in which the expression of *ftz-lac Z* reporter genes in *adf-1* mutant embryos was assessed, it was necessary to unambiguously genotype embryos which had been previously stained. Since it was not feasible in this case to include a marked balancer chromosome, as we

could do in experiments described later, we needed to develop a technique new to our laboratory, single-embryo PCR. Prior to this work, this procedure was only occasionally successful, with less than 50% of embryos yielding PCR product. Now more than 90% of embryos which are treated result in PCR product, allowing us to conduct the studies of reporter gene expression discussed below.

The most crucial aspects in refining the protocol were the initial preparation of the embryos. If $NiCl_2$ were added to the color reaction during antibody staining of embryos, no PCR product would result. We speculate that this is because the Ni^+ competes with Mg^+ in its role as a DNA polymerase coenzyme. The other major factor is freezing embryos at -80° C following proteinase digestion. This freezing and thawing may act to make the embryo more permeable, allowing greater access of PCR reagents to the DNA.

This technique will continue to be of use in our laboratory, both in genetic experiments such as this, and also as a means of confirming the identity of fly stocks without having to prepare large amounts of genomic DNA from adult flies.

The effect of *adf-1* mutation on *ftz* expression:

Two second chromosome balancer chromosomes, CyO-*hblacZ* and CyO-*actinlacZ*, were employed in the examination of Ftz expression in *adf-1* mutants. Both allowed the identification of *adf-1/adf-1* homozygotes by the absence of balancer chromosome-directed *lac* Z expression in anterior of the embryo, or ubiquitously, respectively. CyO-*hblacZ* enabled us to examine the *ftz* striped pattern, while CyO-*actinlacZ* let us examine the expression of *ftz* in the nervous system. Neither the *ftz* striped expression nor the *ftz* nervous system expression

pattern were disrupted in adf^{de60} homozygous embryos. The timing and order in which Ftz stripes appeared in the mutant were the same as those observed in wild type embryos. The pattern of expression in the nervous system was likewise unaffected.

At least two explanations existed for this apparently unchanged *ftz* expression pattern. It was possible that in fact the loss of Adf-1 had no effect on *ftz* expression, and that this result reflected the true situation. It was also possible that the effect on *ftz* expression was subtle, and that we were unable to detect the disturbance in these experiments. In support of the latter explanation it is important to note that mutating *ftz-f1* had an apparently minimal effect on *ftz* expression, and that it was only when the expression of *ftz-lac Z* reporter constructs was examined that the importance of Ftz-F1's influence was apparent. In those mutants, while the expression of the *ftz*-lac A and *ftz*-lac C constructs were unchanged, neither the UPS-*lac Z* construct nor the 323-base pair-*lac Z* construct were expressed (K. Su and L. Pick, unpublished). To determine if the effect of *adf-1* mutation was also more obvious in its effect on *ftz-lac Z* reporters, we crossed *ftz-lac Z* reporter constructs into adf^{de60}/CyO flies, and PCR genotyped the embryos which could express the constructs.

Four *ftz-lac Z* reporter constructs were tested in *adf-1* mutants. The *ftz*-lac A zebra element construct, the *ftz*- lac C full length construct, and the 323-bp- *lac Z* constructs were all expressed normally in adf^{de60} / adf^{de60} embryos. Only the UPS-*lac Z* upstream element construct showed any decrease in the relative number of adf^{de60} / adf^{de60} embryos which could direct reporter gene expression. This decrease is most probably due to sampling error. This experiment is being repeated to confirm or deny this apparent anomaly. Thus, in contrast to *ftz-f1*, it seems that the mutation of a*df-1* has no effect on the expression of *ftz*, nor on subdivisions of the *ftz* promoter.

Future Directions:

While *adf-1* mutation alone has little effect on *ftz* expression, the possibility remains that its role is redundant *in vivo*. Examination of embryos which are simultaneiously mutant for multiple *ftz* regulatory factors would allow us to determine if Adf-1 could act additively or synergistically with any of those factors. One immediate candidate for such a test is Ftz-F1, which interacts with the same *ftz* proximal enhancer sites as Adf-1. Double mutants for *ftz-f1* and *adf-1* could reveal a cooperative role for Adf-1 in the regulation of *ftz*. To explore this possibility, the adf^{de60} *adf-1* null mutation would have to be bred into flies which could generate *ftz-f1* germline clones (Perrimon et al., 1996). The cross strategy outlined in Figure 33 would allow the production of embryos from *ftz-f1* mutant ovaries, removing the maternally deposited Ftz-F1 (Chou and Perrimon, 1996; Chou and Perrimon, 1992). One-quarter of these embryos would be *adf-1* mutant as well. Note that the genotype given for females in the F2 generation is the genotype of the germline cells from which the embryos arise, and not the genotype of the somatic cells. PCR genotyping of individual embryos which were expressing Ftz stripes would allow us to determine if doubly mutant embryos could express Ftz normally.

Another related experiment would be to determine if the expression of either the *ftz-lac A* zebra element reporter or the full-length *ftz-lac C* reporter construct is affected in doubly mutant *adf-1, ftz-f1* embryos. Expression of the upstream element reporter construct is absent in *ftz-f1* mutants (K. Su and L. Pick, unpublished). Examination of these double mutant lines which carried these reporters might also highlight some hitherto-masked effect of *adf-1* mutation on *ftz* expression.

It is possible in *adf-1* mutants that the effect on *ftz* is not a loss of expression but a decrease in the level of expression. One *in situ* hybridization was performed by Kai Su in our laboratory to check if this was the case. No obvious difference in the levels of staining were detected. This did not rule out the possibility, since *in situ* hybridization is not quantitative. Examination of *adf-1* mutants by confocal microscopy and computer-assisted image analysis, with the collaboration of Dr. Reinitz's laboratory, might reveal some slight difference in Ftz staining intensity.

It is equally possible at this stage to conclude that Adf-1 has in fact no effect on the expression of *ftz*. If that is the case, two questions immediately arise. The first is why Adf-1 interacts with the *ftz* proximal enhancer. The other question concerns the role that *adf-1* plays in *Drosophila* embryogenesis.

The data which indicated that Adf-1 binds to the *ftz* proximal enhancer seem fairly convincing. Its purification from a binding site 6 affinity column (Han et al., 1998) could be interpreted as the unintentional isolation of a nonspecific DNA binding protein. Still, its presence in complexes formed in mobility shift assays with binding sites of the proximal enhancer is more difficult to attribute to nonspecific binding (Han et al., 1998). However, the fact that anti-Adf-1 antibody abolishes the formation of a complex with every binding site tested (Han et al., 1998), in contradiction of the EMSA competition data for those complexes (Han et al., 1993), could suggest that the antibody does not specifically recognize Adf-1 and that the presence of Adf-1 in those complexes is therefore not certain. The strongest evidence that Adf-1 interacts with binding site 6 is its ability to activate transcription from concatamerized site 6 in yeast (Han et al., 1998). Nevertheless, its ability to activate transcription from the 323 bp element, in which site 6 is in its natural context, appears much weaker. Thus, while Adf-1 may in fact be able to recognize and interact with sites 6, 8, and 9 *in vitro*, in the embryo its

interaction with the *ftz* enhancer could be obstructed by the action of proteins such as Ftz-F1 which have a stronger affinity for the site that it recognizes. This would reconcile the DNA interaction data with the fact that mutation of *adf-1* has no effect on *ftz* expression.

If Adf-1 does interact with the *ftz* proximal enhancer *in vivo*, perhaps its role as a transcriptional activator has been supplanted by activators with a stronger affinity for the enhancer. Its lack of effect on *ftz* expression could indicate the presence of some other factor besides Ftz-F1 which is able to compensate for the loss of Adf-1. Given that Ttk has been identified genetically as a repressor of *ftz* (see Introduction), perhaps the unknown fourth protein which interacts with binding site 6 (see Figure 4) is the factor which renders Adf-1 redundant.

That *adf-1* is crucial in some stage of *Drosophila* embryogenesis is evident from the lethality of its mutation. The exact role that this factor plays is still unclear. The primary role of *adf-1* in the embryo cannot be the regulation of *adh*, since the role of *adh* in embryogenesis is probably minimal. The expression of *adh* RNA in the embryo is extremely low when compared to its expression in larvae, pupae and adults (Corbin and Maniatis, 1989; Savakis et al., 1986). Our attempts to detect Adh protein in the embryo were unsuccessful, suggesting that Adh may act only in older flies and not have a prominent role in the embryo. Finally, mutation of *adh* is not lethal (Aaron, 1979; Grell et al., 1968; O'Donnell et al., 1975), which means that some other essential gene pathway must be disrupted by adf-1 mutagenesis to cause embryonic lethality. One possibility which recently arose is the *tinman* mesoderm and heart patterning gene pathway (Jagla et al., 1997; Yin et al., 1997). A yeast one-hybrid screen performed in Dr. Frasch's laboratory has identified Adf-1 as a possible Tinman cofactor (X. Xu and M.

Frasch, personal communication), suggesting that Adf-1 may have a role in heart and muscle patterning.

The regulation of *ftz* expression is a complex process. Demonstration of the role of individual factors in the overall regulatory scheme often brings up as many questions as it answers, as the present study shows for *adf-1*. In time, however, all of the pieces of this puzzle will be clear, and the way in which they fit together will bring us one step closer to understanding the mechanisms of embryogenesis in the fruit fly, and in humans.

Column Matrix	Specific Activity			Purification	Protein Yield	Unit Yield
		load	flowthrough			
DEAE	*fEBP 1:*	186	243	1.3X	86%	112%
	fEBP 2:	91	96	1.05X		90%
		load	pooled fractions			
P11	*fEBP 1:*	480	168	3.5X	1.4%	4.7%
	fEBP 2:	118	407	3.5X		4.8%
CM	See Figure 7					
		load	0.5 M eluate			
SP	*fEBP 1:*	243	1987	8X	2.9%	22%
	fEBP 2:	96	1540	16X		42%
Phenyl	No oligonucleotide 2 binding activity was recovered					
		load	0.5 M eluate			
S.S. DNA	*fEBP 1:*	167	571	3.4X	27%	39%
	fEBP 2:	177	779	4.3X		50%
		load	pooled fractions			
S-200	*fEBP 1:*	70	790	11X	12.5%	141%
	fEBP 2:	47	1559	33X		430%

Table 1: Quantification of tests of protein purification methods. Samples were assayed by EMSA and dried gels were exposed to a PhosphorImager screen. Data were analyzed using ImageQuant software. Units are defined as the amount of activity necessary to retard 0.05 fmol probe. Specific activity is given in U/mg.

	Total Protein	Total Activity	Specific Activity	Cumulative Purification	Cumulative Unit Yield
Nuclear extract	499.7 mg	330861 U	662 U/mg	--	--
S-200 Sephacryl	98.7 mg	3540172 U	35868 U/mg	54 X	1070%
SP Sepharose	12.8 mg	3300473 U	256447 U/mg	387 X	998%
O2 column #1	1.3 mg	320500 U	242803 U/mg	367 X	97%
O2 Column #2	0.06 mg	70635 U	1177242 U/mg	1778 X	21%

Table 2: Quantification of large-scale purification of fEBP 2. Fractions from each stage of the purification were assayed for O2 binding proteins by EMSA. Dried gels were exposed overnight to a PhosphorImager screen, and analyzed using ImageQuant software. Units are defined in Table 1.

	Specific Activity	Cumulative Purification	Cumulative Unit Yield
Nuclear extract	119 U/mg	--	
SP Sepharose	2795 U/mg	23 X	106%
65% Ammonium Sulfate	1836 U/mg	0.6 X	28%
FPLC Superose	5165 U/mg	43 X	23%

Table 3: Quantification of FPLC column pool for purification of fEBP 2. Samples were assayed by EMSA for O2 binding activity, and dried gels were exposed overnight to a PhosphorImager screen. Data was analyzed using ImageQuant software.

fEBP 1	Total Protein	Total Activity	Specific Activity	Purification	Yield
Nuclear extract	4.3 mg	180 U	42 U/mg		
20% AS	0.5 mg	99 U	195 U/mg	4.6 X	55%
30% AS	0.8 mg	38 U	50 U/mg	1.2 X	21%
40% AS	1.4 mg	19 U	14 U/mg	0.3 X	10%
50% AS	1.9 mg	46 U	25 U/mg	0.6 X	23%
60% AS	1.8 mg	43 U	24 U/mg	0.6 X	24%

fEBP 2	Total Protein	Total Activity	Specific Activity	Purification	Yield
Nuclear extract	4.3 mg	858 U	200 U/mg		
20% AS	0.5 mg	514 U	1008 U/mg	5.0 X	60%
30% AS	0.8 mg	283 U	368 U/mg	1.1 X	33%
40% AS	1.4 mg	111 U	82 U/mg	0.4 X	13%
50% AS	1.9 mg	217 U	117 U/mg	0.5 X	25%
60% AS	1.8 mg	345 U	191 U/mg	0.9 X	40%

Table 4: Quantification of the precipitation of fEBP 1 and 2 by varying amounts of ammonium sulfate. Ammonium sulfate was added to five aliquots of nuclear extract in a total volume of 3 ml no-salt HEMG to acheive concentrations corresponding to 20%, 30%, 40%, 50% or 60% saturation. Samples were centrifuged and resuspended and assayed for the presence of O2 binding activiity. Dried gels were exposed overnight to a PhosphorImager screen and analyzed with ImageQuant.

Results of F2 test cross:
41 complemented le 60
11 partially complemented le 60
23 did not recover ability to complement le 60
75 *total excisions tested*

Table 5: Results of F2 test crosses for H2 revertants. Flies were tested for the recovery of complementation of the le 60 *adf-1* null allele. Complementation was assessed by the presence of straight wings, indicating two non-CyO chromosomes. Full complementation was determined by the appearance of straight wings in 50% of non-Gla offspring of the F2 cross.

P element insertion line 27-1:

Mated to le 60:

Glazed eyes:	223 (77%)
Normal eyes:	65 (22%)
	288

Mated to H2:

Glazed eyes:	142 (73%)
Normal eyes:	50 (26%)
	192

P element insertion line 76-2:

Mated to le 60:

Glazed eyes:	244 (87%)
Normal eyes:	36 (12%)
	280

Mated to H2:

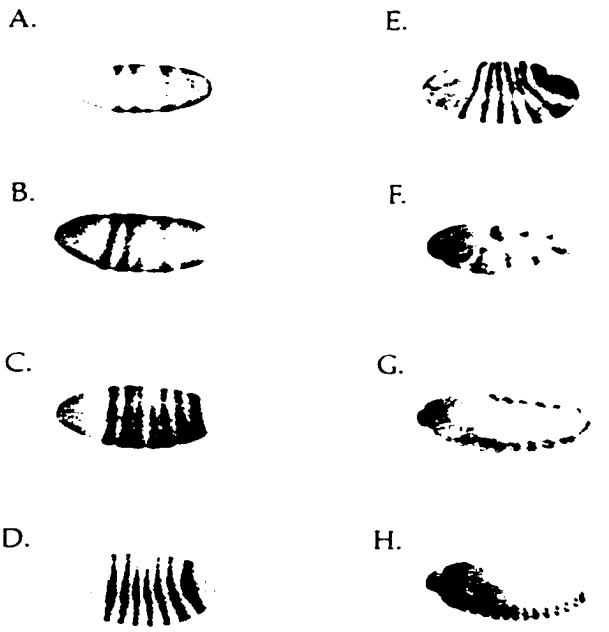
Glazed eyes:	191 (85%)
Normal eyes:	33 (14%)
	224

Table 6: Result of crosses to determine if $P^{[adf\text{-}1+]}$ rescued putative *adf-1* mutant alleles le 60 and H2. Normal eyes indicate two mutant copies of *adf-1*, and should appear only if rescue was successful.

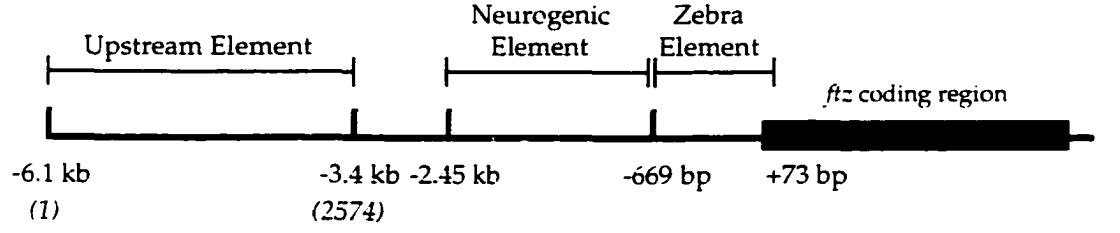
Expressing ftz-lac C:		
CyO/CyO:	13	39%
le 60/CyO:	12	36%
le 60/le 60:	8	24%
	33	
Expressing ftz-lac A:		
CyO/CyO:	12	30%
le 60/CyO:	17	42%
le 60/le 60:	11	27%
	40	
Expressing ftz-lac A (variant pattern):		
CyO/CyO:	8	36%
le 60/CyO:	10	45%
le 60/le 60:	4	18%
	22	
Expressing UPS-lac Z:		
CyO/CyO:	21	42%
le 60/CyO:	24	48%
le 60/le 60:	5	10%
	50	
Expressing 323-lac Z:		
CyO/CyO:	6	38%
le 60/CyO:	8	28%
le 60/le 60:	7	33%
	21	

Table 7: Results of PCR genotyping of le 60/CyO embryos expressing *ftz-lac Z* reporter constructs. Embryos were stained with anti-β-galactosidase, and individual embryos which expressed the construct were genotyped by PCR using the primer pair M3/Mrev.

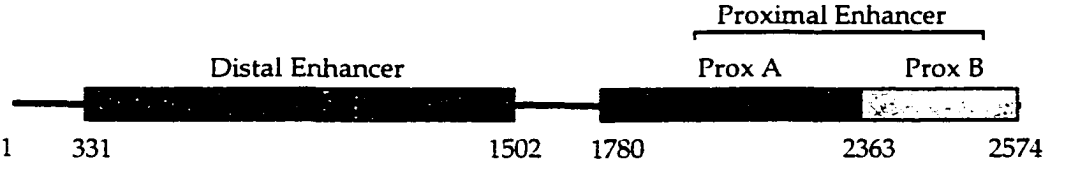
Figure 1: Expression of Ftz in wild type embryos. Embryos of 0-16 hours were collected and stained with polyclonal rat anti-Ftz antibody *A, B, C-* Establishment of Ftz stripes. *D-* Peak Ftz striped expression. *E-* Beginning of germ band extension , with narrowing of Ftz stripes. *F-* Fading of Ftz stripes as germ band extension progresses. *G-* Expression of Ftz in the nervous system in late germ band extension. *H-* Fading of Ftz in the nervous system at the completion of germ band retraction.



A. *ftz* regulatory regions



B. *ftz* upstream element



C. Protein binding sites of ftz proximal enhancer

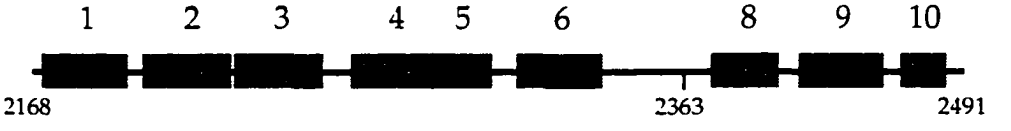


Figure 2: A schematic diagram of the *ftz* 5' regulatory region. (A) Division of the *ftz* 5' regulatory region into zebra, neurogenic, and upstream elements. Based on Hiromi, *et. al*, 1985. (B) The upstream element, showing in greater detail the distal and proximal enhancers, and the division of the proximal enhancer into Prox A and Prox B. Based on Pick, *et. al.*, 1990. (C) The nine protein binding sites in the *ftz* proximal enhancer, with the division between Prox A and B at base 2363 indicated. Based on Han, *et. al.*, 1993.

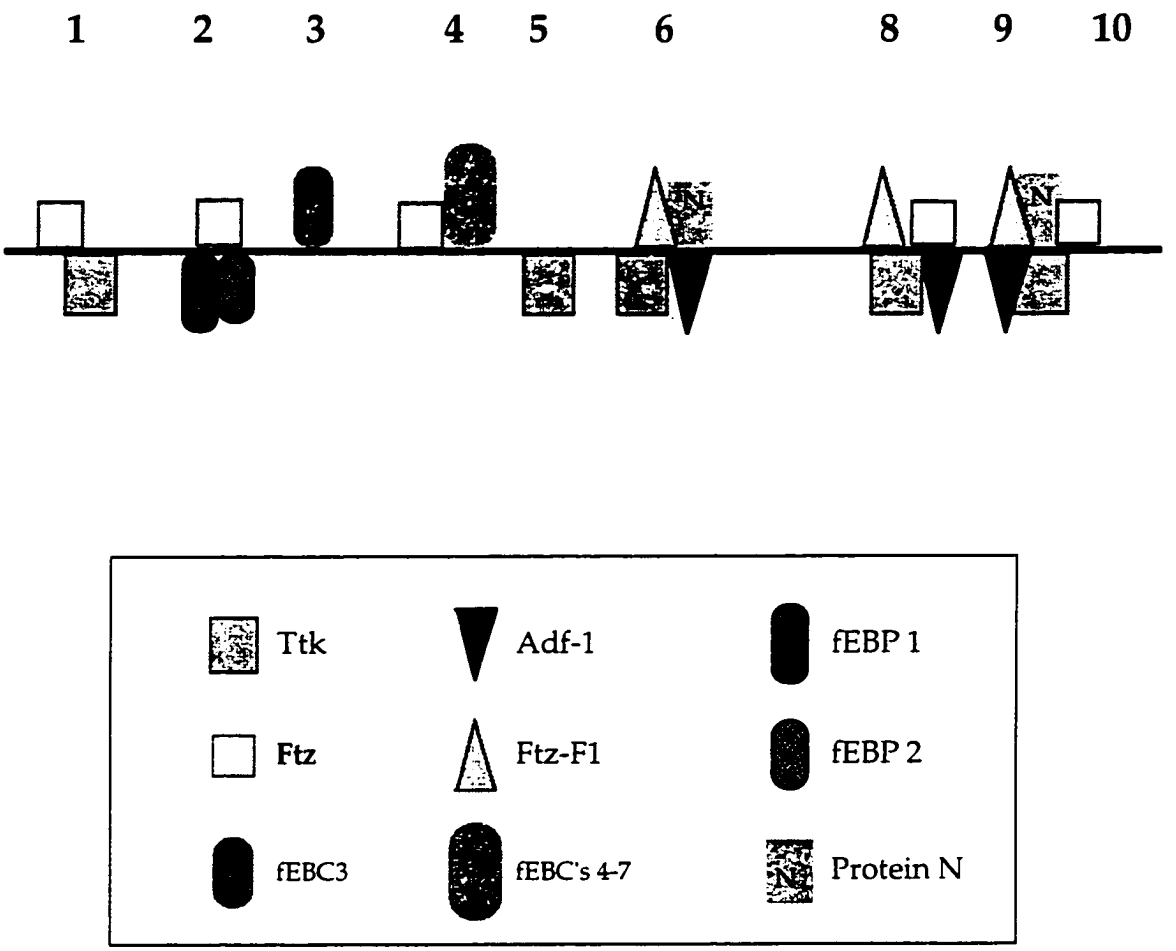


Figure 3: A schematic diagram of the proteins which interact with the nine protein binding sites of the *ftz* proximal enhancer. Note that the four unidentified proteins which interact with site 4 have been combined into one symbol. "Protein N" refers to the unidentified protein which interacts with at least sites 6 and 9, and possibly with 8. Based on Han, *et. al.*, 1993 and Han, *et al.* 1998.

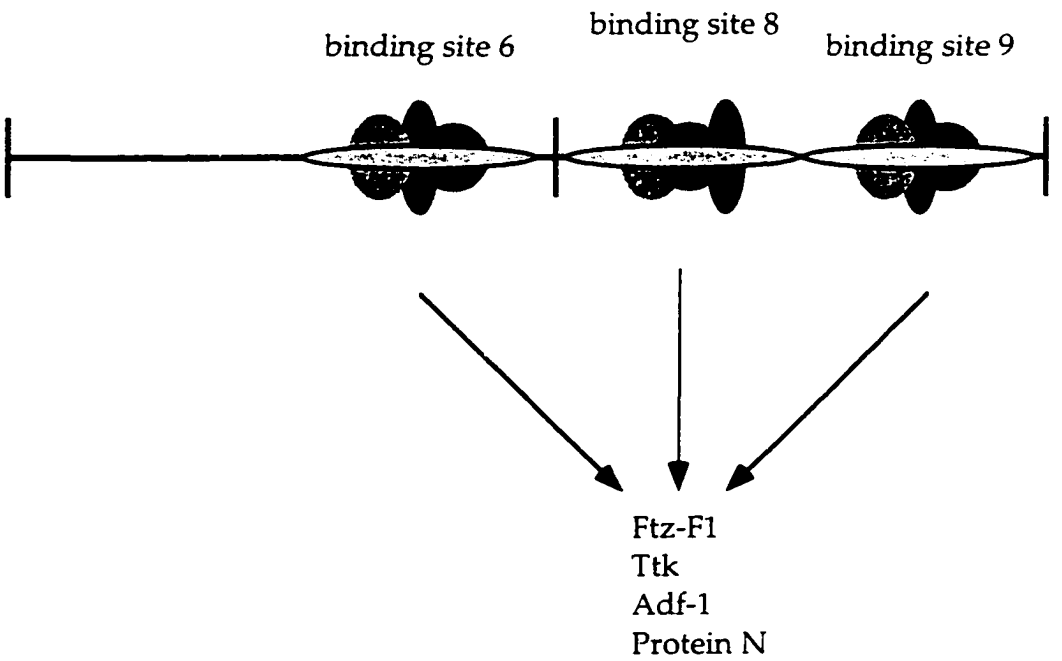


Figure 4: Schematic of the three redundant binding sites of the *ftz* proximal enhancer. Each interacts with the same set of nuclear proteins: Ftz-F1, Ttk, Adf-1 and Protein N, which has not been identified. Based on Han, *et al.* 1993, Han, *et al.* 1998.

Figure 5: *Panel A*- Freezing-induced degradation of the proteins interacting with protein binding site 2 is more pronounced in younger embryos. Autoradiograph of EMSA demonstrating the effect of embryo freezing on the levels of fEBP 1 and 2. ^{32}P labeled-O2 was incubated with nuclear extract prepared from 0-7 hour fresh embryos (lane 1), 0-7 hour embryos previously frozen at -20°C in 50% glycerol (lane 2), 0-10 hour fresh embryos (lane 3), 0-10 hour frozen embryos (lane 4), or 0-16 hour frozen embryos (lane 5). Both activities, particularly fEBP 1, lost DNA-binding activity following the freezing of younger embryos. Lane 6 shows a reaction using 0-9 hour embryos which had been frozen for over one year at -20°C, showing that long-term freezing degraded both activities even in older embryos. Approximately 10 μg protein was used in each reaction. fEBP 1 and 2 are indicated by closed arrows, open arrow indicates a non-specific DNA binding protein.

Panel B - fEBP 1 and 2 are more strongly expressed in 0-9 hour embryos. ^{32}P labeled O2 was incubated in an EMSA with nuclear extract prepared from 0-2.5 hour embryos (lane 1), 0-6 hour embryos (lane 2), or 0-9 hour embryo (lane 3), all of which were prepared from fresh embryos. fEBP 1 and 2 were most active in 0-9 hour embryos. Comparison of lanes 4 and 5 of panel A indicate that their activity decreases again in embryos of 0-16 hours.

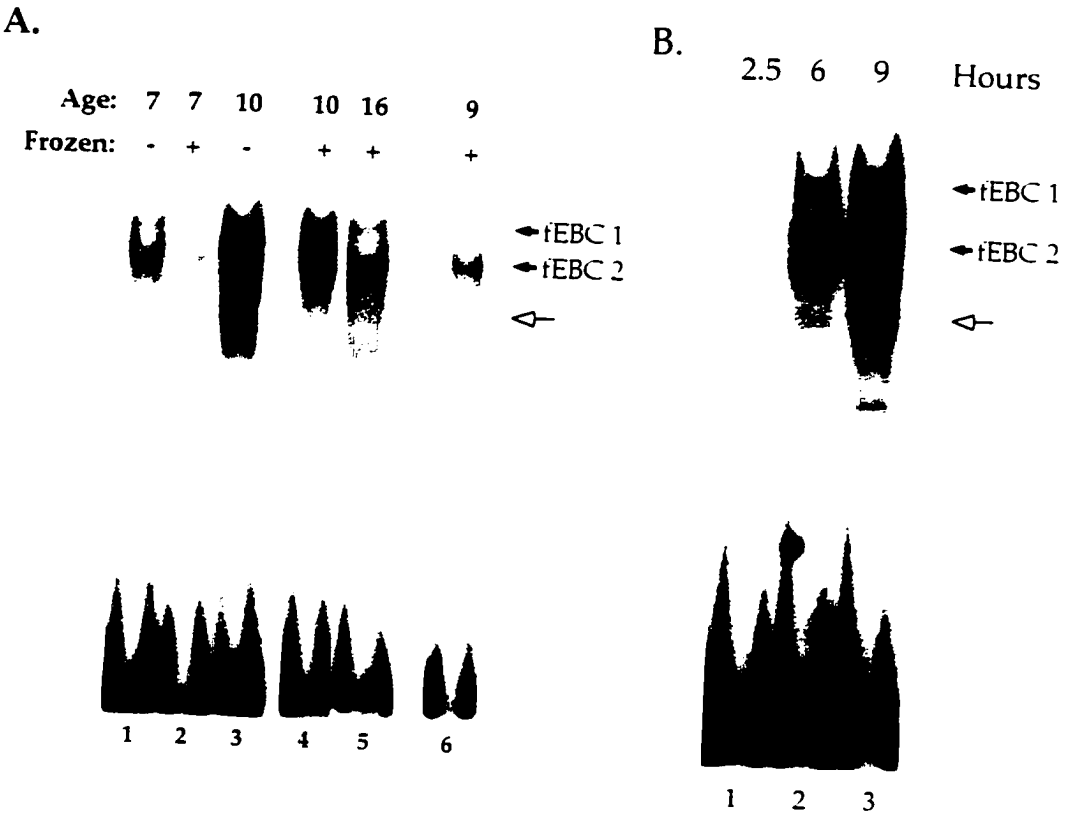


Figure 6: Ftz protein is not detectable in either fEBP 1 or 2. Radiolabeled O2 was incubated with approximately 5 μg of nuclear extract in a mobility shift reaction in the presence of 1 μl of anti-Ftz antibody (α–Ftz, lane 2), or 1 μl of pre-immune serum (PIS, lane 3). No alteration in the interaction of either fEBP 1 or 2 with O2 was noted in the presence of antibody.

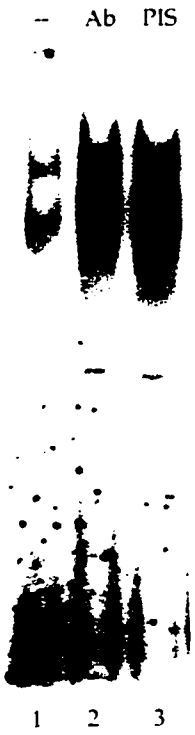


Figure 7: CM Sepharose does not interact with fEBP 1 and 2. 12.5 µg of protein pooled from DEAE Sepharose column flowthrough was loaded onto a CM Sepharose column, washed with 80 mM HEMG-KCl, and eluted with 0.25 M, 0.5 M and 1 M HEMG-KCl steps. Protein was detected only in the flowthrough and 1 M elution pools. Lane 1- EMSA of the load, lane 2- flowthrough, lane 3- 1 M eluate pool, adjusted to 0.1 M KCl prior to assay. Approximately 10 µg protein was used per assay. Neither fEBP 1 or 2 (indicated by arrows) were found to interact with the column.

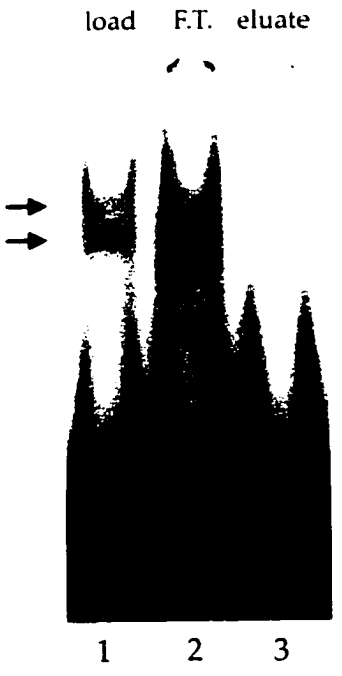


Figure 8: At least two potential inhibitors of the interaction of fEBP 1 and 2 with O2 are separated from them by size-filtration chromatography. *Panel A*- PhosphorImager-quantified results of EMSA in which fEBP 1 or 2 pools collected from an S-200 size filtration column were assayed alone (top row) or in combination with selected fractions across the protein peak of the column (indicated by fraction number in first column). Results are given as units of DNA-binding activity. A potential inhibitor of fEBP 1 was detected in fractions 34-46 by a drop in fEBP DNA-binding units. An inhibitor of fEBP 2 binding activity was detected in fractions 52-54, and potentially another inhibitor in the same fractions which inhibited fEBP 1 activity.
Panel B- Graph of the protein concentrations of the fractions recovered from the S-200 size filtration column. The fractions which were pooled for fEBP 1 and fEBP 2 are indicated.

A.

	fEBP1 pool	fEBP 2 pool
---	32,050 U	125,162 U
fraction 34	15,575 U	84,609 U
fraction 38	22,043 U	88,610 U
fraction 42	19,959 U	44,984 U
fraction 46	19,313 U	93,449 U
fraction 50	38,362 U	37,353 U
fraction 54	39,454 U	32,080 U
fEBP1 pool	67,283 U	50,026 U
fEBP 2 pool	47,015 U	281,366 U
fraction 82	31,721 U	241,365 U

B.

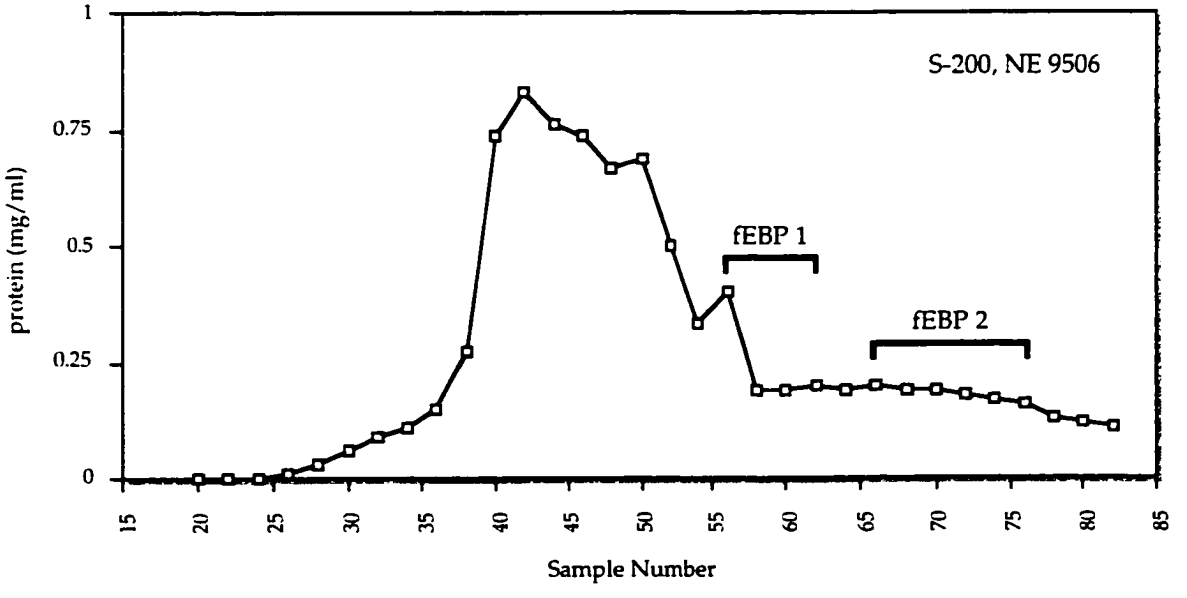
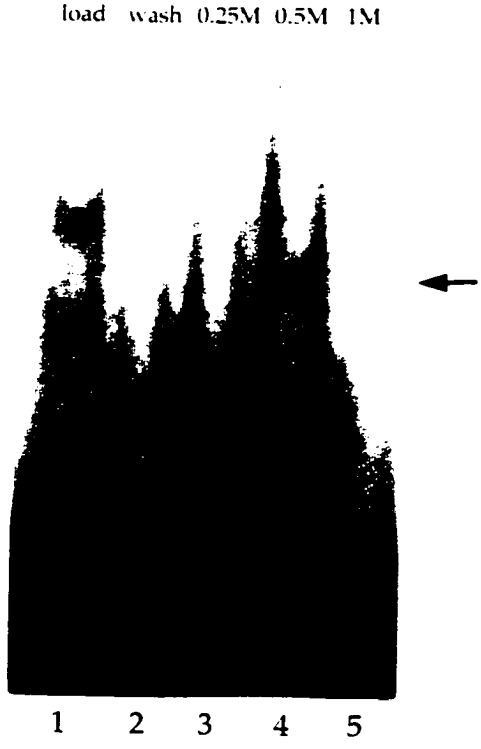


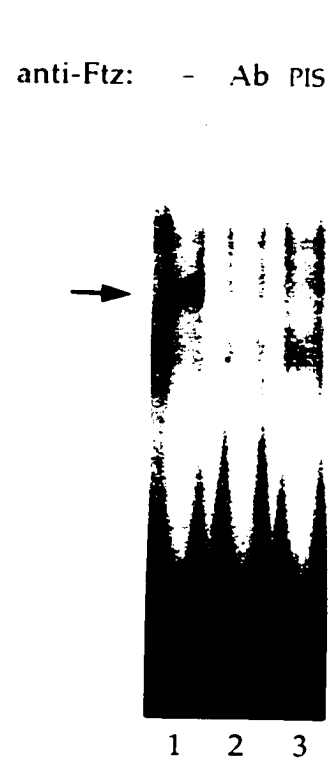
Figure 9: A possible third protein interacting with oligonucleotide 2 is generated by elution of a Cibachron Blue Sepharose column. *Panel A-* 12.5 µg of flowthrough from a DEAE column containing fEBP 1 and 2 was loaded onto a Cibachron Blue Sepharose column and washed with 0.1 M KCl/HEMG, and then eluted sequentially with 0.25 M , 0.5 M and 1 M KCl. Shown is an autoradiograph of an EMSA performed on an aliquot of each elution (the 1 M eluate was adjusted to 0.1 M KCl prior to assay, lane 5). fEBP 1 and 2 were detectable in the column load (lane 1), but were not found in any eluted fraction. The 0.5 M eluate (lane 4) was found to form a complex with O2 with a mobility intermediate to fEBP 1 and 2.

Panel B- Autoradiograph of EMSA on complex 3, which was performed in the presence of 1 µl of anti-Ftz antibody (lane 3) or 1 µl of pre-immune serum (PIS, lane 4). Both PIS and anti-Ftz abolished the complex (indicated by an arrow), indicating that this disruption was non-specific.

A.



B.



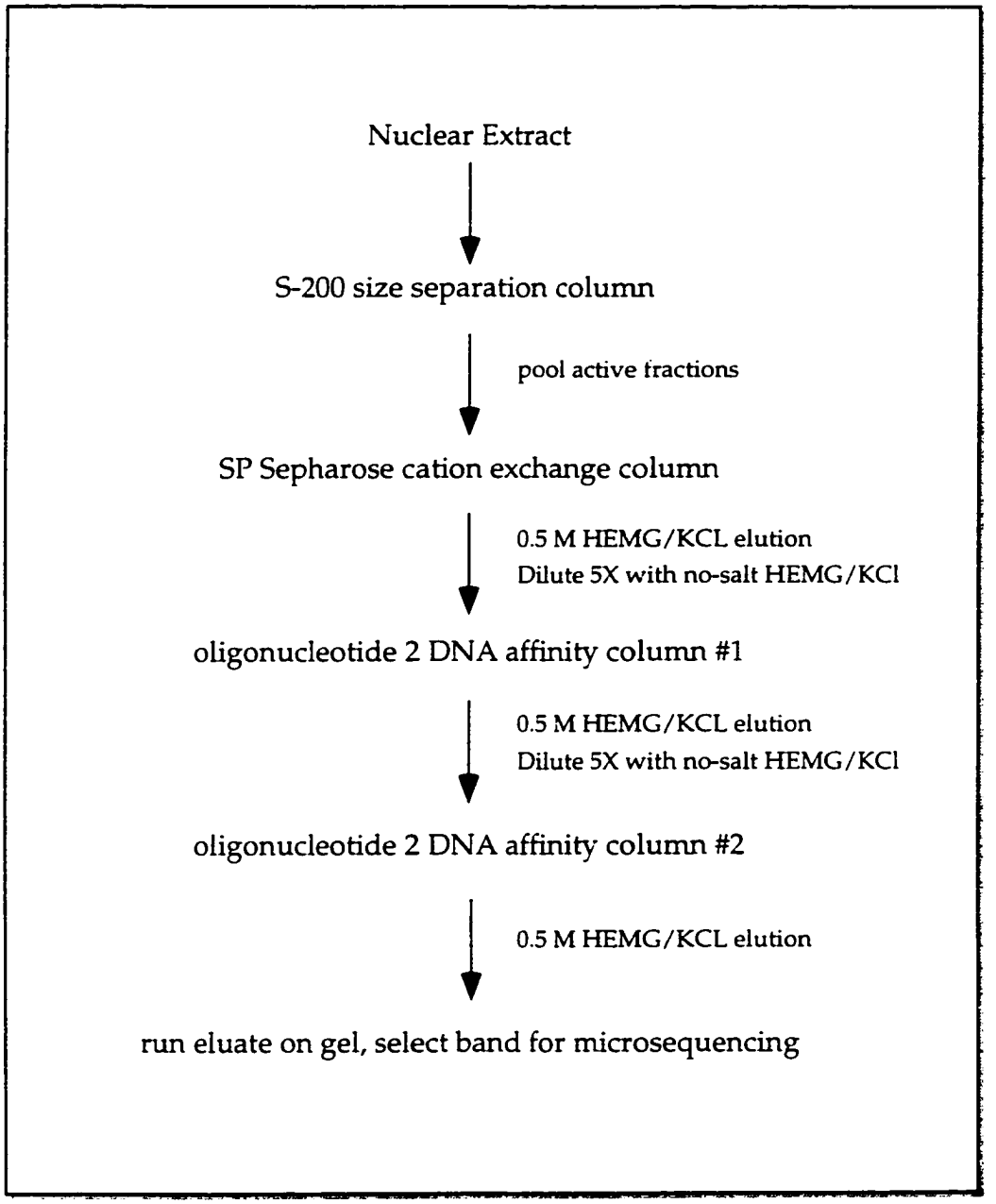


Figure 10: Diagram of the scheme designed for the purification of fEBP 1 and 2

Figure 11: Size chromatography of fEBP 1 and 2. 125 mg of nuclear extract was loaded onto a 500 ml S-200 Sephacryl column and washed with 0.1 M HEMG-KCl. Every other fraction was assayed for protein content and fractions containing protein were tested by EMSA for oligonucleotide 2 binding activity. Lane 1- nuclear extract, 5 µg. Lanes 2-9- fractions 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, and 62, approximately 10 µg protein per assay. fEBP 1 and 2 are indicated by arrows.

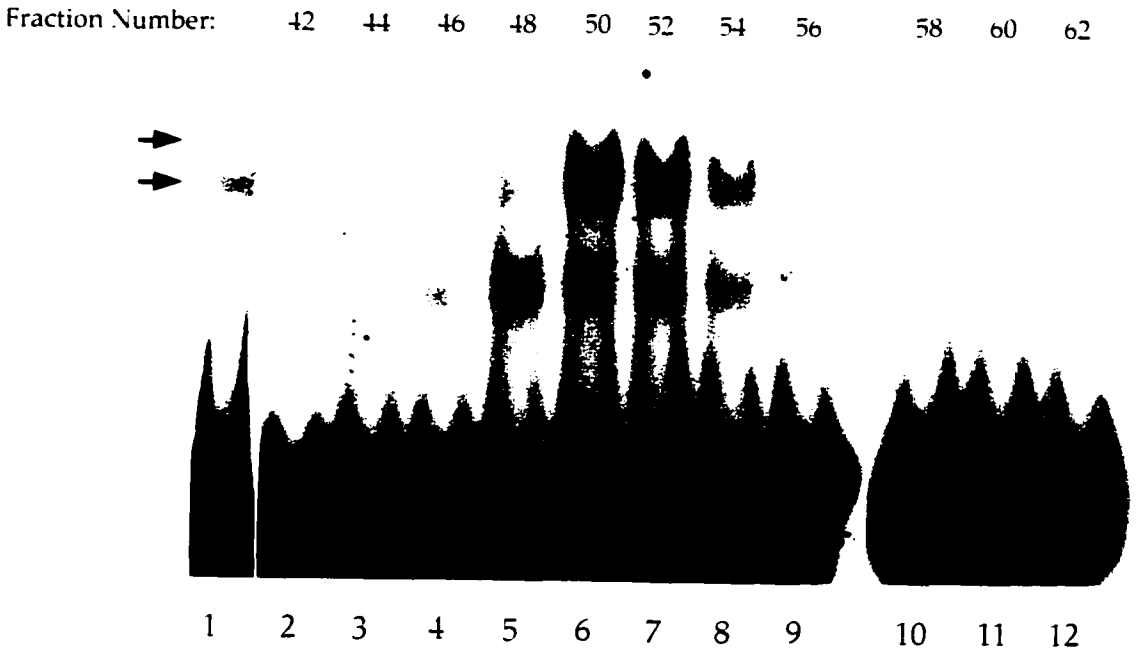


Figure 12: Large-scale attempt at purification of fEBC 1 and 2. EMSA was performed on sample from each step of the purification process. Samples were stored on ice at 4°C prior to assay. Lane 1- nuclear extract, 7.8 µg. Lane 2- S-200 pooled activity peaks, 5.5 µg. Lane 3- SP Sepharose 0.5 M KCl eluate, adjusted to 0.1 M KCl prior to assay, 1.6 µg. Lane 4- DNA affinity column, first round, 0.5 M eluate, adjusted to 0.1 M KCl prior to assay, 0.66 µg. Lane 5- DNA affinity column, second round, 0.5 M eluate, adjusted to 0.1 M KCl prior to assay, 0.2 µg. No nonspecific competitors were included in these assays, so that units in the later fractions would not be masked. fEBP 1 apparently degraded, even in nuclear extract, fEBP 2 is indicated by an arrow.

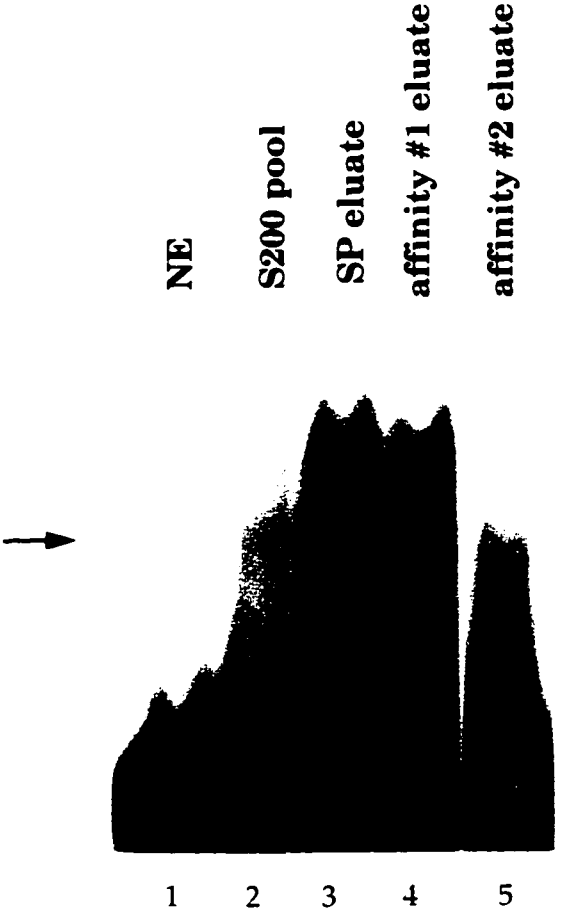


Figure 13: Silver stained SDS gel of large-scale purification of fEBC 1 and 2. Samples from each step of the purification process were separated on a 10% SDS-polyacrylamide gel and silver stained. Following silver staining, the gel was briefly immersed in Coomassie Blue to increase contrast. Lane 1- nuclear extract, 45 µg. Lane 2- S-200 column pooled activity peaks, 15 µg. Lane 3- SP Sepharose 0.5 M KCl eluate, 5 µg. Lane 4- DNA affinity column, first round, 0.5 M KCl eluate, 1.3 µg. Three major bands at ~55 kDa, ~40 kDa and ~32 kDa are indicated by arrows. At least three others in the same lane are not so indicated.

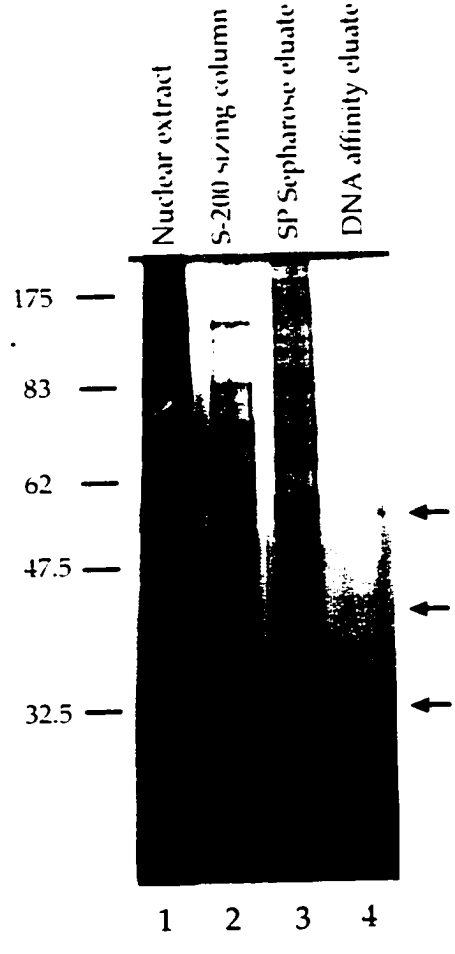


Figure 14: Ultraviolet crosslinking of oligonucleotide 2 to proteins which interact with it. Nuclear extract and the pooled 0.5 M eluate of both rounds of affinity chromatography were incubated with bromodeoxyuridine-substituted oligonucleotide 2. Following ultraviolet irradiation, the samples were separated on a 10% SDS-polyacrylamide gel, and the dried gel was exposed to film. Lane 1- nuclear extract, 1.8 µg. Lane 2- DNA affinity column first round eluate, 0.75 µg. Lane 3- DNA affinity column second round eluate, 0.96 µg. One major band at approximately 55 kDa increased in intensity from nuclear extract to the second round of affinity chromatography.

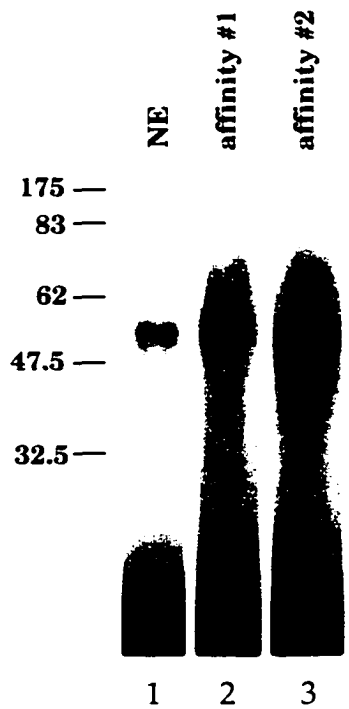


Figure 15: *Panel A*: Sequence of mutant oligonucleotides M-0-1, and M-0-9 prepared by Wei Han (Han, 1994). Boldface type indicates the bases which are altered in each oligonucleotide.

Panel B: EMSA testing the specific interaction of mutant oligonucleotides with nuclear extract or bacterially produced Ftz protein. Lanes 1 and 2- oligonucleotide 2 with nuclear extract (lane 1) or Ftz protein (lane 2). Lanes 3 and 4- oligonucleotide M-0-1 with nuclear extract or Ftz, respectively. Lanes 5 and 6- oligonucleotide M-0-9, with nuclear extract or Ftz, respectively. fEBP 1 and 2 are indicated by arrows. Open arrows indicate additional complexes formed in EMSA using M-O-9. Approximately 10 μg of protein was used per assay.

A.

O2: AGCTTGACAGGAGCAATTAA
ACTGTCCTCGTTAATTTCGA

M-O-1: AGCTTGACAGGAGC**TTC**TAA
ACTGTCCTCG**AAG**ATTTCGA

M-O-9: AGCTTGAC**GAA**AGC**TTC**TAA
ACTG**CTT**TCG**AAG**ATTTCGA

B.

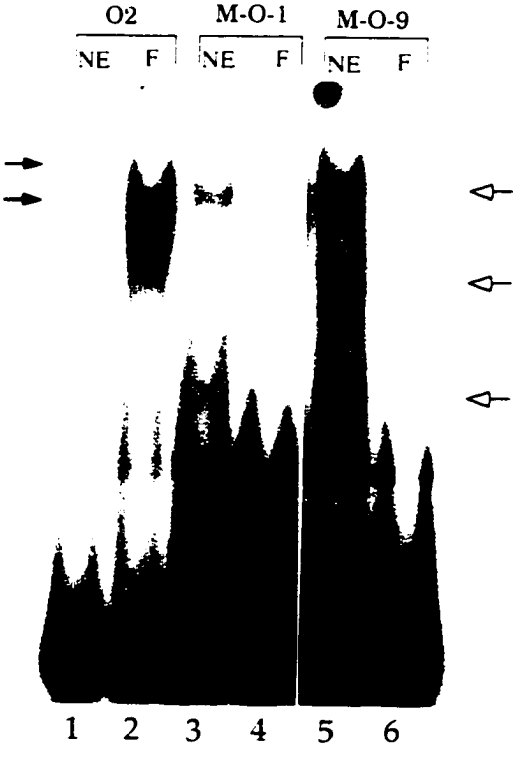


Figure 16: Southwestern blot testing the binding of various mutated forms of oligonucleotide 2. Nuclear extract (55 μg per lane) and SP Sepharose 0.5 M HEMG-KCl eluate (32 μg per lane) were separated on a 10% SDS polyacrylamide gel and transferred to nylon membrane. Proteins on the gel were renatured and hybridized to radiolabeled probe. Lanes 1 and 2- oligonucleotide 2 hybridized to nuclear extract (lane 1) and SP Sepharose eluate (lane 2). Lanes 3 and 4- oligonucleotide M-0-1 hybridized to nuclear extract and SP eluate respectively, lanes 5 and 6- oligonucleotide M-0-9 hybridized to nuclear extract and SP eluate respectively. No change in the proteins' interaction with probe (indicated by closed arrows) was seen when O2 was mutated. Open arrow indicates an additional protein which interacted with M-O-9.

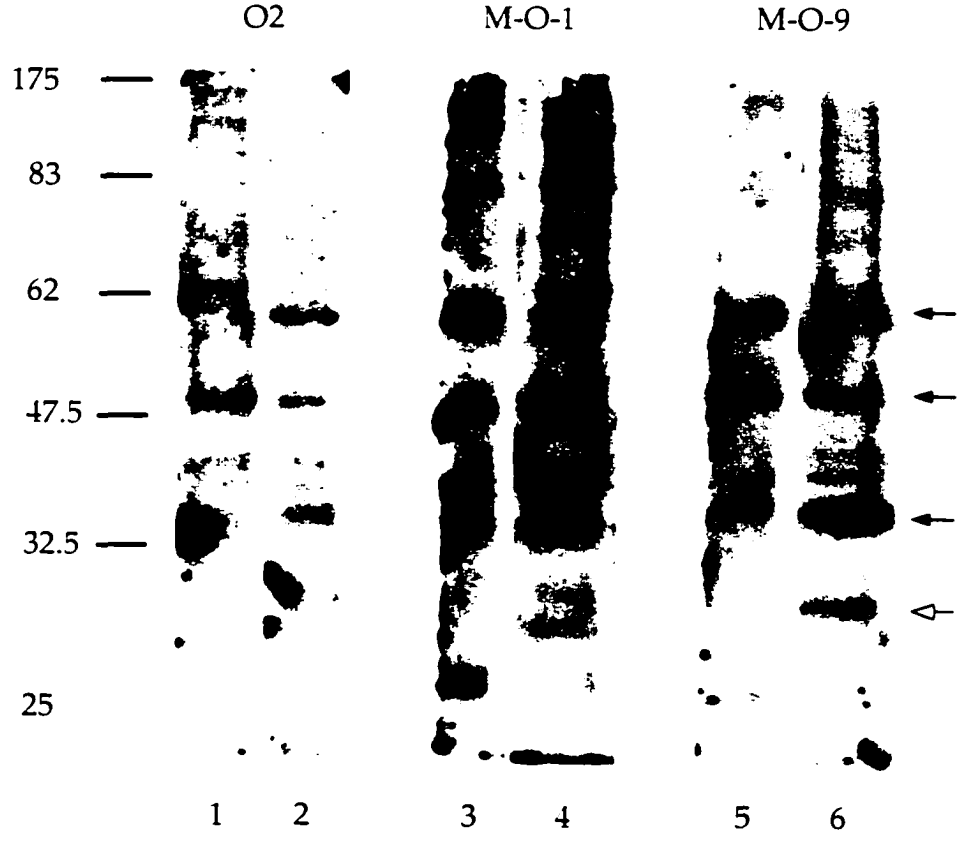


Figure 17: FPLC Superose size fractionation. Nuclear extract was loaded onto SP Sepharose, eluted by 0.5 M HEMG-KCl, precipitated with 65% saturation ammonium sulfate (AS), resuspended in no-salt HEMG and loaded onto a Superose-12 FPLC size separation column. Every other fraction was tested for protein, and those containing protein were assayed by EMSA for oligonucleotide 2 interacting proteins. Lane 1- AS precipitated SP Sepharose eluate, lanes 2-18 fractions 13-45 (every other one). fEBP 2 is indicated by an arrow. Approximately 10 µg of protein per assay was used. fEBP Fractions 19-31 were pooled for EMSA titration of specific activity, which is shown in Table 3.

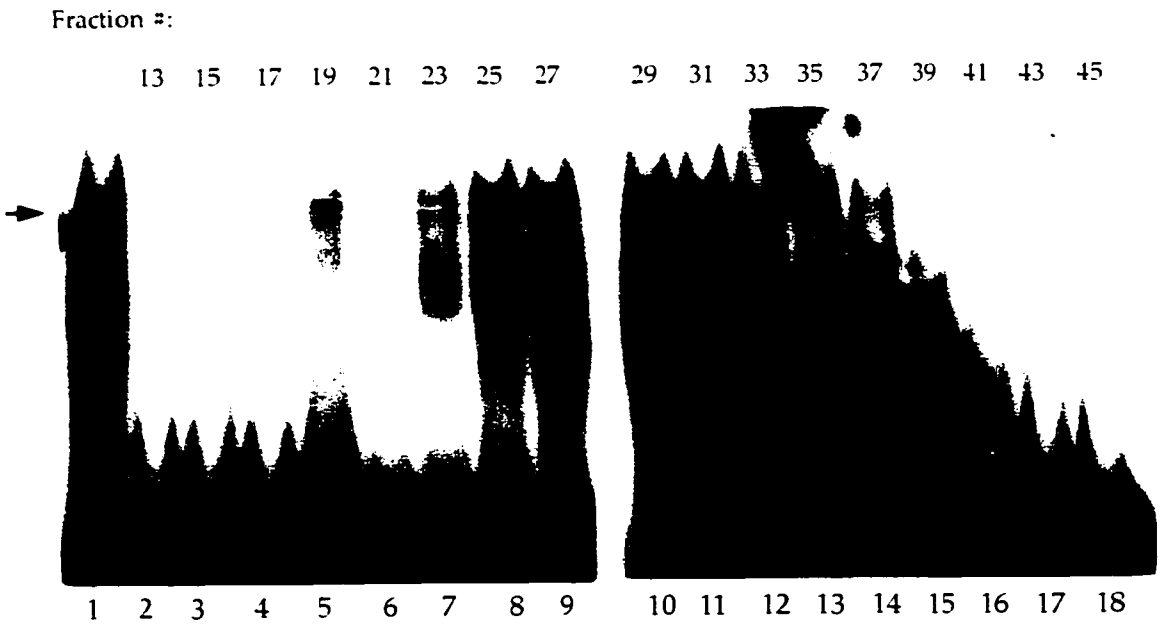


Figure 18: Simplified diagram of the scheme used to disrupt the *adf-1* locus. Flies carrying a P element inserted near the locus were mated to flies carrying an immobilized copy of P element transposase. F1 progeny carrying the transposase were mated to flies with two balancer second chromosomes, in order to remove the transposase from further generations, and to suppress recombination at the point of P element mobilization. F2 progeny whose eye color indicated that they had experienced P element excision or relocalization were mated to flies carrying a deficiency in the area of the *adf-1* locus. Their offspring were evaluated for lethality of the P element mobilization.

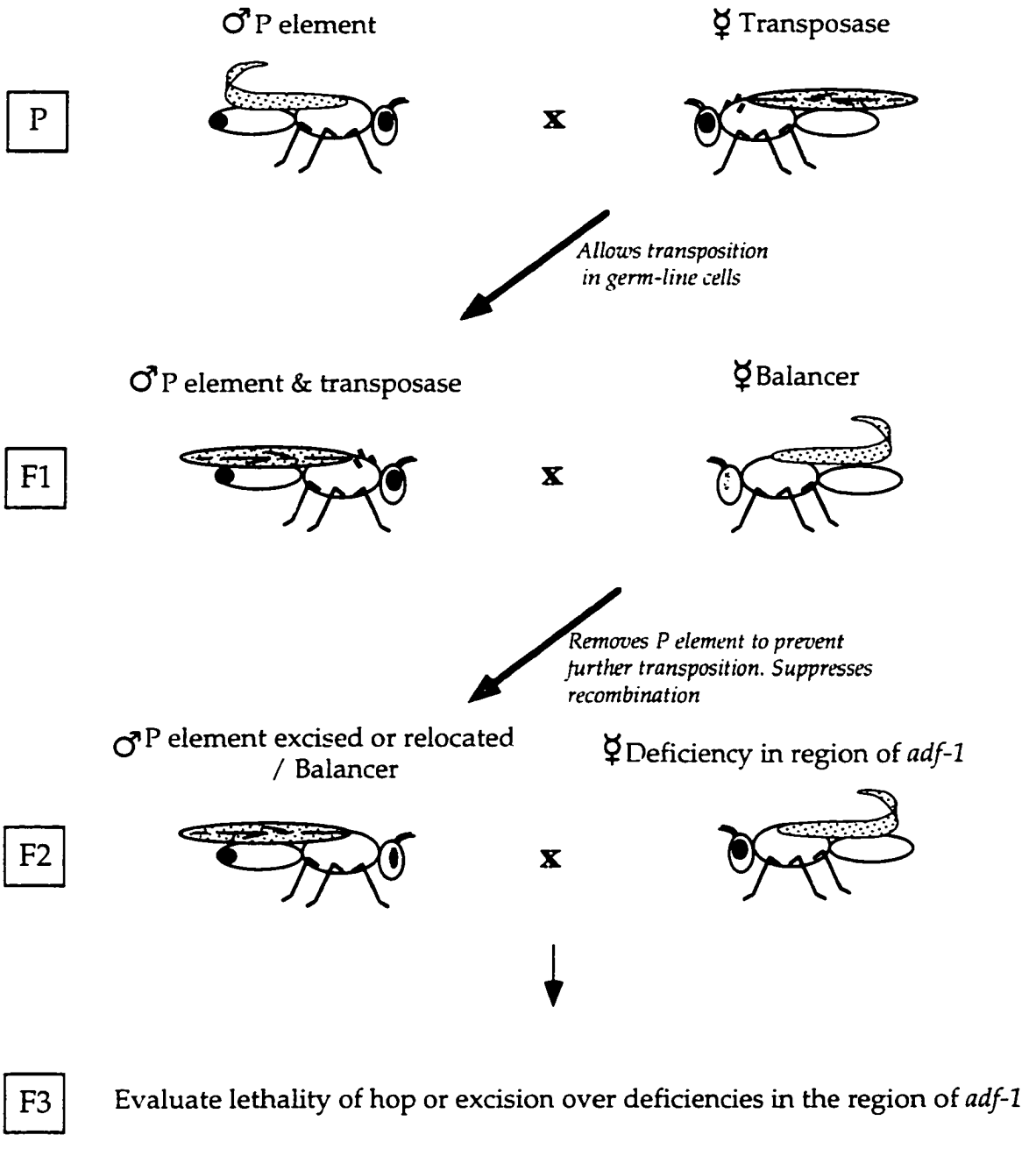
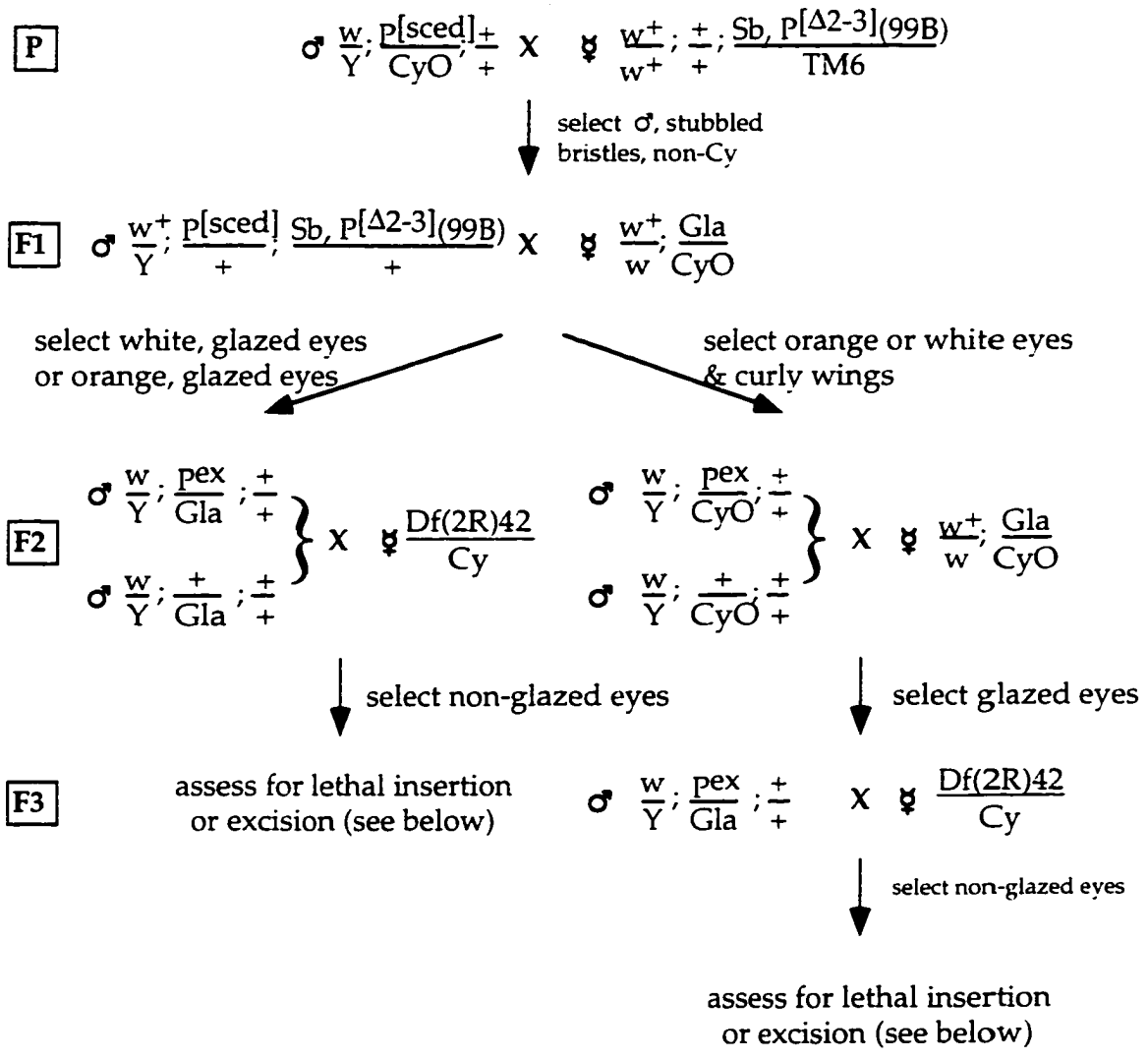


Figure 19: *Panel A*- Diagram of the scheme used to isolate P element-induced mutations in the *adf-1* locus. Symbols used are as follows: $P^{[sced]}$: P-element inserted within 3 kb of *adf-1* start site *(Sullivan, 1993)*. This P element contains the *miniwhite* gene, which gives orange eyes, as a marker *(Bier, 1989)*. CyO: second chromosome balancer carrying the Curly wing marker. $P^{[\Delta 2\text{-}3]}$: chromosome carrying immobilized copy of the P element transposase gene *(Robertson, 1988)*. This chromosome carries the Stubble (Sb) bristle marker. TM6: third chromosome balancer. w: mutant form of *white* gene (results in white eyes). Gla: second chromosome balancer with the Glazed eye marker. P^{ex}: indicates a P element mobilization. Df(2R) 42: Chromosome carrying a deletion in chromosome 2, from 42 C2-C8 to 42 D2-D3. Note that two genotypes among the offspring of the second cross are phenotypically identical, and both are therefore mated to flies carrying the deficiency.
Panel B - The possible combinations of markers in the offspring of the F2 cross of Pex to Df(2R)42. The combination of normal eyes and straight wings should occur either if the fly carried a copy of chromosome II which had never contained a P element (+/Df(2R)42), or if the P element rearrangement is not lethal over the deficiency (P^{ex}/Df(2R)42). Thus, the absence of these flies indicates that a lethal event has occurred in the region of chromosome 2 uncovered by the deficiency.

A.



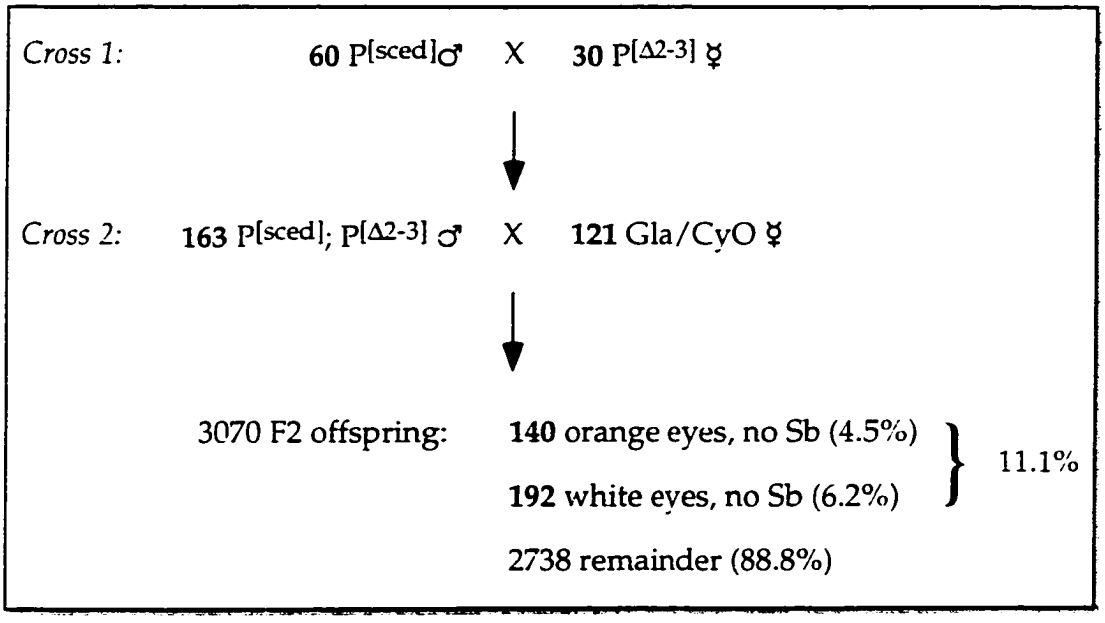
B. Offspring of F2 cross:

	eyes	wings	viable?
pex / Cy	normal	curly	yes
Gla / Cy	glazed	curly	yes
Gla / Df(2R)42	glazed	straight	yes
+ /Df(2R)42	normal	straight	yes
pex / Df(2R)42	normal	straight	only if P element rearrangement is not lethal

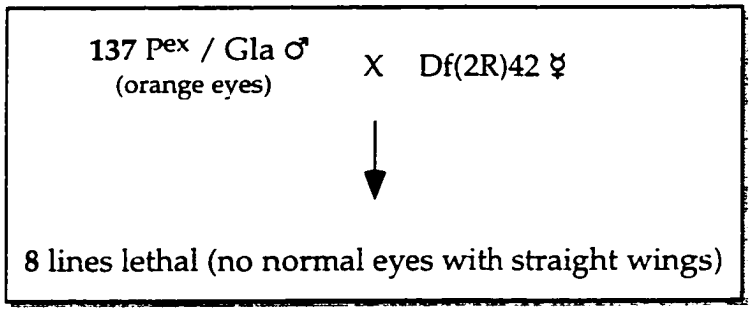
Figure 20: Results of the *adf-1* mutagenesis crosses. *Panel A-* 60 $P^{[sced]}$ males were crossed to 30 females carrying the $P^{[\Delta 2\text{-}3]}$ transposase. 163 male offspring, which had both constructs, were mated to 121 Gla/CyO females. Of the 3070 offspring recovered from this cross, 140 had lost the transposase (as indicated by the lack of the Sb marker) and had orange eyes. Orange eyes indicated retention or relocation of the P element. 192 had white eyes and no Sb. White eyes indicated loss of the P element.

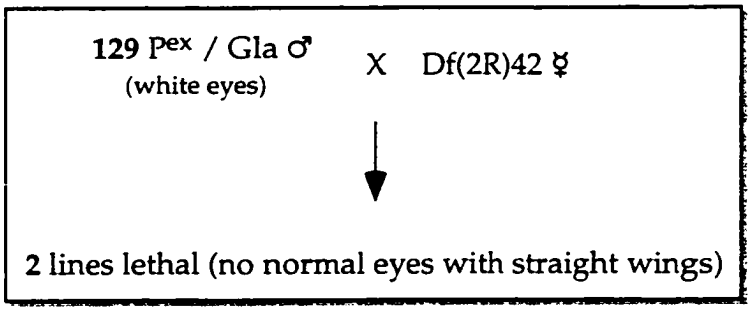
Panel B- 137 orange-eyed flies and 129 white-eyed flies were individually mated to Df(2R)42 females. Note that a few lines were lost in the crosses necessary to substitute the Gla marker for the CyO marker in some of the F2 offspring (see Figure 19). 8 lines from orange-eyed fathers and 2 from lines with white-eyed fathers were found to be lethal over the deficiency.

A.



B.





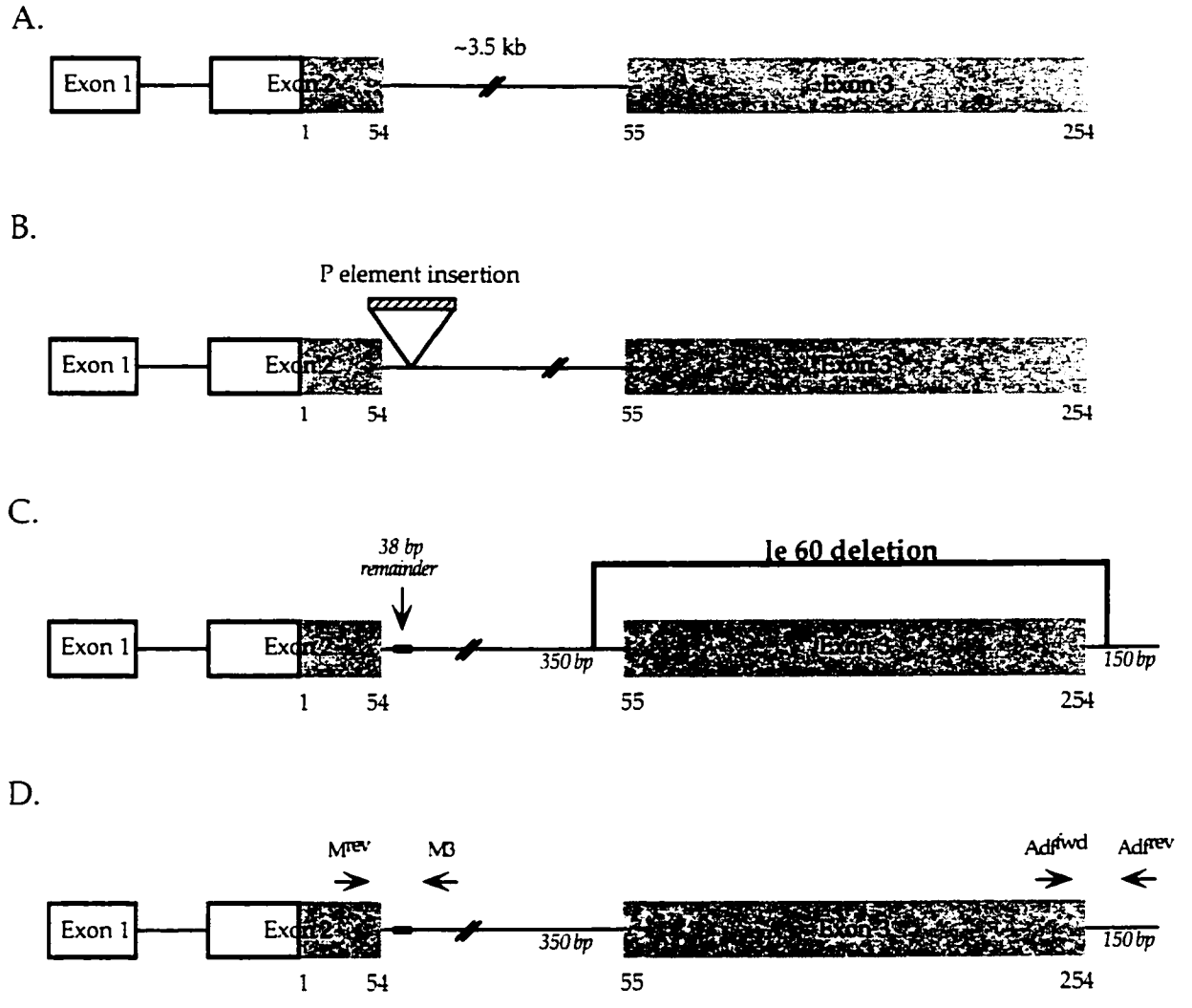


Figure 21: Diagrams of the *adf-1* coding region (information supplied by J. DeZazzo, Tully laboratory). *A.* The three exons of the *Adf-1* mRNA. The protein coding region is shaded gray. Amino acids are numbered underneath. *B.* The original P element insertion (*nalyot*) identified by the Tully laboratory in *adf-1*, 147 bases 3' of the second exon. *C. Adf-1* null allele, le 60, generated by P element excision. The le 60 deletion comprises about 1600 bases, from 350 5' of the third exon to 150 bases 3' of the poly-A site. Note the extra 38 bases which remained at the original P insertion site. *D.* PCR primers M3/Mrev used to diagnose le 60 chromosome, and primers Adf-fwd/Adf-rev used in tests of embryo PCR.

P^{ex} / CyO ♂ X le 60 / CyO ♀

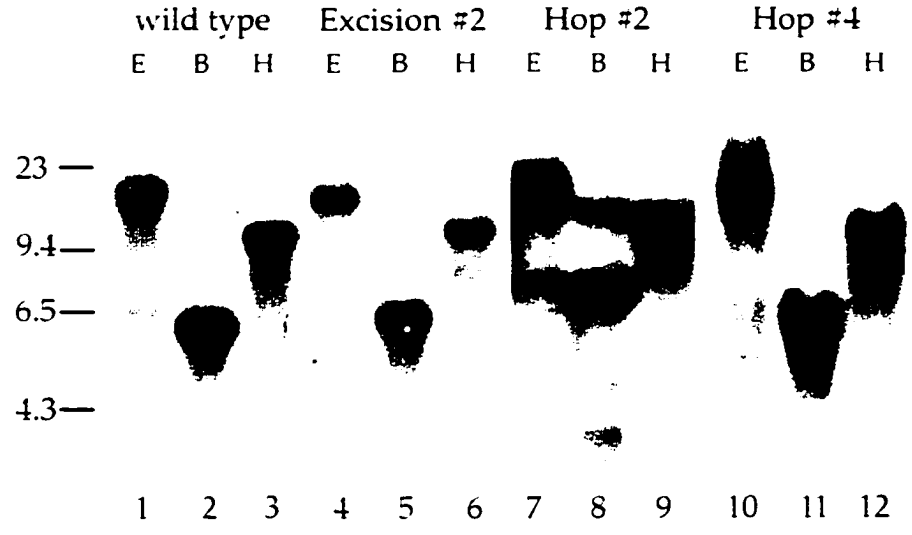
↓

Offspring:	Expected percent of population: With complementation	No complementation
CyO / CyO: lethal	0%	0%
le 60 / CyO: curly wings, viable	33%	50%
P^{ex} / CyO: curly wings, viable	33%	50%
P^{ex} / le 60: straight wings	33%	0%

Figure 22: Predicted results of the cross to determine if le 60 complements any of the 10 lines which are lethal over Df(2R)42 (denoted P^{ex}). If le 60 and the P element rearrangement do complement each other, indicating that different genes are affected in the two lines, 33% of the progeny of the cross should have straight wings, and 66% should have curly wings. If the two fail to complement, no straight-winged flies will be seen.

Figure 23: Southern blot to confirm disruption of the *adf-1* locus. *Panel A*- Approximately 15 µg of genomic DNA from adult flies from wild type and putative adf-1 mutant lines was prepared and digested with either Eco RI, Bam HI or Hind III. Digested DNA was separated on a 0.8% agarose gel and transferred to a nylon membrane. Membranes were hybridized to radiolabeled DNA corresponding to the *adf-1* coding region, prepared from cDNA, washed, and exposed to film. Lanes 1-3 - wild type DNA digested with Eco RI (E), Bam HI (B), or Hind III (H), respectively. Lanes 4-6 - *adf-1* mutant allele E2 digested with Eco RI (E), Bam HI (B), or Hind III (H). Lanes 7-9 - *adf-1* mutant allele H2 digested with Eco RI (E), Bam HI (B), or Hind III (H). Lanes 10-12 - *adf-1* mutant allele H4 digested with Eco RI (E), Bam HI (B), or Hind III (H).
Panel B- Map of approximate sizes of bands generated by restriction endonuclease digestion (provided by J. Dezazzo).

A.



B.

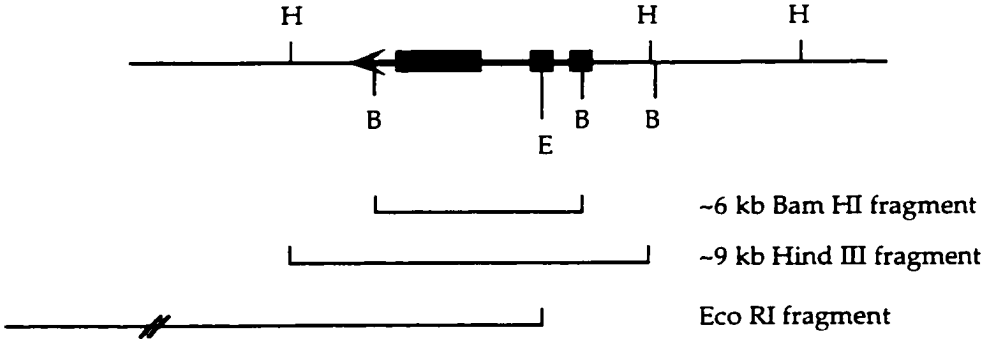
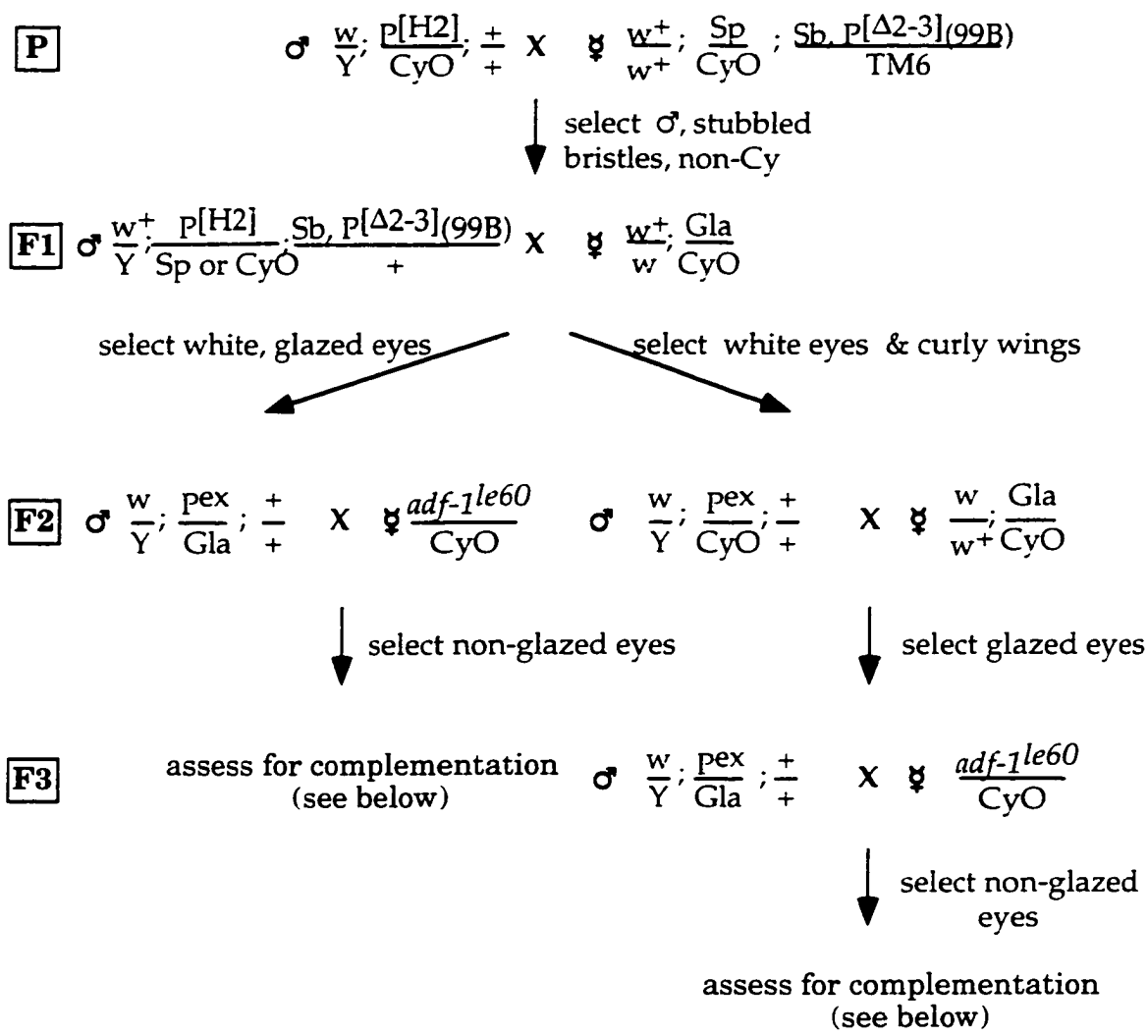


Figure 24: *Panel A*- Diagram of the scheme used to excise the P element from the H2 *adf-1* lethal insertion line. $P^{[H2]}$ refers to the P element inserted into the *adf-1* locus. All other symbols are described in Figure 19.

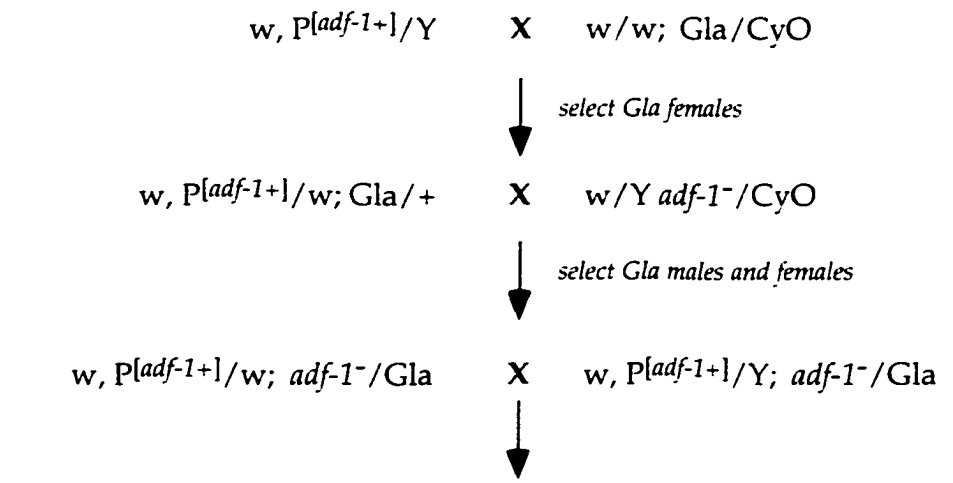
Panel B- The four possible marker combinations in the offspring of the F2 cross of Pex/Gla to adf^{de60}/CyO. The percentage of adult offspring expected in the case of precise excision, which is predicted to restore the *adf-1* gene to functionality, is indicated.

A.



B. Offspring of F2 cross:

	eyes	wings	viable?	Expected percent of non-Gla offspring:
Gla / le 60	glazed	straight	yes	–
Gla / CyO	glazed	curly	yes	–
P^{ex} / CyO	normal	curly	yes	50%
P^{ex} / le 60	normal	straight	only if excision of P element was exact	50%



	Expected percent of population	
Offspring:	Rescue	No Rescue
Gla / Gla: lethal	0%	0%
adf-1⁻/ Gla: glazed eyes, viable	72%	100%
adf-1⁻/adf-1⁻: normal eyes	27%	0%

Figure 25: Scheme to determine if a P element carrying the *adf-1* gene ($P^{[adf-1]}$) can rescue the lethality of putative *adf-1* mutant alleles le 60 and H2. le 60 and H2 are indicated by the symbol *adf-1⁻*, since the cross scheme is the same for both. Rescue was evaluated by the presence or absence of normal eyes in the F2 generation. Normal eyes would be seen only if the P element carrying the *adf-1* gene could rescue lethality of the homozygote H2/H2 or le 60/le 60.

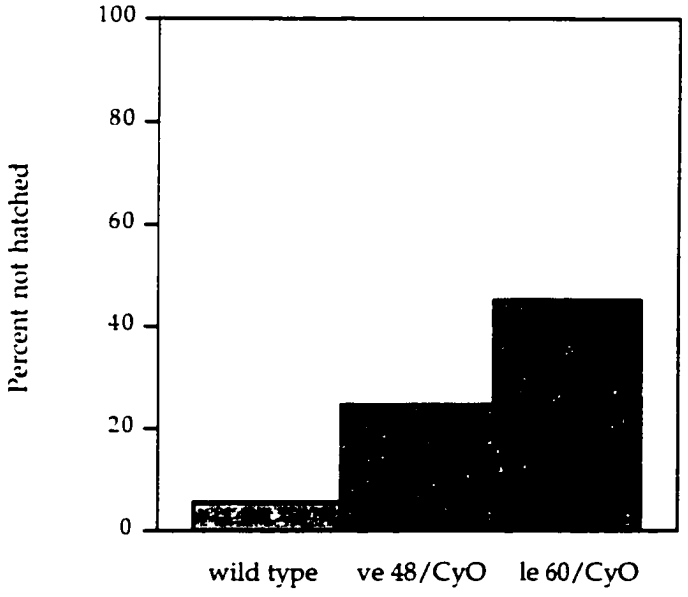


Figure 26: Hatching rates of wild type, ve 48/CyO control, and le 60 *adf-1* null embryos. Embryos were placed on agar plates, aged 36 hours, and nonhatched embryos were counted.

n = 590 for wild type, percent not hatched was 5.2%.

n = 460 for ve 48/CyO, percent not hatched was 24.3%.

n = 610 for le 60/CyO, percent not hatched was 44.9%.

Figure 27: Expression of *ftz* in adf^{de60} / adf^{de60} homozygotes. Embryos from adf^{de60} / CyO-*hb-lac Z* embryos were hybridized *in situ* to both *ftz* and *lac Z* RNA probes. Only embryos which did not express *lac Z* were identified as adf^{de60} homozygotes. *A*- example of expression pattern of *hb-lac Z* reporter gene in anterior 1/3 of embryo. *B, C, D*- Establishment of *ftz* stripes. *E*- Peak *ftz* striped expression. *F*- Beginning of germ band extension and narrowing of *ftz* stripes. *G-H* - gradual loss of *ftz* stripes. *I*- Expression of *ftz* in nervous system.

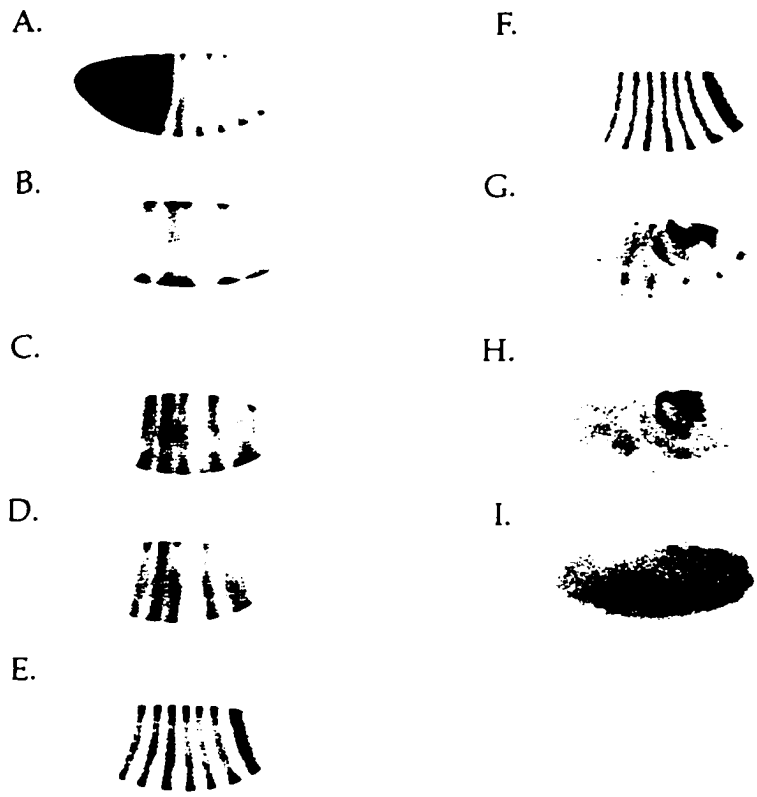
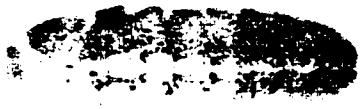


Figure 28: Expression of Ftz in the nervous system of wild type embryos and adf^{de60}/ adf^{de60} homozygotes. 0-16 hour wild type and adf^{de60}/CyO-*actinlacZ* embryos were collected and stained with polyclonal rat anti-Ftz antibody and *in situ* hybridized with *lac Z* probe. *Panels A-D* show wild type embryos, *panels E-H* show adf^{de60}/ adf^{de60} homozygotes. *A and E-* late germ band extension (stage 10). *B and F-* peak germ band extension (about stage 11). *C and G-* beginning of germ band retraction (early stage 12). *D and H-* late germ band retraction (late stage 12). *I-* Example of a stage 11 embryo stained for Ftz and *lac Z*.

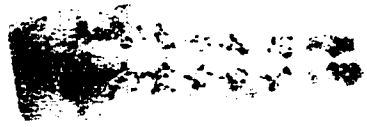
Wild Type

le 60/le 60

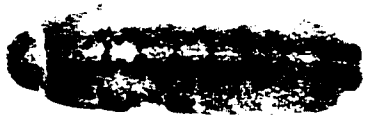
A.



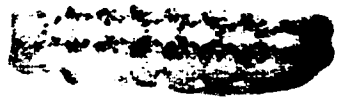
E.



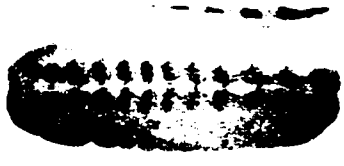
B.



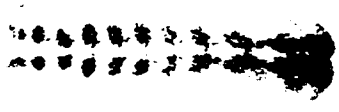
F.



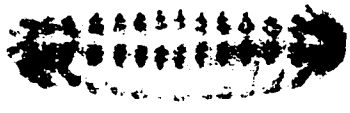
C.



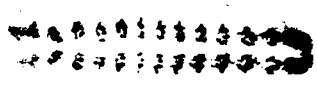
G.



D.



H.



I.

Figure 29: Establishment of protease digestion conditions for reliable single-embryo PCR. Single embryos were dechorionated, fixed, devitellinized, and placed into protease-detergent solution for two hours. Following boiling to inactivate the protease, PCR reagents were added to the embryos, and PCR reactions were carried out. *Panel A:* Lane 1- PCR results from wild type genomic DNA, using primers Adf-fwd/Adf-rev, resulting in a 485 bp product. Lanes 2-5 - DNA from PCR on intact embryos. Lanes 6-9 - DNA from PCR on embryos which were manually broken prior to proteinase treatment. Lanes 10-13 - DNA from PCR on embryos which were repeatedly pipetted during proteinase treatment. The percent of embryos which resulted in PCR product is 50% in all three cases. *Panel B:* Lane 1- PCR results from adf^{le60}/CyO genomic DNA, using primers M3/M-rev, resulting in two products of 520 and 558 bp. Lanes 2-7 - DNA from PCR on embryos which were intact during proteinase treatment and immediately frozen at -80°C for one hour, and then boiled. The percent of embryos which result in PCR product is over 90%.

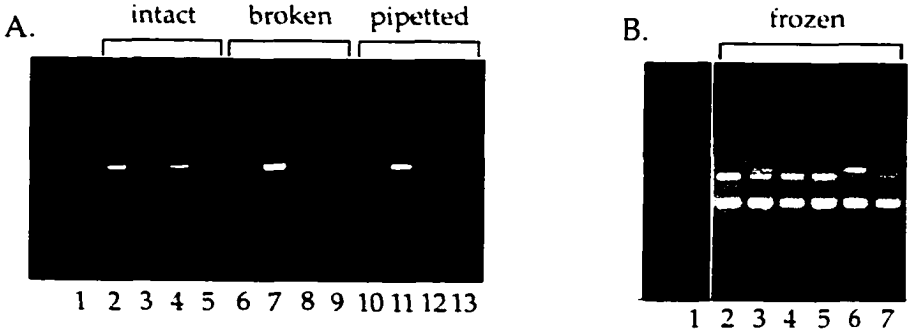


Figure 30: Establishment of PCR reaction conditions for reliable single-embryo PCR. *Panel A*- The effect of various $MgCl_2$ concentrations. Lane 1- adf^{de60}/CyO DNA control, using primers M3/M-rev. Lane 2- single embryo, 1.5 mM $MgCl_2$. Lane 3- single embryo, 1.625 mM $MgCl_2$. Lane 4- single embryo, 1.75 mM $MgCl_2$. Lane 5- single embryo, 1.875 mM $MgCl_2$. Lane 6- single embryo, 2 mM $MgCl_2$. *Panel B*- Effect of embryo staining using DAB-H_2O_2-$NiCl_2$ color reaction on PCR. Lane 1- wild type DNA control, using Adf-fwd/Adf-rev primers. Lanes 2-6 - PCR from single stained embryos, using Adf-fwd/Adf-rev primers. *Panel C*- Effect of embryo staining using DAB-H_2O_2 color reaction. Lane 1- wild type DNA control, using Adf-fwd/Adf-rev primers. Lanes 2-9- PCR from single stained embryos using Adf-fwd/Adf-rev primers.

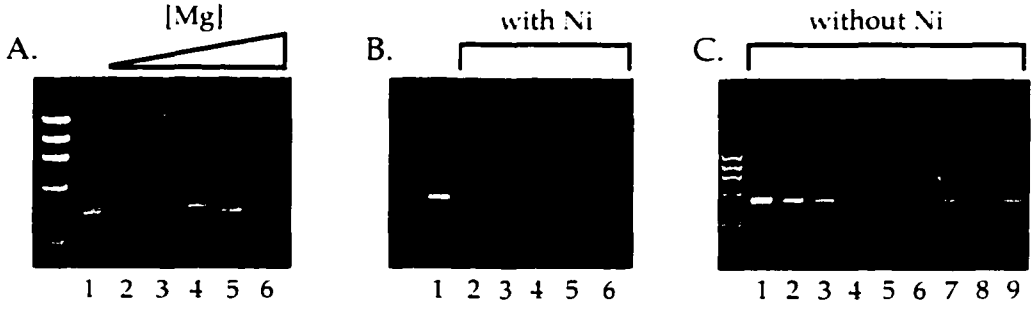
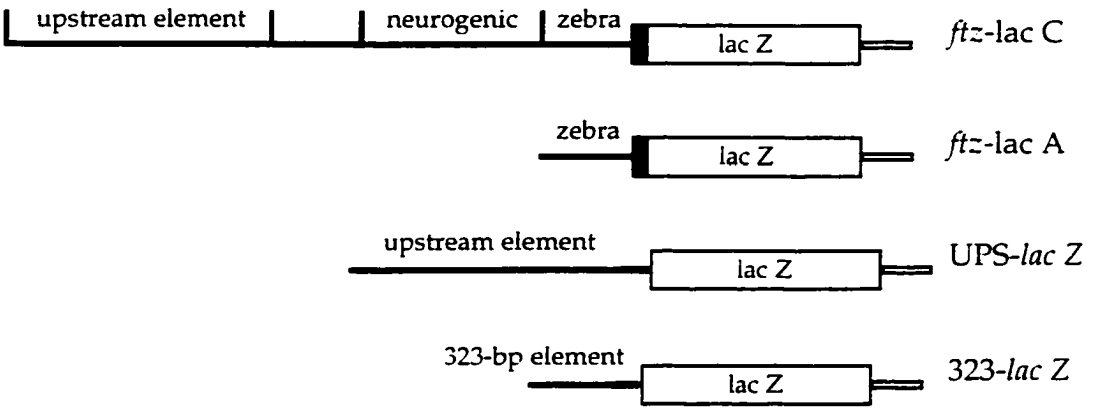


Figure 31: Expression of *ftz-lac Z* reporter genes in adf^{le60}/CyO embryos. Panel A- schematic of the reporter genes. *ftz*-lac C and *ftz*-lac A are redrawn from Hiromi (Hiromi, 1985). UPS-*lac Z* is redrawn from Pick (Pick, 1990). 323-*lac Z* is redrawn from Han (Han, 1993). The latter two both use the hsp 70 basal promoter (indicated by speckled box) B- *ftz*-lac C. C- *ftz*-lac A. D- *ftz*-lac A, variant pattern. E- UPS-*lac Z*. F- 323-*lac Z*. G- PCR genotyping of *ftz*-lac A embryos. Lanes 1 and 5- CyO homozygotes. Lane 2- heterozygote. Lanes 3 and 6- $adf\text{-}1^{le60}$ homozygotes. Lower band (open arrow) present in every lane but lane 4 is an internal reaction control.

A.



B.

E.

C.

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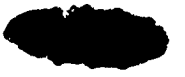
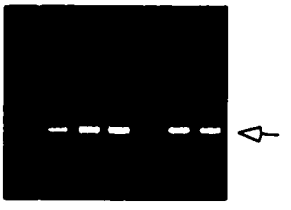


D.

G.



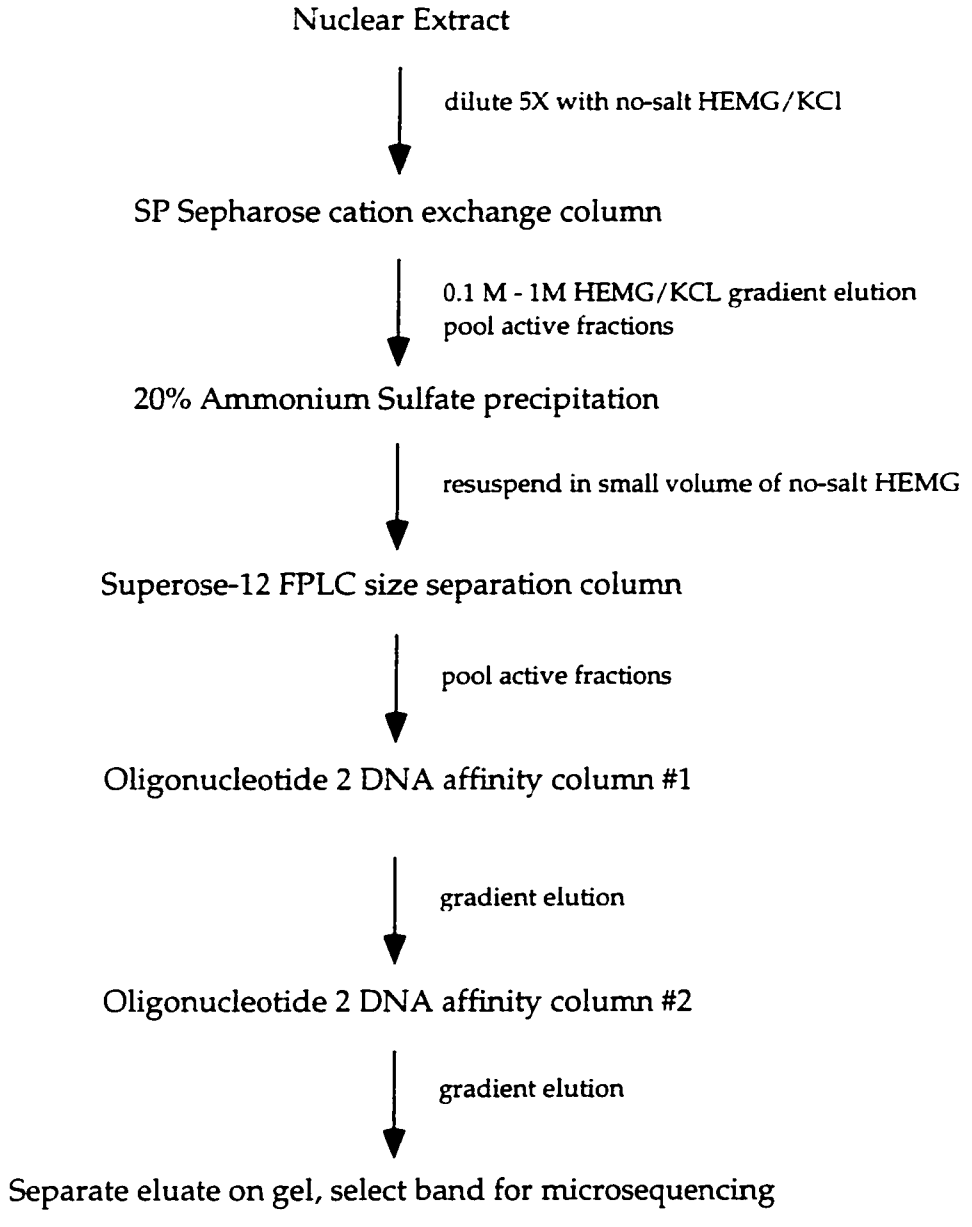


Figure 32: Proposal for revision of the purification scheme of fEBC 1 and 2

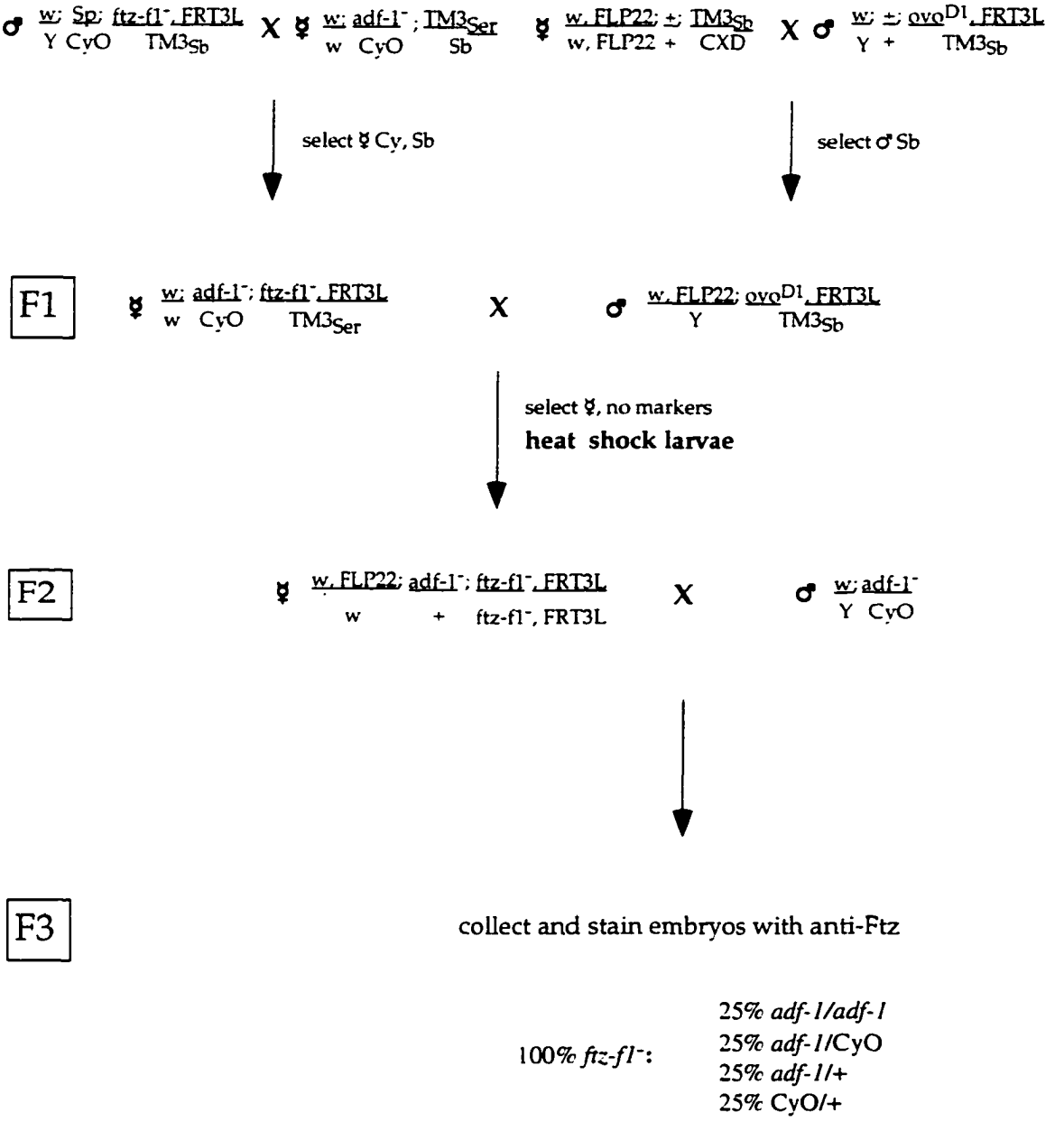


Figure 33: Scheme of crosses used to generate adf-1/ftz-f1 double mutant embryos

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IMAGE EVALUATION
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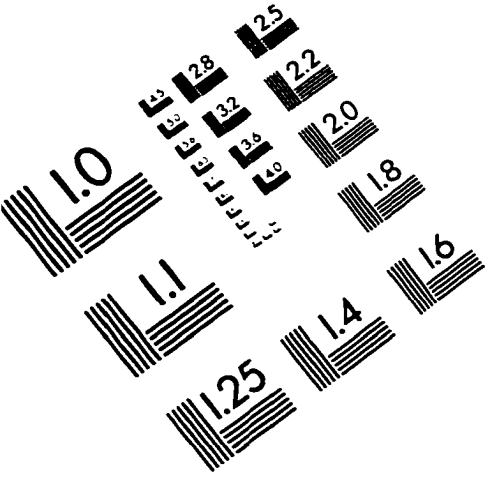

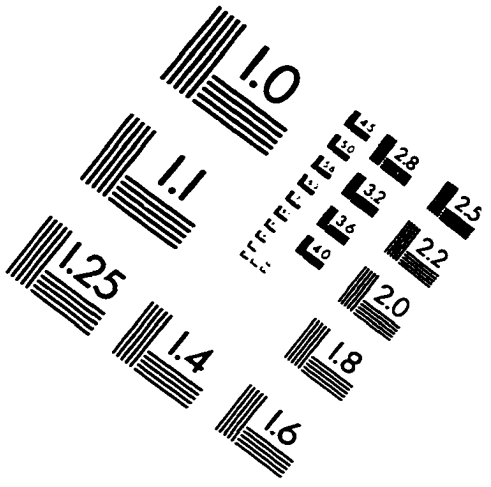

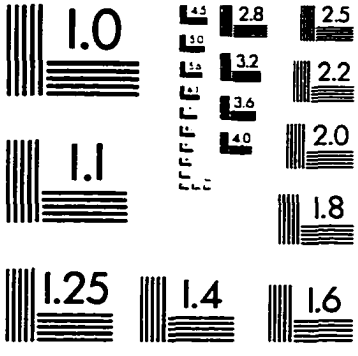

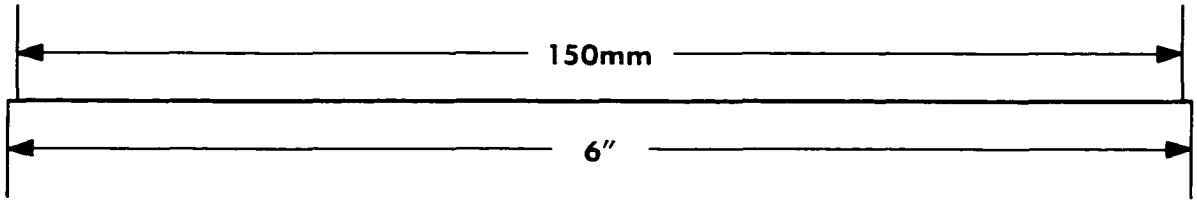

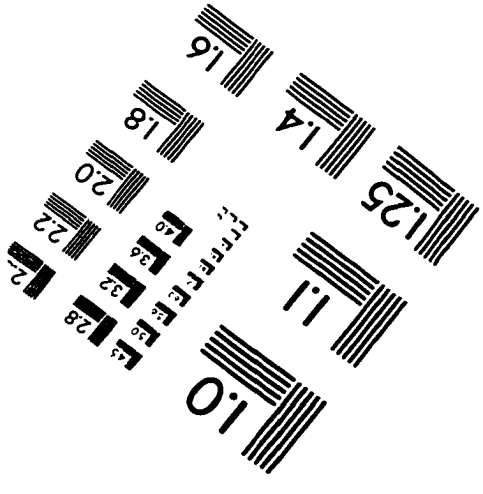

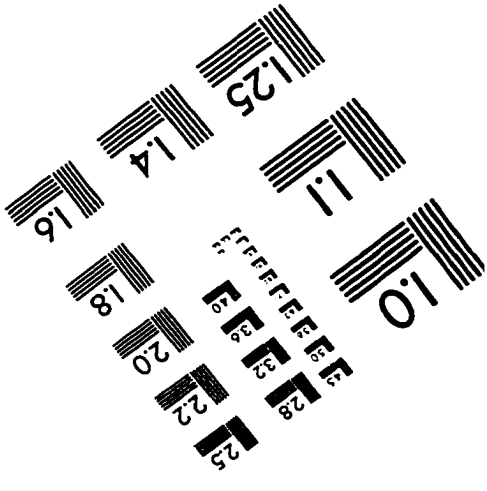

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