Transcriptional Control of the *Drosophila* Segmentation Gene *fushi tarazu*

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To Mike,

whose undying love and support, extraordinary inner strength and unfathomable faith in me have made this accomplishment possible.

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Abstract

The *Drosophila* segmentation gene *fushi tarazu* (*ftz*) is expressed in a characteristic pattern of seven stripes during early embryogenesis. The promoter sequences sufficient to direct this stripe pattern are located within the 670 base pairs (bp) proximal to the *ftz* transcriptional start site. When we extract nuclear proteins from 0-12 hour *Drosophila* embryos, we find sequence-specific DNA binding proteins that recognize multiple sites within the 670 bp zebra stripe promoter. This observation suggests that the control of *ftz* zebra stripe expression may require a complex array of transcriptional regulators. The results of our P element-mediated transformation experiments using *ftz* promoter/ß-galactosidase fusion genes confirm this notion. They demonstrate that the zebra stripe promoter contains multiple, distinct activator and repressor recognition elements responsible for the formation of *ftz* stripes. The transformation studies also reveal that a pattern of general activation, that is, a continuous band of gene expression throughout the germ band, can be generated when repressor recognition sites are deleted from the fusion gene promoter and activator sites are retained. This result strongly supports a mechanism for *ftz* stripe formation involving general transcriptional activation and localized repression.

Studies with constructs in which individual protein binding sites have been deleted or added to the *ftz* promoter correlate protein recognition elements with regulatory functions. We characterized two distinct interband repressor sites and two distinct general activator sites with this approach. One activator site recognizes the product of the homeobox gene *caudal* (*cad*); the other contains a DNA sequence motif found in the *cis*-activators of a number

of *Drosophila* genes. As would be expected for general activators, both these sites are able to mediate expression throughout most of the germ band. We also demonstrate that when multiple copies of two distinct repressor recognition sites are independently ligated to a portion of the *ftz* promoter, they transform the continuous band of gene expression generated by a group of endogenous *cis*-activators into the characteristic seven stripe pattern of *ftz* expression. Finally, we find that multiple copies of these repressor elements are more capable of mediating repression than single copies.

Table of Contents

Preface

Before entering the doctoral program at Caltech, my curiosity was struck by two intricately related biological processes that are essential for the development of a living organism. The first process is the determination of fates of embryonic cells prior to the specification of their pathways of differentiation, and the second is the control of gene expression during organismal development, in particular, the molecular mechanisms governing transcriptional regulation. Through my selection of a thesis project, I attempted to satisfy my strong interest in both these areas. Consequently, I chose to examine how the complex patterns of transcription that are responsible for specifying cell fate during development might be generated in the early embryo.

The process of segmentation in *Drosophila melanogaster* is an excellent system in which to study this problem. First, the early events that determine cell fate along the anterior-posterior axis of the *Drosophila* embryo have been extensively studied on the genetic and molecular level, and so any data gathered in this system can be analyzed and interpreted in the context of a great deal of additional knowledge and insight. Second, it is clear from the information gathered thus far, that the molecular processes governing cell fate along the longitudinal axis of the embryo are controlled by genes with spatially and temporally distinct expression patterns; furthermore, a large number of these genes have been cloned and characterized in detail. Finally, methods are available to reintroduce altered genes into the fruit fly so that the promoter regions controlling position-specific expression can be identified. The identification of *cis*-acting control regions (and subsequently the proteins that interact with them) is a productive first step towards unraveling the molecular details of regulated gene expression in embryogenesis.

INTRODUCTION TO THESIS

The Genetic Regulatory Network Controlling the Longitudinal Pattern of the *Drosophila* Embryo

Introduction

The spatial organization of the *Drosophila* embryo originates prior to fertilization, when maternally-derived morphogenetic determinants become localized in the developing oocyte (reviewed in Akam 1987; Ingham 1988). After fertilization, a cascade of genetic interactions take place in response to these localized morphogens. As a result, increasingly precise spatial domains become defined within the embryo. By the cellular blastoderm stage, a cell's fate is already restricted to a certain body segment and this developmental fate is determined in accordance with its position along the anterior-posterior (A-P) axis. Thus, the body plan of the fruit fly is established before any morphological signs of segmentation appear.

The regulatory network that defines spatial domains along the A-P axis involves the regulation of gene expression in a position-specific manner. Maternal and zygotic pattern forming genes and their products regulate one another both within a given gene class and among the various classes in a hierarchy of interactions. The maternally expressed genes are found at the top of this hierarchy since they are responsible for triggering the regulatory cascade (reviewed in Manseau and Schupbach 1989). There are three different groups of maternal genes that deposit spatial cues along the longitudinal axis of the oocyte: the anterior pattern organizer genes, the posterior pattern organizer genes and the genes required for normal establishment of the termini (Nusslein-Volhard, et al. 1987; Schupbach and Wieschaus 1986). Each of these maternal activities controls the formation of a particular subset of pattern elements in defined (yet overlapping) regions: the anterior group organizes the head and thoracic pattern, the posterior group is essential for

the development of abdominal pattern, and the terminal gene class specifies the acron and telson. It is clear from the phenotypes of embryos lacking maternal factors that interactions between the three maternal activities must occur at some level. For instance, in the absence of either the anterior or the terminal activity, the abdominal pattern elements invade the "empty" region of the embryo, suggesting that anterior and terminal activity negatively modulate the extent and spatial distribution of the abdominal domain (Degelmann, et al. 1986; Mlodzik, et al. 1987; Winslow, et al. 1988).

During oogenesis, mRNA molecules that code for the spatial information of the anterior and posterior group become localized in the developing oocyte (Frohnhofer and Nusslein-Volhard 1987; Lehmann and Nusslein-Volhard 1986; Lehmann and Nusslein-Volhard 1987). After fertilization, spatial cues emanate from these localized mRNA sources at each pole of the embryo. The anterior cue is believed to be the *bicoid* (*bcd*) protein (Frohnhofer and Nusslein-Volhard 1986) and the posterior cue, the product of the *nanos* (*nos*) gene (Sander and Lehmann 1988). The third maternal gene system, the terminal group, also appears to depend on a single morphogenic "signal," in this case the product of the *torso* (*tor*) gene (Strecker, et al. 1989; Klingler, et al. 1988).

By the beginning of the syncitial blastoderm stage, these maternal signaling molecules are dictating the expression pattern of zygotic gene products. Within the anterior organizing system, an anterior to posterior gradient of *bicoid* protein is being translated into the more distinct expression domain of the *hunchback* (*hb*) gene, a principal member of the gap class of segmentation genes (Driever and Nusslein-Volhard 1988a; Driever and Nusslein-Volhard 1988b; Driever and Nusslein-Volhard 1989). The *bcd*

protein contains a homeodomain, and is thus able to regulate transcription by specifically binding to DNA. By binding upstream of the *hb* gene, the *bcd* protein activates *hb* transcription in a broad domain in the anterior half of the blastoderm embryo (Driever and Nusslein-Volhard 1989). It has been shown that higher affinity *bcd* binding sites within a promoter activate gene expression further down the *bcd* protein gradient than low affinity sites (Driever, et al. 1989). The *bcd* protein may, in fact, utilize this mechanism to activate distinct domains of transcription of multiple zygotic genes.

The posterior morphogen, *nanos*, does not appear to behave as a classical gradient morphogen like *bcd*, although diffusion or transport of *nos* from the posterior pole is necessary for the development of the abdominal pattern (Lehmann and Nusslein-Volhard 1987; Sander and Lehmann 1988). Instead of activating domains of gap gene expression *nos* represses the translation of the maternal *hb* mRNA in the posterior half of the embryo (Hulskamp, et al. 1989; Irish, et al. 1989; Struhl 1989). *hunchback* protein disrupts abdominal patterning and so by eliminating it, *nos* allows the abdominal segments to be specified by other morphogens.

Finally, the *torso* terminal gene morphogen is also believed to affect the expression of subordinate genes, in this case genes that act downstream of *torso* in the terminal pathway (Strecker, et al. 1989; Klingler, et al. 1988). The zygotic terminal gene *tailless* (*tll*) (Strecker, et al. 1986) is one such gene. The evidence suggesting that *tor* acts through *tll* to control body plan is that mutations in the *tll* gene are able to neutralize the phenotypic effects of a hyperactive *tor* allele. In other words, the effects of over-expression of *tor* can be suppressed by the under-expression of *tll*. Regarding its mode of action, studies on *tor* protein indicate that *tor* functions as a ubiquitous surface

receptor that is activated by a spatially restricted ligand (Casanova and Struhl 1989).

This cascade of genetic interactions continues with the regulation of gene expression by the gap segmentation genes. There are three other principal members of the gap class, in addition to *hb*; they are *Kruppel* (*Kr*), *knirps* (*kni*) and *giant* (*gt*) (Jurgens, et al. 1984; Lehmann and Nusslein-Volhard 1987; Nusslein-Volhard and Wieschaus 1980; Petschek, et al. 1987; Wieschaus, et al. 1984a; Wieschaus, et al. 1984b). The expression of these four genes span the greater part of the axis of the embryo; *hb* is primarily expressed in the gnathal and thoracic regions, *Kr* in the thoracic and anterior abdominal regions, *kni* in the abdominal region and *gt* in the posterior abdominal region (Knipple, et al. 1985; Nauber, et al. 1988; Preiss, et al. 1985; Tautz, et al. 1987). When protein expression patterns are examined in embryo lacking a single gap gene, the expression domain(s) of the neighboring gap gene(s) expands; thus, negative regulation of neighboring gap genes is thought to be involved in establishing wild-type expression domains (Gaul and Jackle 1987; Jackle, et al. 1986).

Gap genes also regulate the expression of the pair-rule class of segmentation genes (Carroll and Scott 1986; Frasch and Levine 1987; Ingham, et al. 1986). A characteristic feature of the pair-rule genes is their periodic or stripe pattern of expression (Gergen and Butler 1988; Hafen, et al. 1984; Ingham, et al. 1985; Ish-Horowicz, et al. 1985; Macdonald, et al. 1986). How the aperiodic pattern of gap gene expression is translated into the periodicity of pair-rule gene expression is a difficult puzzle to solve, clearly more difficult than that of translating maternal graded information into discrete regions of zygotic gene expression (Carroll 1990). The crux of the problem is that gap

genes are expressed in broad domains spanning several pair-rule stripes and an individual pair-rule gene is both active and inactive within a single domain. Thus, the presence or absence of a gap gene cannot by itself determine whether or not a stripe is formed. The following clue may help solve this problem: Although the regions of gap gene transcription appear to be sharply bounded, the regions of gap protein expression overlap. The presence of these overlap regions suggests that domains of double gap gene expression may be providing cues for subsequent pattern formation, along with the domains of single gap gene expression (Gaul and Jackle 1989; Pankratz, et al. 1989; Stanojevic, et al. 1989).

The genetic evidence suggesting that the gap genes do indeed regulate the initial periodicity of pair-rule gene expression comes from the examination of pair-rule expression patterns in mutant embryos. When mutations in particular maternal genes are combined to create ubiquitous gap gene expression, the pair-rule gene expression is correspondingly uniform (Gaul and Jackle 1989). Hence, it appears that unless some differential information is provided by the gap genes, pair-rule periodicity cannot be generated.

Studies with embryos lacking functional gap genes reveal that the pair-rule genes can be classified into at least two groups: those that appear to respond directly to gap gene spatial cues and those that appear, for the most part, to be regulated independently of the three principal gap genes (reviewed in Carroll and Vavra 1989; Howard 1988). When the expression patterns of the pair-rule genes *hairy* (*h*), *even-skipped*(*eve*) and *fushi tarazu* (*ftz*) are examined in *Kr* and *kni* mutant embryos, significant pattern alterations are observed in all cases (Carroll and Scott 1986; Ingham, et al. 1986). The *hairy*

and *eve* patterns are unique and appear unrelated whereas *ftz* is expressed in patterns that are complementary to those of *hairy* expression (Carroll, et al. 1988; Howard 1988; Ingham, et al. 1986). From these observations it was suggested that alterations of *ftz* expression patterns in these gap mutant embryos are indirect and, instead, mediated primarily by the *hairy* gene product, whereas *hairy* and *eve* patterns are, at least in part, formed directly by gap genes. Thus, *hairy* and *eve* are likely to be directly involved in the translation of aperiodicity into periodicity, whereas *ftz* probably responds primarily to established periodic cues.

Although expression patterns in mutant embryos provide useful information on the epistatic interactions among the segmentation genes (and clues regarding the directness of these interactions), they cannot conclusively demonstrate whether a particular gap gene controls *hairy* and/or *eve* expression by selective repression, by selective activation or by a combination of both mechanisms. Nonetheless, expression patterns in mutant embryos clearly show that, consistent with the presence of overlap regions of gap gene expression, pair-rule regulation involves multiple control mechanisms such that combinations of gap genes affect pair-rule expression in a manner different than a single gap gene (Gaul and Jackle 1989). For instance, it makes a difference whether a blastoderm cell experiences the expression of *hb* alone, *Kr* alone or *hb* and *Kr* at the same time, suggesting that the overlap of *hb* and *Kr* expression observed in wild-type embryos is indeed functional. In addition, gap gene expression is graded toward the margins of the broad domains, and it seems very likely that this, too, is functionally relevant (Gaul and Jackle 1989; Pankratz, et al. 1989; Stanojevic, et al. 1989). Therefore, it not

only matters which gap genes are expressed in a particular region of the embryo, but also at what level they are expressed.

The molecular evidence suggesting that gap gene products may be directly regulating the transcription of other pattern forming genes comes from the fact that several of the principal gap genes contain homologies with the zinc-finger DNA binding motif (Rosenberg, et al. 1986; Tautz, et al. 1987). Consistent with that observation, both *hb* and *Kr* proteins have been shown to possess sequence-specific DNA-binding activities, and both gene products have recognition sites in the promoter of the pair-rule gene *eve* and the gap gene *hb* (Stanojevic, et al. 1989; Treisman and Desplan 1989); *Kr* has also been shown to bind to its recognition sequence in the *kni* promoter region (Pankratz, et al. 1989). These protein-DNA interactions are likely to be responsible for the cross talk that takes place among the gap genes and between the gap and pair-rule classes.

Studies on the regulation of the *eve* promoter best illustrate the points stated thus far on the control of pattern formation by the gap genes. First, P element-mediated transformation of *eve* promoter fusion genes provides evidence that individual *eve* stripes are regulated by separable *cis* sequences, as would be expected if the *eve* pattern is generated by the gap genes (Goto, et al. 1989; Harding, et al. 1989). Second, the *hb* and *Kr* recognition sequences lie within these *cis* elements, supporting the notion that *hb* and *Kr* form *eve* stripes by regulating *eve* transcription (Stanojevic, et al. 1989).

A model can be postulated from the *eve cis*-element binding data to explain how *hb* and *Kr* might act independently and in concert to activate and repress *eve* expression (Figure 1). This model is based on the fact that the

different upstream elements of *eve* shown by P-transformation to be required for different *eve* stripes, contain different distributions of *hb* and *Kr* binding sites. For instance, the element required to generate stripe 2 contains three *hb* sites and six *Kr* sites, whereas the stripe 3 element contains over 20 *hb* binding sites. Also of interest is the fact that two of the three *hb* sites in the stripe 2 element overlap with the *Kr* sites. It was therefore postulated that the stripe 2 element or the *3hb/6Kr* site element activates transcription when there are high levels of *hb* protein, as is the case in the nuclei expressing stripe 2, and that the stripe 3 element which contains 20 *hb* binding sites promotes transcription under conditions of low *hb* protein concentration, as is the case in the nuclei that generate stripe 3. The model also considers the mechanism for the repression of *eve* expression in the nuclei of the interband region between stripes 2 and 3. It postulates that even in the presence of high levels of *hb* protein in the 2/3 interband region, the *3hb/6Kr* site element can repress transcription when sufficient *Kr* protein is present in the nuclei to either compete with *hb* for binding or mask the activity of the *hb* protein through protein-protein interactions. (This model assumes that the stripe 2 *cis*-regulator overrules the stripe 3 *cis*-regulator in the interstripe 2/3 region.)

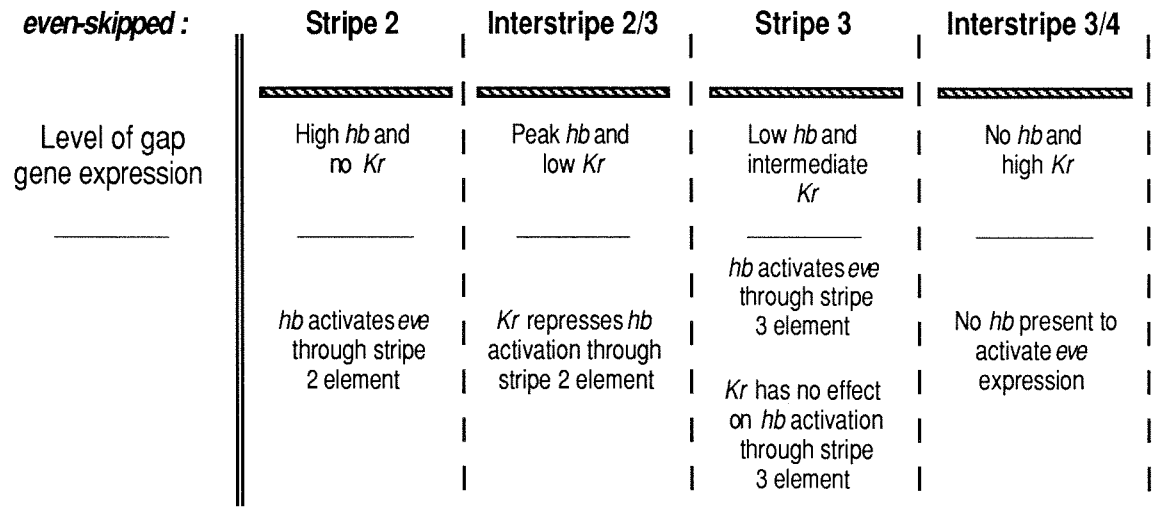

Figure 1. Model for the Generation of *eve* Stripes 2 and 3

Genetic studies support this model for the generation of *eve* stripes (Frasch and Levine 1987). They indicate that *hb* exerts a positive effect on the expression of *eve* stripes 2 and 3, whereas *Kr* represses the expression of stripe 2. In *Kr* mutant embryos, the posterior margin of stripe 2 expands posteriorly since no *Kr* protein is present to repress *hb* activation through the stripe 2 element. It is likely that *eve* stripes 4-7 are generated in an analogous manner by the gradients of the *Kr* and *kni* gap gene products.

The regulation of the stripe pattern of pair-rule gene expression depends not only on the products of the gap genes but on the interactions between the pair-rule genes themselves (Carroll and Vavra 1989; Ingham and Gergen 1988). Examination of gene expression patterns in both single and double pair-rule mutant embryos provide clues as to which of the pair-rule gene interactions might be direct and which might be indirect (Carroll and Vavra 1989). These genetic studies suggest that *hairy* and *eve* regulate *ftz*, *eve* and *runt* regulate *h*, *eve* and *hairy* regulate *runt*, and *runt* regulates *eve*. In

addition, they indicate that *runt* does not regulate the *ftz* pattern, *hairy* does not regulate the *eve* pattern, and *ftz* does not regulate *h*, *runt* or *eve* expression.

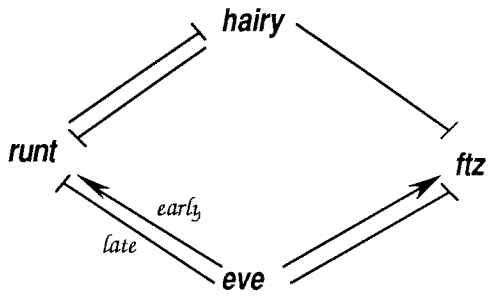

Figure 2. Diagram of Pair-Rule Gene Interactions

A mathematical model has been developed (based on experimental data) that illustrates how these interactions among the pair-rule genes might refine their own initially coarse periodic pattern, generated by the gap genes, into a final pattern of sharp stripes (Edgar, et al. 1989). In this model, each blastoderm nucleus acts as a bistable switch which can operate equally well in either of two transcriptional states (e.g., *hairy* on and *ftz* off in one state and *hairy* off and *ftz* on in the opposite state). By modulating transcription of pair-rule genes, certain combinations of gap gene products impel the switch (i.e., the pair-rule gene network) toward one final state, while other slightly different combinations of gap genes impel it toward the other state. (For example, see Figure 1. Only slight differences in gap gene expression exist between nuclei at the posterior border of stripe 2, where eve is on, and the anterior border of interstripe 2/3, where *eve* is off.) However, the model illustrates that it is the interactions between the pair-rule genes themselves that refine and stabilize the coarse pattern laid down by the gap genes. Once the switch is stabilized by the pair-rule genes, it becomes resistant to large

perturbations such as the loss of gap gene expression. In this manner, the switch can hold constant a pair-rule stripe pattern long enough for it to have an impact on the fate of the blastoderm nucleus.

The best characterized interaction among the pair-rule genes is the repression of *ftz* expression by the *hairy* gene product (Carroll, et al. 1988; Howard and Ingham 1986; Ish-Horowicz and Pinchin 1987). As one would predict for a negative regulator of *ftz*, *hairy* protein is detected, for the most part, in nuclei and cells out of phase with those expressing *ftz* (Carroll, et al. 1988). The first indication that *hairy* negatively regulates *ftz* expression came from the examination of the *ftz* pattern in *hairy* mutant embryos (Howard and Ingham 1986). In those embryos, the *ftz* stripes are broader than in wild type embryos, and they failed to resolve completely. Studies using P element-mediated transformation and *ftz* promoter fusion genes indicate that sequences capable of mediating the repression of *ftz* by *hairy* are located within the 670 base pairs proximal to the *ftz* transcriptional start site, the promoter region shown to be sufficient for generating *ftz* stripes (Hiromi and Gehring 1987; Hiromi, et al. 1985).

The interaction between *hairy* and *ftz* was further characterized by determining the effects of ectopic *hairy* expression in the cells which normally comprise the *ftz* stripes (Ish-Horowicz and Pinchin 1987). Induction of a fusion gene (*hsp70-h* gene), which places *hairy* under heat-shock control, results in the rapid and irreversible repression of *ftz* transcription. The kinetics of *ftz* protein disappearance in the heat-shocked embryos strongly argues that *hairy* acts as a transcriptional repressor of *ftz*, and not through some intermediate regulatory gene.

The structure of the *hairy* protein provides clues as to its mechanism of action (Rushlow, et al. 1989). In particular, the *hairy* protein includes a domain that shows extensive similarity to a domain of the proto-oncogene N-*myc* that may be involved in DNA binding and/or protein dimerization. This protein domain, which has been proposed to form two amphipathic helices separated by an intervening loop (the helix-loop-helix domain), is found in a number of other regulatory gene products including the *daughterless* protein, the myogenic regulator *MyoD*, and two immunoglobulin enhancer binding proteins (Murre, et al. 1989). Since *in vitro* studies have not demonstrated the capacity of *hairy* or N-*myc* to bind directly to DNA, it is likely that their mechanism of action involves protein-protein interactions with other transcription factors rather than protein-DNA interactions. They may regulate transcription by forming heterodimers with DNA binding proteins in a manner analogous to the proto-oncogene c-*fos* (Kouzarides and Ziff 1989; Sassone-Corsi, et al. 1988). (c-*fos* only shows sequence-specific binding when complexed with the *jun*/AP1 protein.)

The *hairy* protein is only one of a number of gene products that regulate the generation of *ftz* stripes (Hiromi and Gehring 1987; Hiromi, et al. 1985; this thesis). Prior to the repression of *ftz* expression in the interstripe regions (by repressor molecules such as *h*), *ftz* transcripts are found in a broad continuous band throughout most of the embryo (Hafen, et al. 1984). General transcriptional activators are required to mediate this initial pattern of *ftz* expression. Thus, *ftz* stripe formation requires both general activator molecules and localized repressor molecules.

The work presented in this thesis includes a detailed characterization of the *cis*-regulators required for both the activation and repression of *ftz* transcription. The first chapter describes an *in vitro* study that was designed to identify these transcriptional regulators in a cell-free system. In this study, extracts from embryonic nuclei are shown to contain sequence-specific DNA binding proteins that recognize multiple sites within the zebra stripe promoter. Included in the study are experiments using staged embryo extracts (Appendix 1). The second and third chapters contain promoter deletion data, obtained *in vivo*, that provide the framework for a model of how *ftz* transcripts become expressed in a characteristic seven stripe pattern. The role of localized transcriptional repression (and general activation) in the formation of *ftz* stripes is clearly demonstrated in these chapters. In addition, the highly resolved stripe pattern of *ftz* expression, as well as the initial continuous pattern, have been recreated *in vivo* using *ftz/lacZ* fusion genes with synthetic oligonucleotides of *cis*-regulatory sites (described in Chapter 3). Inferences can be made from these results about the molecular nature of *ftz* repression. Finally, controlled manipulations of this reconstructed system will allow us to gain further insights into the molecular mechanisms governing position-specific gene expression in the early embryo.

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CHAPTER 1

Biochemical Analysis of the *fushi tarazu* and *Ultrabithorax* Promoter Regions

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Chapter 1

Introduction

In recent years, geneticists and molecular biologists have made progress towards understanding the basic principles involved in the establishment of spatial organization of the *Drosophila* embryo, in particular the establishment of segment pattern and identity. Two classes of zygotically active genes have been found to control this process: segmentation genes that affect segment number and polarity (Nusslein-Volhard, et al. 1980), and homeotic genes that affect segment identity (Lewis, 1978). With the recent cloning and molecular characterization of a number of these genes (Scott, et al. 1983; Kuroiwa, et al. 1984; Weiner, et al. 1984, Bender, et al. 1983; Akam, 1983), and with the development of an efficient *Drosophila in vitro* transcription system (Parker, et al. 1984), it now seems possible to study the transcriptional regulatory mechanisms involved in the differential control of gene expression during early embryogenesis.

In this study a representative gene from each of the two classes has been chosen for careful analysis: the segmentation gene *fushi tarazu* (*ftz*) and the homeotic gene *Ultrabithorax* (*Ubx*). The *ftz* gene is located within the cluster of genes known as the *Antennapedia* complex, approximately 30 kb to the left of the *Antennapedia* (*Antp*) locus (Wakimoto, et al. 1981; Scott, et al. 1983). The *Ubx* gene is one of the homeotic genes within the bithorax complex and it is located in the left half of this complex (Lewis, 1978; Bender, et al. 1983). Both genes are initially transcribed during the blastoderm stage of development (Kuroiwa, et al. 1984; Weiner, et al. 1983; Akam, et al. 1985). Their proper temporal and spatial expression throughout embryogenesis is

critical for establishing the correct segmental organization of the *Drosophila* larva and adult fly. Based on P-element mediated transformation experiments, it is reasonable to assume that the restricted expression of the *ftz* gene is under transcription control (Hiromi, et al. 1985). It is likely that the *Ubx* gene is also controlled at the level of transcription initiation.

The biochemical nature of positional information that restricts *ftz* and *Ubx* gene expression early in development is unknown. The products of maternal effect genes or early zygotic genes are the most likely candidates. Whether these gene products themselves become localized in the developing embryo to regulate *ftz* and *Ubx* expression or whether they respond to other factors acting as positional cues is a compelling question for developmental biologists. This problem can be approached by identifying and purifying the *trans*-acting regulators of *ftz* and *Ubx*, and determining their spatial distribution very early in embryogenesis.

A preliminary biochemical analysis of the transcriptional regulation of the *ftz* and *Ubx* genes is presented in this chapter. The promoters of the two genes were studied *in vitro* using nuclear extracts prepared from *Drosophila* Kc cells and 0-12 hr embryos. DNAse I footprinting (Galas, et al. 1978) and *in vitro* transcription assays (Parker, et al. 1984) were used to identify potential regulatory sites contained within the promoters of both genes.

Footprint Analysis and Competition Experiments on *ftz* and *Ubx* Promoter Regions

Footprinting analysis on the *ftz* and *Ubx* promoters were performed using crude and partially fractionated Kc cell or embryonic extracts, and a number of sequence-specific DNA binding proteins were identified. There are at least 12 binding domains within approximately 400 base pairs (bp)

upstream of the transcriptional start site and at least one within the untranslated leader region of the *ftz* promoter (Figure 1A and data not included). The *ftz* promoter contains three TATA-like elements located upstream from the start point of transcription. Protein(s) present in the extracts bind to all three TATA boxes as shown by the lack of DNAse I cleavage in those regions of the footprint. The protein(s) binding to those regions co-elute with the previously identified B factor (Parker, et al. 1984) on a Biorex 70 column (data not included), suggesting that binding protein(s) that recognize the TATA boxes of the histone, actin 5C, and hsp 70 promoters also recognize the *ftz* promoter. No consensus sequences have been identified in the other binding domains although it is still not known whether each footprint domain represents the binding of a unique protein or protein complex.

Footprint analysis of the *Ubx* promoter reveals binding domains within approximately 200 bp from the start point of transcription, including a cluster between -50 bp and -162 bp (Figure 1B). One footprint domain between -100 bp and -162 bp appears to be due to multiple DNA binding proteins because two distinct footprinting activities can be separated when the nuclear extract is chromatographed on cation exchangers. Note the qualitatively different footprinting pattern in this region when crude embryonic extract is used as material for the footprinting reaction (Figure 1B, lanes 4-6) compared to the pattern obtained when the extract has been passed over Biorex 70 and DNA cellulose columns (Figure 2B, lanes 2 and 3). When protein from a Kc cell nuclear extract is concentrated using a Biorex 70 column, an additional binding domain is observed (Figure 4B). It is located in an AT-rich region between 26 bp and 42 bp upstream from the start point of transcription. This

region may serve the same function as a TATA box since there is no canonical TATA box in the *Ubx* promoter.

The footprinting analysis has been extended by competition experiments in which unlabeled promoter DNA is added in great excess to a labeled promoter fragment prior to the binding reaction. Such a footprint-competition experiment can determine whether a particular promoter fragment specifically binds proteins that recognize the labeled DNA. Competition for binding can be detected by the loss of clearing in a footprint domain of the labeled fragment. Studies with promoters showing similar patterns of expression, e.g., competition experiments with different segmentation gene promoters and the *ftz* promoter, are of particular interest because such shared DNA binding proteins may be reasonable candidates for transcription factors.

A 300-fold molar excess of *ftz* promoter DNA is unable to compete for proteins that recognize the *Ubx* promoter (Figure 1B, lane 12). On the other hand, only a 30-fold molar excess of unlabeled *Ubx* promoter DNA is necessary to eliminate regions of clearing on the *Ubx* footprint (Figure 1B, lane 7). The latter observation confirms that proteins are binding to the DNA in a stoichiometric fashion. Similar results were obtained with a labeled *ftz* promoter fragment (data not included); unlabeled *ftz* DNA competes for binding proteins whereas unlabeled *Ubx* DNA does not.

Effects of *ftz* and *Ubx* Promoter Deletions on *in vitro* Transcription

ftz and *Ubx* promoter activities were investigated *in vitro* using nuclear extracts prepared from *Drosophila* Kc cells and 0-12 hr embryos. DNA templates were truncated several hundred base pairs downstream from the

start point of transcription and the run-off transcripts were analyzed on polyacrylamide gels. The start sites observed *in vitro* for both promoters correspond to the start sites mapped *in vivo* (*ftz*, Paul MacDonald, personal communication; *Ubx*, Peter Harte, personal communication). The *ftz* promoter is very active in Kc cell nuclear extracts (Figure 2A). The level of activity is approximately equivalent to the actin 5C gene promoter activity. The *ftz* promoter is also efficiently transcribed in extracts made from 0-12 hr embryos although the number of transcripts per gene is several fold less than that observed in a Kc cell nuclear extract. The *Ubx* promoter is significantly less active than the *ftz* promoter (Figure 2B); it is approximately ten-fold weaker than the *ftz* promoter in Kc cell extracts, and transcripts are generally undetectable in embryonic extracts. The RNA products made from these templates are clearly transcribed by RNA Polymerase II since the reactions are completely sensitive to 2 μg/ml α-amanitin (Figure 2B).

Progressive 5' deletion sets were constructed for both the *ftz* and *Ubx* promoters using Bal 31 exonuclease. The efficiencies of these altered promoters were assayed in the *in vitro* transcription systems to investigate the role of upstream sequences in promoter function. Deletion of binding sites upstream of -40 in the *ftz* promoter reduced transcription by no more than three-fold in Kc cell extracts (Figures 3A and B) and generally no effect was observed in embryonic extracts (Figure 3C). The three-fold reduction in Kc cell extracts was only seen when the ratio of template concentration and extract concentration were carefully balanced (Figure 3A). In all extracts tested, the *ftz* promoter was non-functional when the TATA box closest to the transcriptional start site was removed.

The results obtained from transcriptional analysis of the *Ubx* promoter deletions are similar to the results obtained with the *ftz* promoter. Deletion of binding sites upstream of -47 had no affect on *in vitro* transcription in a Kc cell extract (Figure 4A). When the AT-rich region located between -26 and -42 was deleted, the *Ubx* promoter was inactive in the Kc cell *in vitro* transcription system. The latter observation adds support to the notion that the dA•dT-rich domain at -26 may serve as a TATA box for the *Ubx* promoter.

Discussion

Sequence-specific DNA binding proteins that recognize the *fushi tarazu* and *Ultrabithorax* gene promoters can be extracted from Kc cell and embryo nuclei. Since the information necessary for generating the alternate-parasegment-specific pattern of *ftz* expression is encoded in the 740 bp sequence upstream of the *ftz* translational start site (Hiromi, et al. 1985), it seems likely that at least a subset of the proteins that bind to the *ftz* promoter plays a role in controlling the spatial distribution of *ftz* transcripts in embryogenesis. The location of *Ubx* promoter binding domains within 200 bps of the start point of transcription also suggests that they are involved in the transcriptional control of *Ubx*. Competition studies indicate that no DNA binding proteins have been identified that recognize sites on both promoter regions.

The deletion data demonstrate that crude extract preparations provide factors that function by binding to the promoter region, either very close to the start point of transcription or within the coding region of the *ftz* and *Ubx* genes. These promoter-proximal transcription factors may be present in

higher concentrations in our extracts than in the developing embryo so that the effects of additional transcription factors, i.e., the upstream promoter binding proteins, may not be observed. It is likely that the factors activating these promoters *in vitro* direct the transcription of a number of cellular genes in addition to *ftz* and *Ubx*. Therefore the distribution of these factors on other genes in the cell may allow the upstream binding activities to play an active role *in vivo* that is underestimated *in vitro*. Fractionation of the *in vitro* transcription system should allow us to control the ratios of various factors in a transcription reaction so that we can determine whether our DNA binding proteins play a role in the transcriptional machinery.

Extracts made from 0-12 hr embryos or cultured cells obviously lack the temporal and spatial features of a developing embryo. The binding proteins identified in these extracts may consist of a population of both positive and negative effectors of transcription whereas an individual cell in the developing embryo may contain only a subset of these binding proteins. To address the temporal control of the *ftz* and *Ubx* genes, extracts will be prepared from staged embryos (2 hr intervals) and tested by footprinting and transcription assays (see Appendix 1). *Cis*-acting elements involved in controlling the spatial distribution of transcripts will be identified *in vivo* using P-element mediated germ-line transformations of *lacZ* fusion genes in constructs lacking one or several *ftz* or *Ubx* promoter binding domains (see Chapters 2 and 3).

Once sequence specific binding proteins have been shown to play a role in the temporal or spatial control of these developmentally regulated genes, we will purify them to homogeneity, determine amino acid sequences, isolate the appropriate genes from cDNA libraries with oligonucleotide probes, and

produce monoclonal antibodies. With DNA probes and antibodies specific for these factors, it will be possible to trace their developmental expression in the *Drosophila* embryo. By studying the expression of regulators of developmental genes, insight can be obtained regarding the genetic regulatory hierarchy governing segmental determination.

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FOOTPRINT ANALYSIS AND COMPETITION EXPERIMENTS

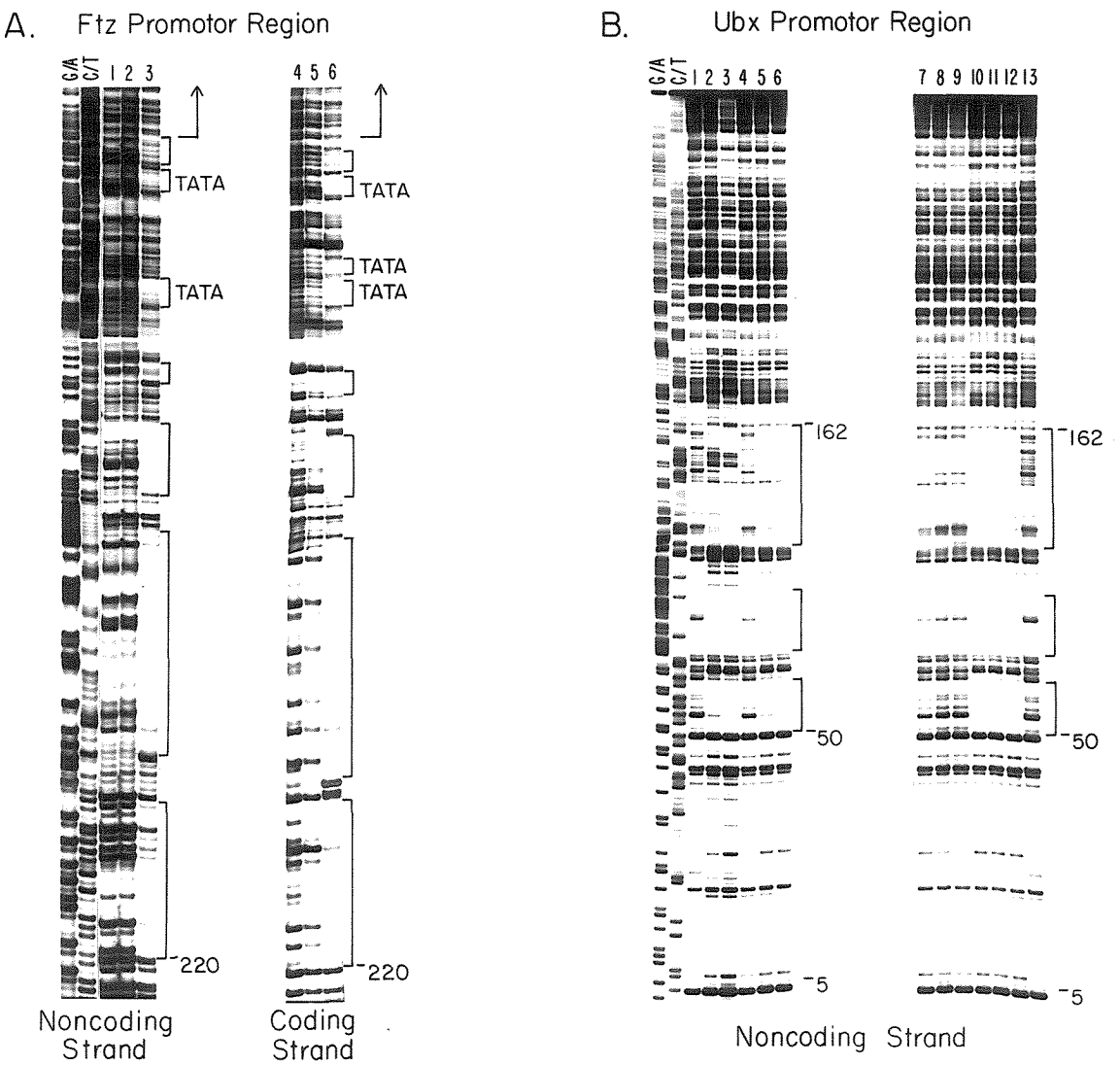

Figure 1.

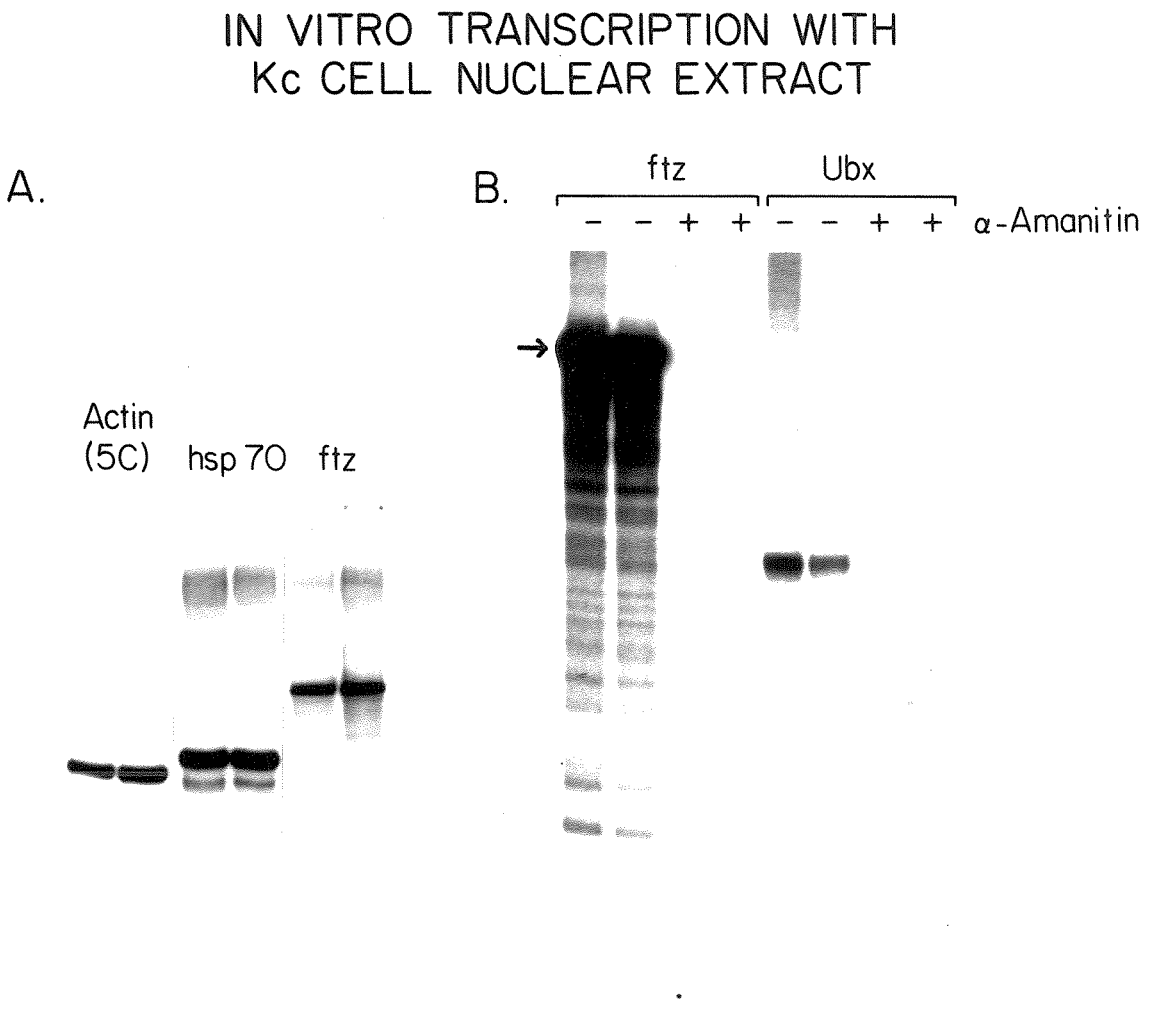
IN VITRO TRANSCRIPTION WITH
Kc CELL NUCLEAR EXTRACT
A.
Actin
(5C)
hsp 70
ftz
B.
ftz
Ubx
− − + + − − + +
α-Amanitin

Figure 2

Ftz Promoter Deletions: Effects on In Vitro Transcription

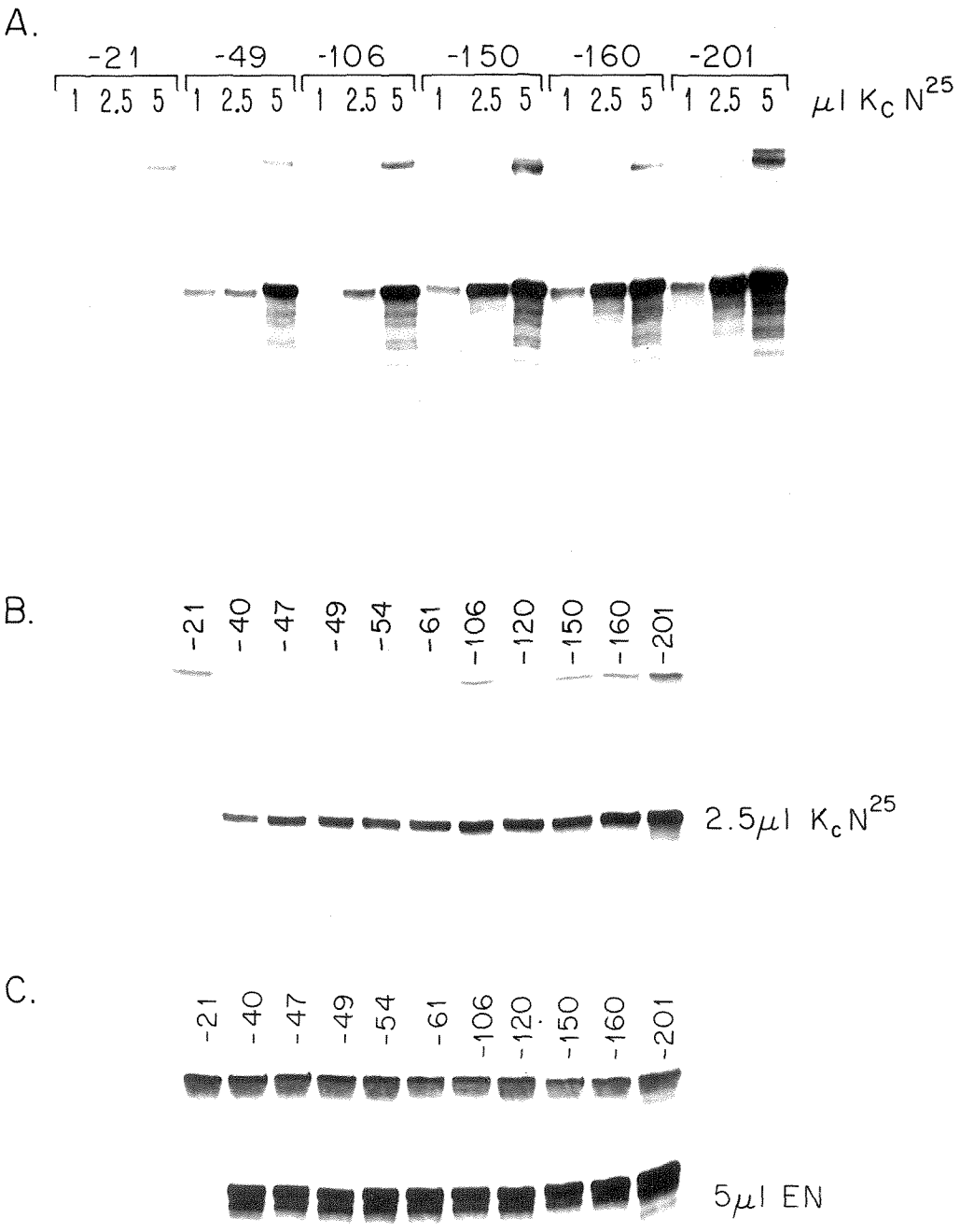

Figure 3.

Ubx Promoter Deletions: Effects on In Vitro Transcription and Footprint Analysis

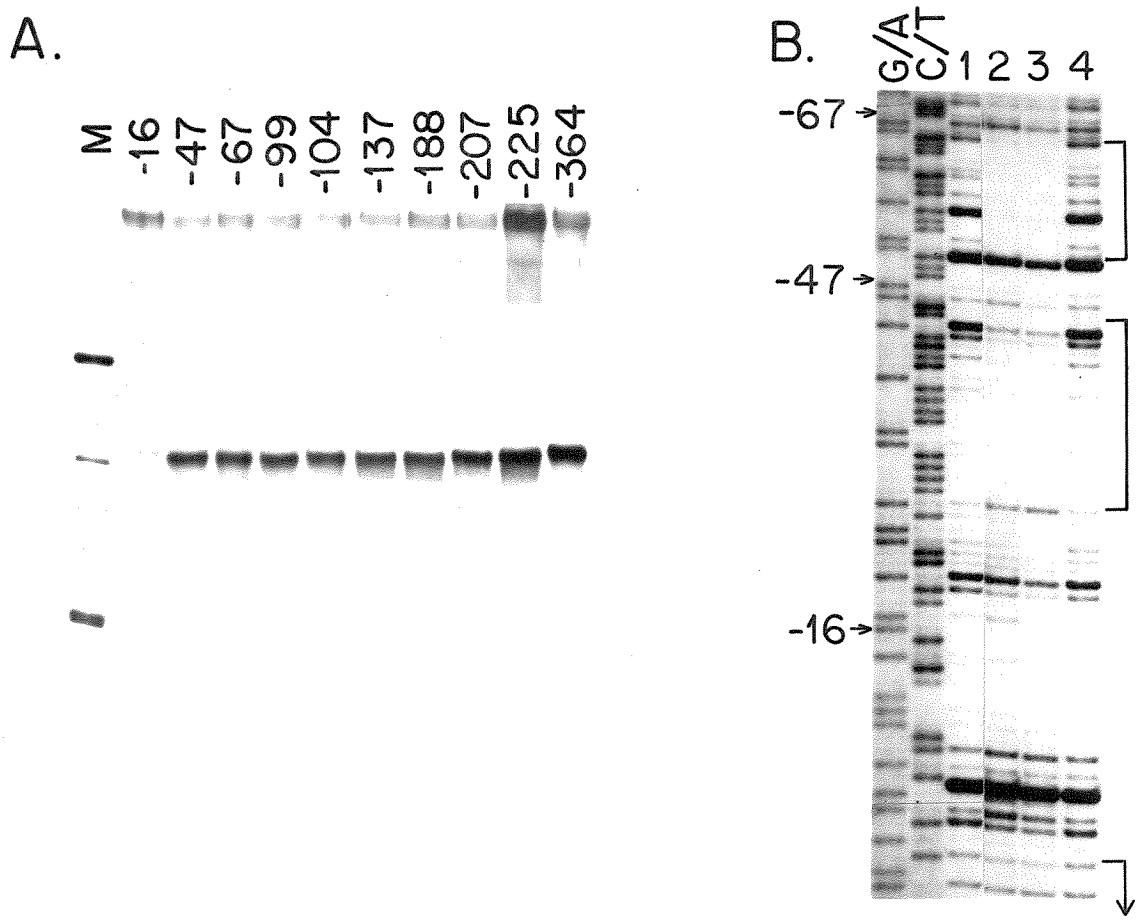

Figure 4.

Figure Legends

Figure 1.

G/A: Chemical cleavage at purine residues of the indicated fragment. C/T: Chemical cleavage at pyrimidine residues at the indicated fragment (Maxam et al., 1980). (A) Footprint analysis of the *ftz* promoter region. Lanes 1, 2, 4 and 5 are controls; no protein was added to the binding reaction. Lanes 3 and 6 contain 20 µl of protein from embryonic nuclei that was concentrated over a Heparin Agarose column (0.4 M KCl step) and a Biorex 70 column (0.8 M KCl step) before it was added to the reaction. (B) Footprint analysis and competition experiments with the *Ubx* promoter region. Lanes 1 and 13 are controls; no protein was added to the binding reaction. Lanes 2 and 3 contain 10 µl and 40 µl of protein, respectively, from embryonic nuclei that was concentrated over a Biorex 70 and a DNA cellulose column before it was added to the reaction. Lanes 4, 5 and 6 contain 2 µl, 7.5 µl and 15 µl of crude embryonic nuclear extract, respectively. Lanes 7-12 contain 15 µl of crude embryonic nuclear extract. Lanes 7, 8 and 9, in addition, contain 30-fold, 100-fold and 300-fold molar excess of unlabeled *Ubx* promoter DNA, respectively. Lanes 10, 11 and 12 contain 30-fold, 100-fold and 300-fold molar excess of unlabeled *ftz* promoter DNA, respectively.

Figure 2.

(A) Comparison of actin 5C, hsp 70 and *ftz* promoter activities *in vitro*. Transcription assays include 5 µg/ml DNA template and 5 µl Kc cell nuclear extract in a final reaction volume of 15 µl. The intense bands on the gels are the products of the run-off transcription reactions. (B) α-Amanitin sensitivity of *ftz* and *Ubx in vitro* transcription reactions. A minus sign (-) indicates that

no α-amanitin was added. A plus sign (+) indicates the addition of 2 μg/ml or 200 μg/ml of α-amanitin to the transcription reaction.

Figure 3.

The number placed above the reaction refers to the deletion end point, i.e., the distance upstream from the start point of transcription. (A) Effect of Kc cell nuclear extract titration on *in vitro* transcription of *ftz* promoter deletions. Transcription reactions include 5 μg/ml DNA template and the indicated amount of extract, in a final reaction volume of 15 μl. (B) *In vitro* transcription of the *ftz* promoter deletion set in Kc cell nuclear extract. 5 μg/ml DNA template and 2.5 μl extract was added to each 15 μl reaction. (C) *In vitro* transcription of the *ftz* promoter deletion set in embryonic nuclear extract. 20 μg/ml DNA template and 5 μl extract was added to each 15 μl reaction.

Figure. 4.

(A) *In vitro* transcription of the *Ubx* promoter deletion set in Kc cell nuclear extract. The number placed above the reaction refers to the deletion end point, i.e., the distance from the start point of transcription. 6 μg/ml DNA template and 5 μl extract was added to each 15 μl reaction. (B) Footprint analysis in the region critical for *in vitro* transcription of the *Ubx* promoter. G/A and C/T: see Figure 1. Lanes 1 and 4 are controls; no protein was added to the binding reaction. Lanes 2 and 3 contain 10 μl and 20 μl of protein, respectively, that was concentrated over a Biorex 70 column (0.4 M KCl step) before it was added to the reaction.

APPENDIX TO CHAPTER 1

Appendix 1: Biochemical Analysis of Extracts Derived from Staged *Drosophila* Embryos

Appendix 1

Biochemical Analysis of Extracts Derived from Staged *Drosophila* Embryos

Introduction

The *Drosophila* segmentation gene *fushi tarazu* (*ftz*) encodes a 1.9 kb polyadenylated transcript that is first detected in syncytial blastoderm embryos during their 10th nuclear division cycle, when the dividing nuclei reach the egg cortex (Weir and Kornberg 1985). It accumulates maximally at the cellular blastoderm stage of development (between 2 and 4 hours after egg deposition), and persists at lower levels throughout gastrulation and germ band extension (Weiner, et al. 1984). This temporal pattern of *ftz* expression is most likely controlled at the transcriptional level since the message turnover rate (T 1/2 = 6-8 minutes) is extremely rapid even when *ftz* transcripts are maximally accumulated in the embryo (Edgar, et al. 1986). Using extracts prepared from staged embryos, we analyzed the composition of sequence-specific DNA binding proteins present at the various stages of embryogenesis. Our intent was to determine whether the presence or absence of these potential regulators of *ftz* transcription correlated with the presence or absence of *ftz* transcripts in the embryo. In addition, we investigated whether the *in vitro* transcriptional activity of the staged embryo extract series could be correlated with the temporal pattern of *ftz* expression.

Results and Discussion

Nuclear extracts were prepared from embryos collected 0-2, 2-4, 4-6, 6-8, 8-10, 10-12, 13-15 and 17-22 hours following egg deposition. DNAse I footprinting (Galas, et al. 1978) was conducted with these staged embryo

extracts to determine the temporal pattern of expression of DNA binding proteins that specifically recognize the *ftz* promoter region (about 600 bp upstream of the transcriptional start point). We found that several of the proteins that bind to the promoter region are slightly more abundant early in embryogenesis whereas others are slightly more abundant late in embryogenesis (data not shown). These fluctuations in protein levels, however, were not significant, nor did they closely correlate with the appearance/disappearance of *ftz* transcripts during embryogenesis. The most noteworthy fluctuation was observed with the protein that binds to the TATA box located 26 bp upstream from the transcriptional start point (Figure 1). The level of this protein peaks in extracts made from 10-12 hour embryos.

The ability of the staged embryo extracts to promote transcription initiation was tested using a soluble *Drosophila* transcription system (Parker and Topol, 1984) and truncated templates containing the *fushi tarazu, runt, hairy, Ubx, hsp 70,* histone, actin and *alcohol dehydrogenase* promoters. The transcriptional efficiency of all the templates increased gradually as the age of the embryos used in the extraction increased to 12 hours (data shown for *ftz* template; see Figure 2B). More specifically, there were no detectable RNA products in any transcription reaction using the 0-2 hr and 2-4 hr extracts. Detectable but low levels of transcripts were synthesized using the 4-6 hr and 6-8 hr extracts. Extracts from 8-10 hr embryos were approximately as transcriptionally active as the 0-12 hr embryo extracts, and extracts derived from 10-12 hr embryos were the most efficient at synthesizing run-off transcripts. Finally, the efficiency of the 13-15 hr extract dropped down to the 4-8 hr extract level and no transcription was observed when the 17-22 hr extract was used.

These results indicate that the transcriptional activities of nuclear extracts prepared from staged embryos do not correlate with the temporal pattern of *ftz* expression; in fact, no gene specific activation can be observed with any of the extracts. Instead, the peak of transcriptional activity for all templates correlates with the peak of RNA polymerase II activity (in the 10-12 hr extract, where the RNA polymerase II level is 50-fold higher than the level in the 0-2 hr extract; data not shown) as well as with the peak concentration of the protein that recognizes the *ftz* TATA box (Figure 1). These observations suggest that the formation of a transcription initiation complex that includes RNA polymerase II and TATA box factor may be the critical, rate limiting step in our soluble transcription system.

Additional support for this hypothesis comes from a comparison of the transcriptional activity of the *ftz* promoter with and without sequences upstream of the TATA box (Figures 2A and B). Only sequences downstream of -40 bp (TATA box located at -26 bp to -32 bp) are necessary to promote the levels and pattern of transcriptional activity observed in the staged extract series, suggesting that *cis*-acting regulatory sequences upstream of the *ftz* TATA box do not play a role in the *in vitro* transcription initiation reaction. Thus, it appears that both the *cis*- and *trans*-acting regulators involved in initiation complex formation are sufficient to promote the maximal levels of *ftz* transcription in our soluble system. In the *in vivo* P-element-mediated transformation system, however, the *ftz* TATA box is not sufficient to promote transcription (see Chapter 2). This discrepancy may be due to drastically different relative concentrations of TATA box binding factor in the cell versus the test tube, and/or the lack of competition between genes utilizing TATA factor *in vitro* compared to the *in vivo* situation.

Experimental Procedures

Embryo Nuclear Extracts

Nuclear extracts of staged embryos were prepared as described in Dearolf, et al. (1989). The protocol described therein was developed by J.T. in an effort to optimize the transcriptional activity of the *ftz* promoter in extracts derived from frozen embryos.

Footprint Analysis

Fragment Preparation: The fragment used in the footprint analysis of the *ftz* TATA box region was isolated from a plasmid containing a *ftz* promoter deletion (generated using Bal 31 exonuclease). The plasmid DNA was labeled by DNA polymerase I (Klenow fragment) filling, at the Bam HI linker site ligated to the deletion endpoint (-128 bp upstream of the start point of transcription). The DNA was subsequently cut at the Sal I site located approximately 500 bp downstream of the translational start site and the labeled fragment was purified following agarose gel electrophoresis.

Footprint Reaction: All footprint reactions were carried out as previously described (Parker and Topol 1984), with two exceptions: the DNAse I reactions were performed on ice rather than at room temperature and added to a final concentration of 50 to 100 μg/ml rather than 2 to 5 μg/ml.

In Vitro Transcription Assays

In vitro transcription reactions were performed essentially according to Parker and Topol (1984) except that the final reaction volume was 15 μl instead of 50 μl (a third of which was the staged nuclear extract). The

template DNAs were digested about 300-700 bp downstream of the transcriptional start site and the run-off transcripts were analyzed on 5% polyacrylamide gels. The deleted *ftz* promoter constructs (Figure 2 and Chapter 1) were generated with Bal 31 exonuclease; Bam HI linkers were ligated to the deletion end points. The resulting DNA was cut with Sal I and cloned into pBR322.

RNA Polymerase II Assays

RNA polymerase II levels were determined essentially as described by Schwartz, et al. (1974).

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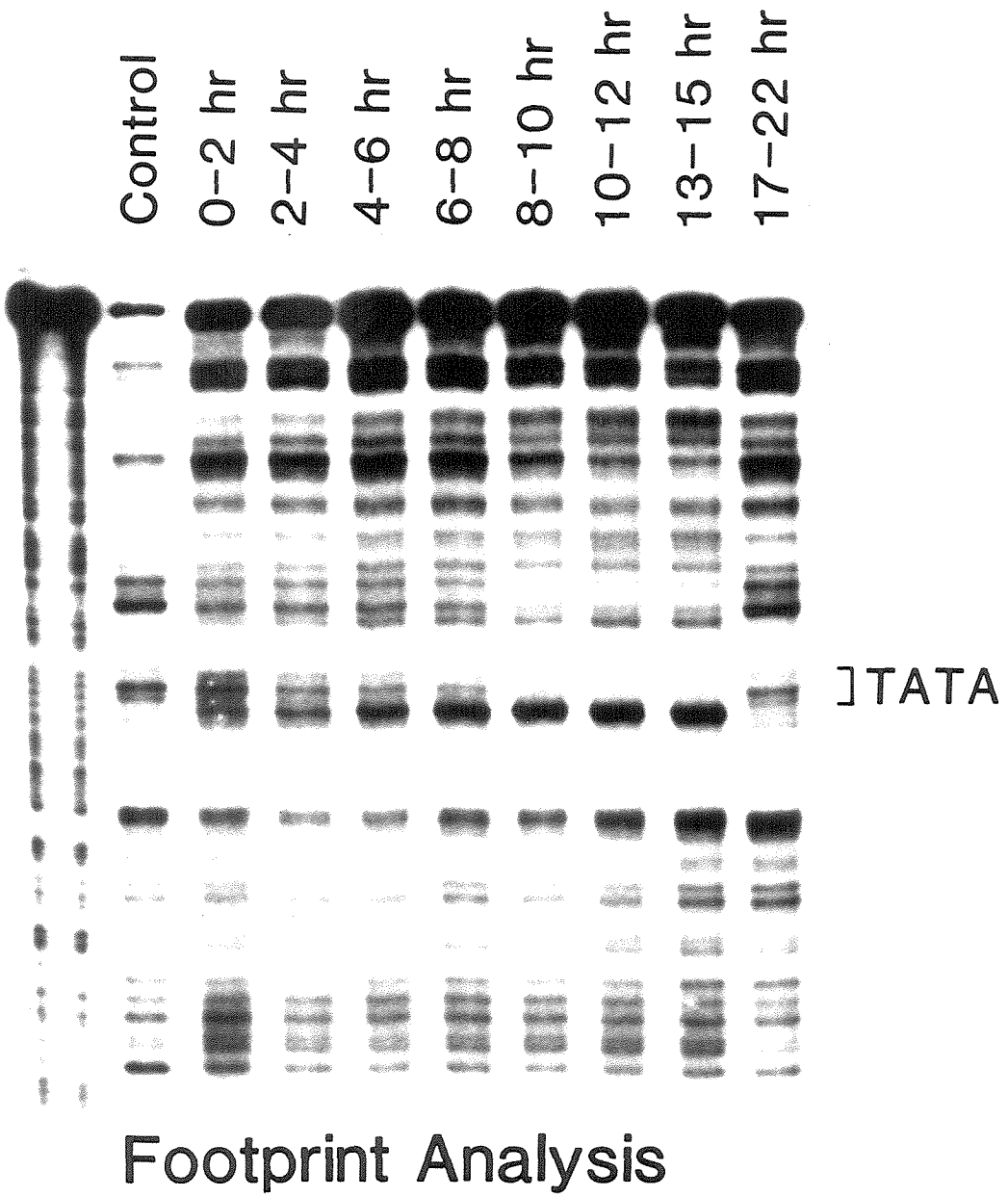
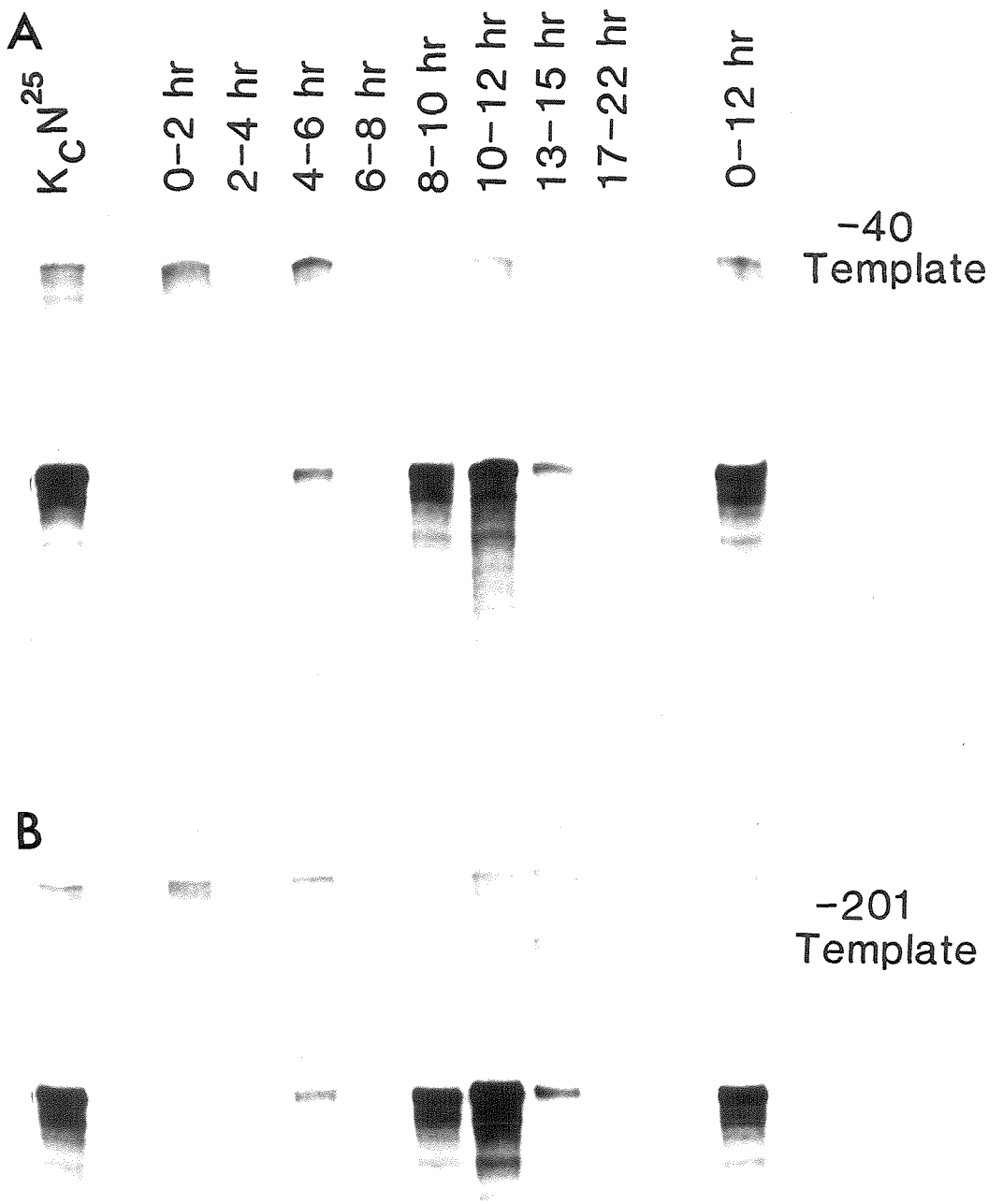

Control
0–2 hr
2–4 hr
4–6 hr
6–8 hr
8–10 hr
10–12 hr
13–15 hr
17–22 hr
]TATA
Footprint Analysis

Figure 1.



In Vitro Transcription

Figure 2.

Figure Legends

Figure 1. Footprint Analysis of the *ftz* TATA-Box Region with Extracts from Staged Embryos

First lane: Chemical cleavage at purine residues G/A. Control lane: No protein was added to the binding reaction. All other lanes: 10 µl of the indicated embryonic extract was added to the binding reaction. Note the lack of DNAse I digested fragments in the region containing the *ftz* TATA-box, as well as the hypersensitive site at the lower edge of the cleared area.

Figure 2. *In vitro* Transcription Reactions with Extract from Staged Embryos

Transcription assays with Kc cell nuclear extract (KcN^{25}) include 5 µg/ml DNA template and 5 µl extract in a final reaction volume of 15 µl. Transcription assays with staged embryo extracts (0-2 hr, 2-4 hr, *etc.*) and 0-12 hour extract include 20 µg/ml DNA and 5 µl extract in a final reaction volume of 15 µl. The intense bands in the center of the gel are *ftz* transcripts synthesized in the run-off transcription reactions. (A) Promoter sequences downstream of -40 bp included on the template. (B) Promoter sequences downstream of -201 bp included on the template.

CHAPTER 2

Transcriptional Control of Drosophila *fushi tarazu* "Zebra Stripe" Expression

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Abstract

The Drosophila segmentation gene *fushi tarazu* (*ftz*) is expressed in a characteristic pattern of seven stripes during early embryogenesis. We have used *ftz/lacZ* fusion genes to determine the effects of deleting relatively small segments of the *ftz* promoter region necessary for this expression. We find that this regulatory region contains multiple activator and repressor elements. The deletion of one particular activator element results in a preferential loss of expression in the posterior stripes, whereas the deletion of other activator elements causes a general reduction in expression throughout the germ band. The removal of repressor elements results in a loss of repression in the odd numbered parasegments. We also find that the *ftz* upstream enhancer element functions primarily in epidermal cells. Our results indicate that *ftz* transcription is activated in each parasegment through the "zebra stripe" promoter region, and is then selectively inhibited in the odd numbered parasegments by repressors which bind directly to elements within this promoter region.

Introduction

Segmentation in Drosophila melanogaster is controlled by a network of interacting genes (reviewed in Akam 1987; Nusslein-Volhard, et al. 1987; Scott and Carroll 1987; Ingham 1988), of which *fushi tarazu* (*ftz*) is one of the best studied. *ftz* is necessary for the development of the even-numbered parasegments (Wakimoto, et al. 1984) and for the correct expression of homeotic and other segmentation genes (Duncan 1986; Howard and Ingham 1986; Ingham and Martinez-Arias 1986). Embryos in which the *ftz* product is either lacking (Wakimoto, et al. 1984) or is indiscriminately expressed (Struhl 1985) develop pair-rule phenotypes, whereby every other parasegment is abnormal.

ftz transcripts and proteins are expressed in a highly regulated manner during embryogenesis (Kuroiwa, et al. 1984; Weiner, et al. 1984; Hafen, et al. 1984; Weir and Kornberg 1985; Carroll and Scott 1985; Doe, et al. 1988; Krause, et al. 1988, Carroll, et al. 1988a). *ftz* transcripts are first detected throughout the embryo after the ninth nuclear division. They are uniformly distributed around the periphery of the embryo from approximately 15% to 65% egg length after the eleventh nuclear division, but resolve into seven discrete stripes by the formation of the cellular blastoderm. *ftz* proteins are detected in cells of the presumptive even numbered parasegments at this stage, and are present until the germ band is fully elongated. *ftz* is also expressed later in neural cells and in the hindgut.

The exact mechanism by which the *ftz* "zebra stripe" expression pattern is generated is not yet known. One approach to studying this problem is to

examine the effects on *ftz* expression of mutations in other segmentation genes (Carroll and Scott 1986; Howard and Ingham 1986; Carroll, et al. 1986; Harding, et al. 1986; Frasch and Levine 1987; Carroll, et al. 1988b). These experiments indicate that the product of the *hairy* (*h*) gene acts in some way to repress *ftz* expression, a result confirmed by *in vivo* overproduction of the *h* protein (Ish-Horowicz and Pinchin 1987). Mutations in other segmentation genes also disrupt *ftz* expression, but the reasons for these disruptions are generally not so clear, because the altered *ftz* patterns could in some cases be due to indirect effects of the mutations. The numerous other early-acting genes, which when mutated do not cause "segmentation"-type phenotypes, in most cases will not be examined by this approach, although it is likely that some directly regulate the transcription of segmentation and homeotic genes (Biggin and Tjian 1988).

A complementary approach is to determine the biochemical and molecular components through which *ftz* is transcriptionally regulated. This approach is especially relevant for the *ftz* gene, because the observed pattern of *ftz* expression is controlled to a large extent at the level of transcription (Edgar, et al. 1987). The first studies of this type have shown that the *ftz* locus contains a complex promoter (Hiromi, et al. 1985; Hiromi and Gehring 1987). Sequences necessary for the correct stripe pattern are located between -669 bp and +73 bp from the startpoint of transcription; this proximal zebra stripe promoter region contains at least 15 protein binding sites (Topol, et al. 1987; our unpublished observations). Additional elements responsible for nervous tissue expression are located between -2.45 kb and -669 bp, and an enhancer element resides between -6.1 kb and -3.4 kb.

We are interested in elucidating the mechanism by which the zebra stripe pattern of *ftz* expression is transcriptionally regulated. In this paper, we use P element-mediated transformation with *ftz/lacZ* fusion genes to examine the effects of removing relatively small amounts of DNA from the proximal promoter region. We find that the zebra stripe promoter region contains multiple activator and repressor elements. The majority of activator elements appear to be recognized by transcription factors present throughout most of the embryo, in nuclei and cells of both the presumptive odd and even numbered parasegments; in addition, one particular activator element functions primarily in the most posterior parasegments. Repressor elements are recognized by factors which negate the action of activators in the odd numbered parasegments. Also, our results indicate that the upstream enhancer element functions primarily in presumptive epidermal cells. From these observations, we propose a mechanism for the transcriptional regulation of the *ftz* zebra stripe pattern.

Results

ftz Transcription Start Site

Transcription of *ftz* DNA *in vitro* using Kc cell and embryonic nuclear extracts indicated that the reported TATA homologies and start point of transcription (Laughon and Scott 1984) could be deleted without noticeably reducing the amount of RNA synthesized (Topol, et al. 1987). These sequences are also unnecessary for the production of functional transcripts *in vivo* (this study). To ascertain the start point of transcription, a primer-extension analysis was performed. The result of this analysis is shown in

Figure 1. Reverse transcription of both poly A^+ and *in vitro* transcribed *ftz* RNA begins at a position 50 bp downstream of that previously reported, -70 bp from the expected start point of translation. The DNA sequence around this initiation site, CTCATTC, is similar to an insect consensus sequence, ATCA(G/T)T(C/T) (Hultmark, et al. 1986). There is a TATATAT sequence present 26 bp upstream of the transcription start point.

Experimental Approach

We have constructed *ftz/lacZ* fusion genes in order to localize individual regulatory elements within the zebra stripe promoter region (Figures 2A-D). Four series of constructs are described here: 5' deletions of the *ftz* promoter, 3' deletions ligated to the *ftz* TATA homology and untranslated leader, a 3' deletion ligated to the *hsp 70* TATA homology and leader, and 5' deletions ligated adjacent to the *ftz* upstream element, which contains the enhancer element. These constructs were introduced into the genome by P element-mediated transformation (Rubin and Spradling 1982), and eight independent transformant lines were established for each construct. The expression pattern of each transformant line was examined by X-Gal staining of embryos. Because β-galactosidase activity is more stable than *ftz* transcripts and proteins, the product of the fusion genes could be detected as late as the completion of germ band retraction. To compensate for the different promoter strengths of the constructs, embryos were stained for different lengths of time. For reference, the level of β-galactosidase activity in transformed lines was also quantitated. Three to five homozygous viable and fertile lines for each construct were selected, and the amount of β-

galactosidase activity in staged embryos was determined spectrophotometrically (Table 1).

Analysis of the Zebra Stripe Promoter

The 5'Δ-669 gene (Figure 2A) is expressed primarily in the mesoderm of each of the even-numbered parasegments (Figures 3A-B; Hiromi, et al. 1985). The first stripe is generally the weakest. In addition, examination of understained embryos indicates that the seventh stripe is consistently either the strongest or one of the strongest stripes (data not shown). Interestingly, this seventh stripe is the widest when *ftz* protein is localized by antibody staining (Carroll and Scott 1985). The 5'Δ-669 construct is also expressed in the ectoderm, but in lower levels relative to EΔ-669 (Figure 2D), which contains both the upstream element and the zebra stripe element (Figures 5A-B). In both cases, ectodermal staining is found in the anterior portion of the even-numbered parasegments. During germ band extension, when mesodermal and ectodermal parasegments are in register, ectodermal stripes primarily align with the anterior part of the mesodermal stripes. Following germ band retraction, parasegments in the mesoderm are shifted approximately one half segment caudally relative to those in the ectoderm (Martinez-Arias and Lawrence 1985; Figure 3B). At this stage, ectodermal staining is localized in narrow bands in the posterior segmental compartments (anterior parasegmental compartments), whereas mesodermal staining appears in broader bands throughout most of the segment just posterior to the ectodermal stripes. This shift in mesodermal staining can also be observed in Figures 3F, 3H, 4C, 4E, and 4G, in which mesodermal stripes are located between the segmental grooves following germ band

retraction. A similar shift has been observed for the expression of *Ubx* (Akam and Martinez-Arias 1985). Staining is also seen on the ventral side anterior to the cephalic furrow, as previously reported (Hiromi, et al. 1985).

Expression of the 5' Deletion Constructs

To determine the effects of deleting short regions of the *ftz* promoter, the expression of each of the 5' and 3' *ftz/lacZ* progressive deletions was compared to that of the fusion gene preceding it in the series. The comparisons are based on examination of X-Gal staining in the germ band of the embryos. Because expression anterior to the cephalic furrow is variable, we do not include the staining in this region in our analysis.

No change in pattern or amount of stain is seen between embryos transformed with 5'Δ-669 and with 5'Δ-535 (not shown). Deleting to -458 bp causes the overall level of expression to decrease compared to -535 bp, as both mesodermal and ectodermal expression levels are reduced in each of the stripes (Figure 3C; Figure 6, Activator Element A1). Deleting to -359 bp additionally reduces the level of expression (Figure 3D; Figure 6, Activator Element A2). Deleting to -276 bp causes expression levels to decrease further, and only mesodermal stain can be detected (Figures 3E-F; Figure 6, Activator Element A3). Interestingly, ectopic mesodermal expression is also observed. A portion of cells in the odd-numbered parasegments express the fusion gene, causing each of the bands during germ band extension to appear broadened (Figure 3E; Figure 6, Repressor Element R1). This ectopic expression is even more noticeable in embryos following germ band retraction (Figure 3F). Deleting to -239 bp (Figures 3G-H; Figure 6, Repressor Element R2) causes cells of the odd-numbered parasegments to stain even more intensely than in 5'Δ-

276 embryos; again, this is most evident following germ band retraction (Figure 3H). Deleting further to -185 bp does not change the staining pattern (Figure 3I), although the total level of expression is lower when assayed spectrophotometrically (Table 1). Deleting to -102 bp causes the level of stain to dramatically decrease (Figure 3J; Figure 6, Activator Element A4). Low levels of mesodermal stain are seen throughout the length of the germ band, but definitive stripes are not observed (Figure 6, Repressor Element R4). Deleting to -40 bp causes the loss of all stain except anterior to the cephalic furrow (Figure 3K; Figure 6, Activator Element A5).

Expression of the 3' Deletion Constructs

The 3' deletion constructs were generated by ligating a series of 3' deleted fragments of the *ftz* zebra stripe promoter to 5'Δ-40. We did not control for the position of the inserted promoter fragment relative to the TATA homology and downstream sequences. To determine whether such a shift could influence *ftz/lacZ* expression, we included the 3'Δ-36 fusion in this study. This construct contains the complete zebra stripe promoter, but with sequences upstream of -36 bp shifted 15 bp, approximately one and one half DNA helical turns (Peck and Wang 1981; Rhodes and Klug 1981). Figure 4A shows that repressor elements are not affected in 3'Δ-36 embryos, as the pattern of stripes is identical to that generated by 5'Δ-669. However, the overall level of activity is reduced approximately 45% (Table 1), indicating that activator elements are sensitive to their distance relative to downstream promoter elements or to their position along the face of the DNA helix.

Deleting to -112 bp does not have a significant effect on the level of stain when compared to 3'Δ-36 embryos, although the most posterior stripe

stains slightly stronger relative to the other stripes (Figures 4B-C; Figure 6, Activator Element A5). Deleting to -172 bp causes a more pronounced alteration in the relative band strengths (Figures 4D-E; Figure 6, Activator Element A4). Stripe 7 is considerably darker than the other stripes, and the expected stripe in parasegment 2 is weak or absent. Stain is generally restricted to the mesoderm. In addition, a slight loss of interband repression is observed, which is most evident following germ band retraction (Figure 6, Repressor Element R4). Ectopic expression is strongest in the posterior odd numbered parasegments, although expression in all odd-numbered parasegments can be seen upon overstaining the embryos (not shown). Deleting to -222 bp causes a further loss of repression (Figures 4F-G; Figure 6, Repressor Element R3). During germ band extension, staining is observed in a continuous band in the mesoderm, with expression stronger in the even numbered parasegments. Following germ band retraction, the ectopic stripes are darker relative to those in 3'Δ-172 embryos. Expression in the posterior parasegments continues to be stronger than in parasegments 1-4. Deleting to -272 has no further effect (Figures 4H-I). Deleting to -347 bp greatly decreases the amount of expression (Figure 4J; Figure 6, Activator Element A3 and Repressor Element R1). In addition, all interband repression appears to be lost. Deleting to -482 bp further reduces the expression of *ftz/lacZ*, particularly in the posterior parasegments. Only a light line of mesodermal stain can be seen in the germ band, with the stain slightly more visible in the region where stripes 4 and 5 would normally be found (Figure 4K; Figure 6, Activator Element A2). Deleting to -576 bp eliminates detectable expression in the germ band, similar to the 5'Δ-40 construct (Figure 4L; Figure 6, Activator Element A1).

To test whether sequences in the *ftz* untranslated leader are necessary for the zebra stripe pattern of expression, *ftz* sequences from -669 to -36 bp were also inserted into HZ50PL (Hiromi and Gehring 1987) which contains the *hsp70* TATA homology and untranslated leader (Figure 2C). As shown in Figure 3L, the striped pattern of β-galactosidase expression is still generated in embryos transformed with this construct, indicating that sequences downstream of -36 bp are not essential for the repression of *ftz* expression in the odd numbered parasegments. However, the overall level of expression is reduced in *hsp70*LΔ-36 embryos compared to the expression of the 3'Δ-36 construct (Table 1), with stripes 3, 4, and 5 consistently the strongest. We do not know whether these alterations are due to the removal of specific *ftz* sequences located close to the cap site or in the untranslated leader, or to an artificial decrease in transcriptional efficiency resulting from the inclusion of heterologous promoter sequences.

The Upstream Enhancer Element Functions Only in Epidermal Cells

The 2.7 kb upstream element contains an enhancer element through which *ftz* increases its own transcription (Hiromi and Gehring 1987). As part of a related series of experiments, we inserted this upstream element 5' to both 5'Δ-669 and 5'Δ-40 (Figure 2D). Although ectodermal expression is found in both EΔ-669 and EΔ-40 embryos (Figures 5A-D), the latter construct is not expressed in mesodermal cells (compare Figures 5G and 5I to Figures 5H and 5J) or in the nervous system (Hiromi and Gehring 1987). This indicates that expression dependent upon the upstream element is restricted to epidermal cells. The EΔ-40/*lacZ* stripes are not found in embryos

homozygous for ftz^{9H34}, a strong allele, verifying that the EΔ-40 expression is the result of *ftz* enhancer activity (not shown).

Discussion

Activator and Repressor Elements

Our results indicate that the *ftz* zebra stripe promoter is a complex composite of multiple activator and repressor elements which contribute to the regulation of *ftz* expression. We find at least five activator and four repressor elements whose removal can be detected in individually stained embryos in the sequences between -669 and -36 bp of the *ftz* transcription unit. The deletion endpoints which remove the function of these elements are presented in Figure 6.

The deletion of activator elements results in a reduction of *ftz/lacZ* expression. This decrease can be seen most clearly in the 5' deletion constructs. The majority of the activator elements appear to allow a similar level of expression in most parasegments, although the level is slightly weaker in the stripes at either terminus of the germ band (for example, see Figures 3D, 3E, 3G and 3I). In contrast, one additional activator element (A2) provides transcriptional activity preferentially in the posterior parasegments, particularly in those cells which comprise the seventh stripe. The positional specificity of element A2 is most noticeable in constructs which contain A2 and only one or two general activator elements (3'Δ-172, 3'Δ-222, 3'Δ-272, and 3'Δ-347). Furthermore, a posterior preference is not seen in fusion genes which lack the A2 activator element but contain general activator elements (3'Δ-482; 5'Δ-359 to 5'Δ-102).

It is important to note that reductions in promoter strength are not due simply to the non-specific removal of *ftz* DNA. Deleting 134 bp between 5'Δ-669 and 5'Δ-535 does not significantly alter the amount of expression, and deleting a repressor element between 5'Δ-276 and 5'Δ-239 actually increases the total amount of expression (Table 1). Hence, significant differences in the level of expression among the 5' constructs most probably represent the effects of deleting *ftz* regulatory elements. The analysis of several activator elements in the 3' deletion series is more complicated because of the nature of the constructs themselves. The deletion of activator elements A4 and A5 in the 3' construct series does not lower the overall level of expression. This is presumably due to the different positions of upstream sequences relative to the TATA homology and start point of transcription among the 3' deletion constructs. Nevertheless, the effects of removing these activator elements can be seen as changes in relative expression levels among the parasegments, rather than as changes in absolute levels, as described above.

The deletion of repressor elements permits ectopic *ftz/lacZ* expression in cells of each of the odd-numbered parasegments. Removing one repressor element from either the 5' or 3' direction causes a mild loss of repression; this effect is seen as a slight broadening of the stripes during germ band extension, and/or as the presence of lightly staining ectopic stripes after germ band retraction (Figures 3E-F, 4E). Deleting a second repressor element results in stronger ectopic expression (Figures 3G-H, 4F-G). Removing at least four of the repressor elements allows the *ftz/lacZ* constructs to be expressed equally in the odd and even numbered parasegments; a continuous band of mesodermal expression is observed in 3'Δ-347 embryos (Figure 4J) and in 5'Δ-

102 embryos (Figure 3J), although the latter expression is sometimes difficult to observe because of the low activation level in these embryos.

It is possible that the *ftz* zebra stripe promoter may contain activator and repressor elements not identified here. The quantitation data show a reduction of approximately 45% in β-galactosidase activity between 5'Δ-239 and 5'Δ-185 (Table 1), suggesting the presence of an activator element which cannot be detected in an examination of individually stained embryos. Also, the presence of multiple activator or repressor elements within an identified regulatory region would not be detected. Further, we have not examined the effects on expression of *ftz* sequences upstream of -669 bp, and cannot rule out the possibility that sequences in the 2.7 kb upstream element may contain regulatory elements other than the enhancer.

Comparison of *ftz* Expression in the Mesoderm and Ectoderm

Ectodermal parasegments are divided during development into anterior and posterior compartments, whereas mesodermal parasegments do not appear to develop these clonal restrictions (Martinez-Arias and Lawrence 1985). It is not surprising that in the mesoderm, the *ftz* zebra stripe promoter is expressed in similar amounts throughout the length of the even-numbered parasegments. In contrast, ectodermal *ftz/lacZ* expression is detected primarily as sharp bands in the anterior part of parasegments (Figures 3A-B, 5A-F), in cells comprising the posterior segmental compartments (Lawrence, et al. 1987; Carroll, et al. 1988a), with only minor staining in the posterior part of parasegments.

The zebra stripe activator elements are transcriptionally active in both ectodermal and mesodermal cells. Deleting activator elements in either the 5' or 3' direction causes a sequential loss of expression in both ectoderm and mesoderm. Detection of ectodermal expression is lost before that of mesodermal expression in the 5' and 3' deletion series (Figures 3E, 4D) because the overall activation is lower in the ectoderm. Our results do not address whether each repressor element functions in both ectoderm and mesoderm, because ectodermal expression is below the level of detectability in those transformants showing a loss of mesodermal repression.

The activity of the upstream enhancer element is detectable only in epidermal cells (Figure 5). Hiromi and Gehring (1987) demonstrated that this activity requires the product of the *ftz* gene itself, but that it does not function in neural cells expressing *ftz*, indicating that at least one additional factor is involved in the transcriptional enhancement. Our results indicate that the enhancer activity is also negligible in cells of the mesoderm, despite the presence of *ftz* product. This observation supports the idea that the *ftz* product alone is insufficient to increase transcription of the *ftz* gene. The *ftz* protein is found in multiple phosphorylated forms in embryos (Krause, et al. 1988), suggesting that tissue-specific modifications could regulate the enhancer-mediated function of this protein. It is not yet known whether *ftz* proteins enhance transcription of the *ftz* locus directly, or whether they act through other factors.

Regulation of Zebra Stripe Expression

Our results suggest that at least two types of *trans*-acting activators and one type of repressors regulate *ftz* transcription through the zebra stripe promoter region. One type of activator recognizes at least the A1, A3, A4 and A5 elements, and is expressed in nuclei and cells throughout the region of the presumptive germ band. The variety of *ftz* protein-binding sequences and DNAse I footprinting patterns throughout embryogenesis (data not shown) suggests that this type of activator contains multiple members. A second type of activator recognizes the A2 element, and functions preferentially in the presumptive posterior parasegments, particularly in the cells which comprise the seventh *ftz* stripe. Interestingly, we do not find clear evidence for activators which act primarily in the anterior parasegments.

The expression of constructs in which repressor elements have been deleted indicates that both types of activators function in nuclei and cells of the odd-numbered, as well as even-numbered parasegments. The deletion of repressor elements allows *ftz/lacZ* constructs to be transcribed in odd-numbered parasegments, at levels proportional to the level of expression in adjacent, even numbered parasegments (see Figure 4G, for example). This ectopic expression could not occur unless trans-acting transcriptional activators were present in similar levels in both the odd and even numbered parasegments.

Our results indicate that the wild-type *ftz* zebra stripe pattern is refined to a large extent by the function of transcriptional repressors, rather than by a loss of activators. This conclusion concurs with those drawn from studies

using an inhibitor of protein synthesis (Edgar, et al. 1986), although we do not detect the loss of polar repression in the most posterior and dorsal anterior parts of the embryo as described in those studies. *ftz* repressors could negate the contributions of activators by several mechanisms. They could act directly on activator proteins, or through *ftz* promoter sequences to block activation. Both possibilities have been identified in cells of higher eukaryotes (Goodburn, et al. 1986; Mitchell, et al. 1987). We observe that *ftz* repressors utilize the latter mechanism; otherwise, deleting the *cis*-acting repressor elements would not result in ectopic expression. However, we cannot rule out that there is a subset of repressors not identified here which act directly on activators. In addition, the lack of ectopic expression in embryos transformed with the *hsp70*LΔ-36 construct, which does not contain any *ftz* transcribed sequences (Figure 3L), indicates that repressors are not involved in differential regulation of *ftz* mRNA stability.

We suggest the following mechanism for the control of *ftz* transcription in early embryos (Figure 7): *ftz* activators are functional before repressors during early embryogenesis (Figure 7A). The majority of activators are expressed in nuclei throughout the presumptive germ band, while others are primarily expressed in posterior nuclei. This enables *ftz* transcripts to be initially expressed in a continuous band of nuclei. As *ftz* repressors become expressed in nuclei which will contribute to the odd-numbered parasegments, *ftz* transcription continues only in nuclei lacking these negative regulators (Figure 7B). Because *ftz* transcripts have a short half-life (Edgar, et al. 1987), the pattern of *ftz* RNAs resolves into the zebra stripe within a short time after the appearance of repressors. At this stage, activators continue to be present in nuclei of both the presumptive odd- and even-numbered parasegments,

although these activators could be the products of different genes than those expressed earlier in development. By the cellular blastoderm stage, *ftz* proteins are translated; these proteins are necessary for the enhancement of *ftz* transcription through the upstream element in epidermal cells (Figure 7C).

Determining the precise *ftz cis*-regulatory sequences would provide a basis for directly identifying activators and repressors of *ftz* transcription. Most of the regulatory regions demonstrated in this paper contain several protein-binding sites, making the identification of the critical recognition elements premature. One exception is the promoter region between -276 and -239 bp, which contains repressor element R2 and only one DNAse I-footprint site (data not shown). A CGGATAA sequence is protected in this region, and also in the regions containing repressor elements R3 and R4. Experiments are currently underway to examine the significance of this heptamer sequence, to localize the other regulatory elements more precisely, and to determine the factors which regulate *ftz* transcription through these sites.

While the results presented in this paper do not identify specific trans-acting factors of *ftz* transcription, they do provide insights into the type of factors involved. Further, they support conclusions concerning the role of certain other segmentation genes in regulating *ftz* expression drawn from previous studies. In particular, the majority of activators appear to be general transcription factors, which may activate a number of zygotically expressed *Drosophila* genes. The identities of these factors are unknown. The factor which recognizes the A2 activator element functions primarily in the posterior parasegments, and may include the product of the *caudal* (*cad*) gene.

The *cad* protein contains a homeodomain and is expressed in a posterior to anterior gradient, (Mlodzik, et al. 1985; Macdonald and Struhl 1986), and mutations in *cad* drastically reduce the level of expression in the posterior *ftz* stripes (Macdonald and Struhl 1986).

Repressors of *ftz* transcription function in a pair-rule pattern, in nuclei and cells which comprise the odd-numbered parasegments throughout the embryo. This result supports the conclusions of previous studies that the product of the segmentation gene *h* directly represses *ftz* expression, and that this interaction occurs at the level of transcriptional control (Howard and Ingham 1986; Carroll and Scott 1986; Hiromi and Gehring 1987; Ish-Horowicz and Pinchin 1987; Carroll, et al. 1988). However, it is unlikely that the *h* product is the only repressor of *ftz* transcription, because the *ftz* zebra stripe expression pattern does resolve partially, albeit more slowly, in *h* mutant embryos. A second possible repressor is the product of the pair-rule gene *even-skipped* (*eve*), which binds to the *ftz* zebra-stripe promoter region *in vitro* at several sites upstream of -185 bp (data not shown). However, the effects of mutations in the *eve* gene on *ftz* expression are more complicated than are mutations in the *h* gene (Carroll and Scott 1986; Frasch and Levine 1987).

Products of the gap segmentation genes have also been postulated to regulate the expression of later acting, pair-rule genes (Carroll, et al. 1988; Ingham, 1988, and references therein). Indeed, studies on the expression of the *h* gene indicate that factors expressed in patterns consistent with gap gene expression act through separate promoter elements to activate *h* transcription (Howard, et al. 1988). We find no evidence for similar interactions with the

ftz promoter, consistent with the interpretation that the effects of gap gene mutations on *ftz* expression are indirect (Ingham, et al. 1986).

Experimental Procedures

Isolation and Synthesis of RNA

The *ftz* DNA template was transcribed *in vitro* with nuclear extracts prepared from 0-12 hr embryos as follows: Dechorionated embryos were frozen in liquid nitrogen, ground with a mortar and pestle, and resuspended in buffer E (60 mM KCl, 15 mM NaCl, 15 mM Hepes pH 7.6, 1 mM EDTA, 8.5% sucrose, 1 mM DTT, 0.1 mM PMSF, 100 μg/ml sodium bisulfite, 5 μg/ml soybean trypsin inhibitor, 250 μg/ml spermidine, 50 μg/ml spermine). This and all further procedures were carried out at 4°C. The solution was homogenized, passed through a Nitex filter, and the nuclei pelleted by centrifugation for 10 min at 10,000 rpm in a Beckman JA20 rotor. Nuclei were resuspended in buffer A (100 mM KCl, 10 mM Hepes pH 7.6, 5 mM $MgCl_2$, 0.1 mM EDTA). Ammonium sulfate was then added to a final concentration of 250 mM, the mixture gently rotated 30 min, and the precipitate pelleted by centrifugation for 1 hr at 100,000 x g. The supernatant was collected, and ammonium sulfate added to a final concentration of 2.0 M. The solution was rotated 30 min, and centrifuged for 15 min at 100,000 x g. The protein pellet was then resuspended in buffer C (10% glycerol, 25 mM Hepes pH 7.6, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT), dialyzed against buffer C, and stored frozen at -80°C until further use.

Primer Extension Analysis

The primer extension assays utilized a synthetic 20-mer oligonucleotide homologous to the *ftz* gene in a position originally estimated to be 100 bp from the start point of transcription. The 5' end of this probe coincides with a Rsa I site in the genomic DNA. The primer was ^{32}P end-labelled using T4 polynucleotide kinase (U.S. Biochemical Corp.). Primer extension was performed according to Wiederrecht, et al. (1987) with the following modifications: 10 μg of poly A^+ RNA, isolated from 0-12 hr embryos (Maniatis et al., 1982) or 1/5 the RNA from an *in vitro* transcription reaction (Parker and Topol, 1984) were used for each assay. RNA and primer were combined, heated at 65°C for 4 min, and annealed at 52°C for 1 hr; the primer was extended using AMV reverse transcriptase (Boehringer-Mannheim) at 37°C for 45 min. To obtain sequencing lanes, the appropriate *ftz* genomic Rsa I fragment was similarly end-labelled and sequenced by the method of Maxam and Gilbert (1980). Both sequenced and extension products were electrophoresed on a 6% acrylamide, 8 M urea gel and exposed to X-ray film overnight.

Construction of *ftz/lacZ* Fusions and P-Element Transformation

The source of the DNA for these experiments was a plasmid containing the *ftz-lac*A fusion gene of Hiromi, et al. (1985) inserted between the Sal I and EcoRI sites of pAT153. This construct contains *ftz* DNA from -669 to + 73 bp from the cap site (the zebra stripe promoter) ligated to the *Escherichia coli lac*Z gene within the second amino acid of the *ftz* coding region. At the 3' end of the *lac*Z gene is a *Drosophila hsp 70* terminator, which includes a

termination codon and polyadenylation signal. 5' deletions were generated by Xba I restriction digestion of this plasmid 5' of the *ftz* promoter, followed by exonuclease III/mung bean nuclease treatment according to the manufacturer's instructions (Stratagene Cloning Systems). Hind III linkers, 10 bp in size (New England Biolabs), were added, the deletion fragments propagated in the Stratagene Bluescript M13+ vector, and subsequently subcloned into a Carnegie 20 vector.

The 3' deletions were generated by restriction digestion of the original plasmid with EcoRI, followed by the identical exo III/mung bean digestion and Hind III linker attachment procedure. These deletion fragments were inserted immediately upstream of the 5'Δ-40 fragment in the Stratagene vector, and these fusions were then subcloned into Carnegie 20. All deletion endpoints were sequenced by the method of Maxam and Gilbert (1980). The *hsp70*LΔ-36 construct was generated by subcloning the -669 to -36 bp fragment from 3'Δ-36 into HZ50PL (Hiromi and Gehring 1987), which contains the basal promoter and untranslated leader of the *hsp70* gene ligated to the *E coli lacZ* gene and *Drosophila hsp70* terminator. The EΔ-669 and EΔ-40 constructs were generated by cloning the appropriate *ftz/lacZ* fusion gene fragments into a Carnegie 20 vector containing the 2.7 kb KpnI-XbaI "upstream element." Germ-line transformation was performed by standard procedures (Rubin and Spradling 1982). 500 μg/ml *ftz/lacZ* construct was coinjected with 100 μg/ml helper plasmid pHSπ (Steller and Pirotta 1985) into ry^{506} embryos. All transformant lines were made homozygous, or balanced over *FM6*, *CyO*, or *TM3* (these fly stocks are described in Lindsley and Grell 1968; ftz^{9H34} is described in Jurgens, et al. 1984). In only 2/207 individual transformant lines were more than one insertion observed.

Detection and Measurement of β-galactosidase Activity

Embryos individually examined by light microscopy were stained for β-galactosidase activity as follows. Dechorionated embryos were fixed with heptane saturated with 25% glutaraldehyde, 25 mM cacodylate buffer pH 7.3, in depression slides for 10 min (Zalokar and Erk 1977). The embryos were rinsed in Ringers solution, manually devitellinized, and stained for an appropriate length of time (1/2 hr to 24 hr, depending on the activity of the *ftz/lacZ* construct) at 37°C in 30% ficoll, 0.3% X-gal, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 10 mM sodium phosphate pH 7.2 (Raghavan, et al. 1986). Ficoll inhibits the precipitation of X-Gal during long incubation periods. Embryos were mounted in glycerol and photographed using Nomarski optics. Embryos were staged according to Campos-Ortega and Hartenstein (1985). β-galactosidase expression was also examined in several transformant lines by antibody staining. Because this method of detection was less sensitive than the enzymatic assay using X-Gal, it was not used to generate the results reported here.

For each transformant line, hundreds of individual embryos were examined. The staining pattern we describe for each construct represents the pattern found in most or all of the independent lines containing that construct. Five of the 207 lines exhibited patterns different than all the others containing the same construct, and are not included in this study. As has been reported by Hiromi, et al. (1985), we found a fraction of the lines to exhibit ectopic staining in glial cells and in cells of the VNS, and observed β-galactosidase expression anterior to the cephalic furrow in all transformant lines. These staining patterns are not discussed here.

For the quantitative measurement of β-galactosidase activity, 3-5 homozygous viable and fertile lines were randomly selected for each construct. Embryos were collected at 25°C for 2 1/2 hr, and aged 3 hr more. At this range of developmental stages, ectopic glial cell and neural precursor cell expression is not yet present. Embryos were dechorionated, homogenized in assay buffer (50 mM potassium phosphate, 1 mM $MgCl_2$, pH 8.0) and centrifuged at 12,000 rpm 15 min at 4°C. The supernatant was frozen at -80°C until further use. For each transformant line tested, the total protein concentration in the supernatant was determined by Bio-Rad protein assay using BSA as a standard. Extract containing 100 μg of total protein was incubated with 5 mM chlorophenol red-β-D-galactopyranoside (CPRG, Boehringer-Mannheim) at 22°C for 2 hr, and the OD_{574} of the solution was determined spectrophotometrically (Simon and Lis 1987).

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Table 1. Expression of *ftz/lac Z* Constructs

Construct	Number of Transformant Lines Quantitated	Units of β-Galactosidase Activity[1]		Level of Derepression
		Mean ± S.D.	Range	
5'Δ -669	5	914 ± 157	744 - 1131	None
5'Δ -535	5	824 ± 255	576 - 1193	None
5'Δ -458	4	544 ± 219	348 - 847	None
5'Δ -359	5	225 ± 66	139 - 315	None
5'Δ -276	3	112 ± 19	91 - 127	Mild
5'Δ -239	5	215 ± 42	174 - 263	Moderate
5'Δ -185	5	119 ± 26	86 - 145	Moderate
5'Δ -102	4	42 ± 35	8 - 87	Strong
5'Δ -40	5	8 ± 9	2 - 18	N.A.[2]
3'Δ -36	4	520 ± 90	457 - 650	None
3'Δ -112	3	449 ± 109	323 - 519	None
3'Δ -172	5	412 ± 52	337 - 463	Mild
3'Δ -222	5	264 ± 112	141 - 415	Moderate
3'Δ -272	5	391 ± 242	119 - 635	Moderate
3'Δ -347	5	97 ± 46	31 - 158	Strong
3'Δ -482	5	46 ± 16	25 - 67	N.A.[2]
3'Δ -576	3	5 ± 9	0 - 16	N.A.[2]
*hsp 70*L Δ -36	4	176 ± 25	142 - 204	None
EΔ -669	3	1566 ± 347	1174 - 1831	---
EΔ -40	5	110 ± 49	43 - 160	---

[1]Each unit of β-galactosidase activity is equivalent to one $O.D._{574}$ unit per mg protein x 10^2. The background value of 47 units for untransformed *ry* embryos was subtracted from each value. The value for each transformant line was obtained from measurement of two separate egg collections.
[2]N.A. indicates that derepression could not be assayed in these transformant lines due to the low levels of β-galactosidase activity.

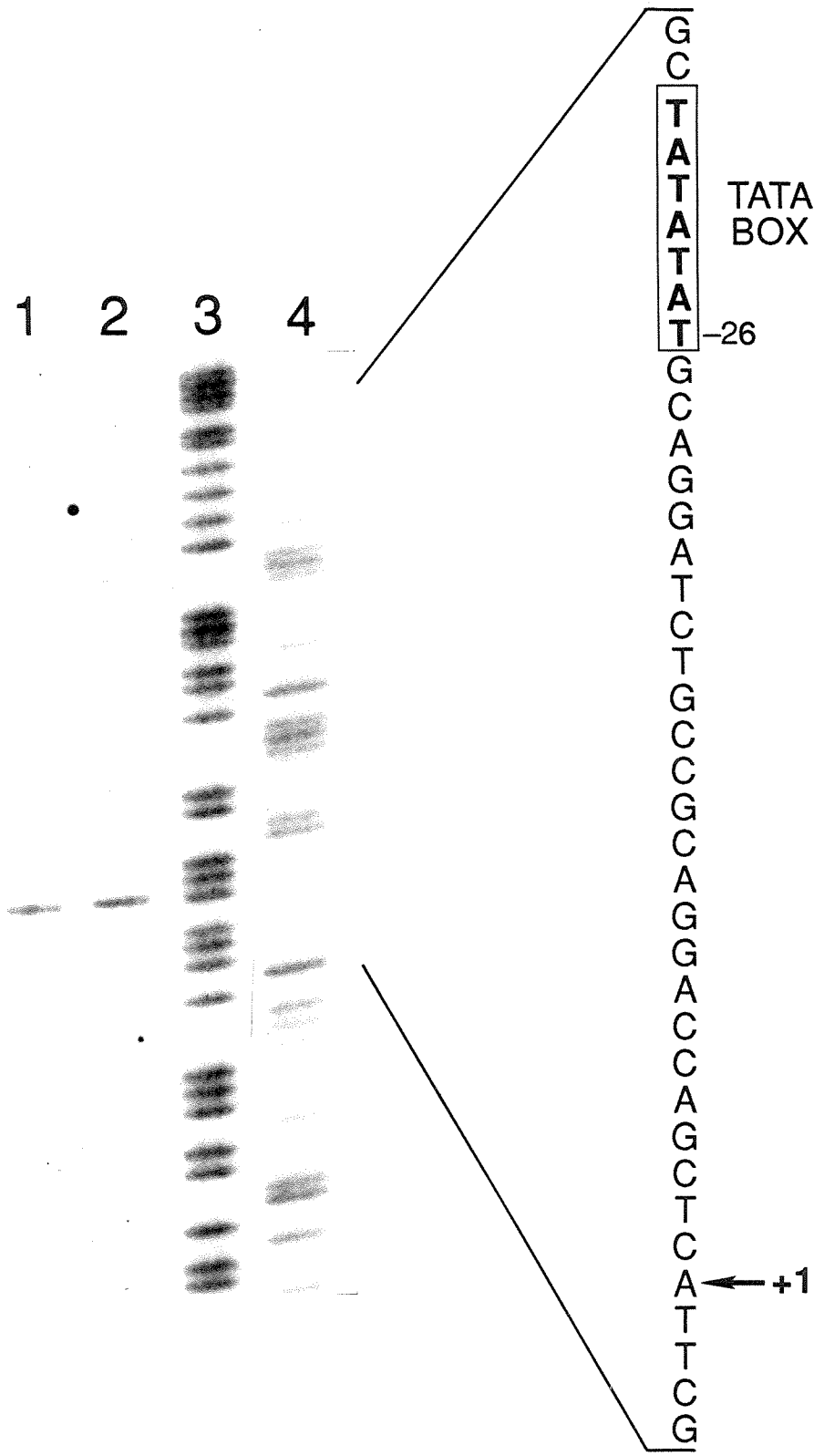

1
2
3
4
GC
TATATAT
TATA
BOX
–26
GCAGGATCTGCCGCAGGACCAGCTCA
+1
TTCG

Figure 1.

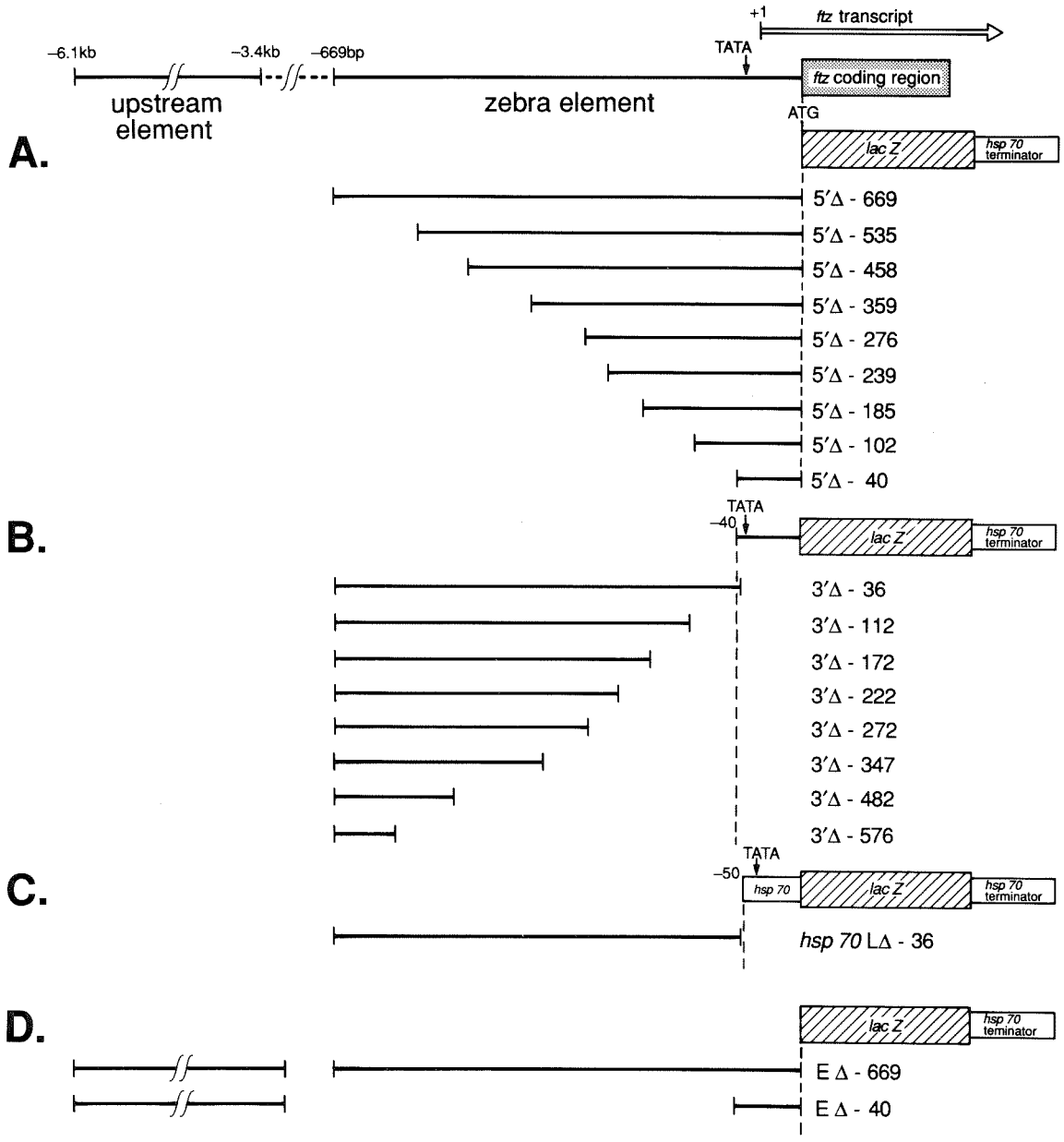

+1
ftz transcript
TATA
-6.1kb
-3.4kb
-669bp
ftz coding region
upstream element
zebra element
ATG
A.
lac Z
hsp 70 terminator
5′Δ - 669
5′Δ - 535
5′Δ - 458
5′Δ - 359
5′Δ - 276
5′Δ - 239
5′Δ - 185
5′Δ - 102
5′Δ - 40
TATA
-40
B.
lac Z
hsp 70 terminator
3′Δ - 36
3′Δ - 112
3′Δ - 172
3′Δ - 222
3′Δ - 272
3′Δ - 347
3′Δ - 482
3′Δ - 576
TATA
-50
C.
hsp 70
lac Z
hsp 70 terminator
hsp 70 LΔ - 36
D.
lac Z
hsp 70 terminator
E Δ - 669
E Δ - 40

Figure 2.

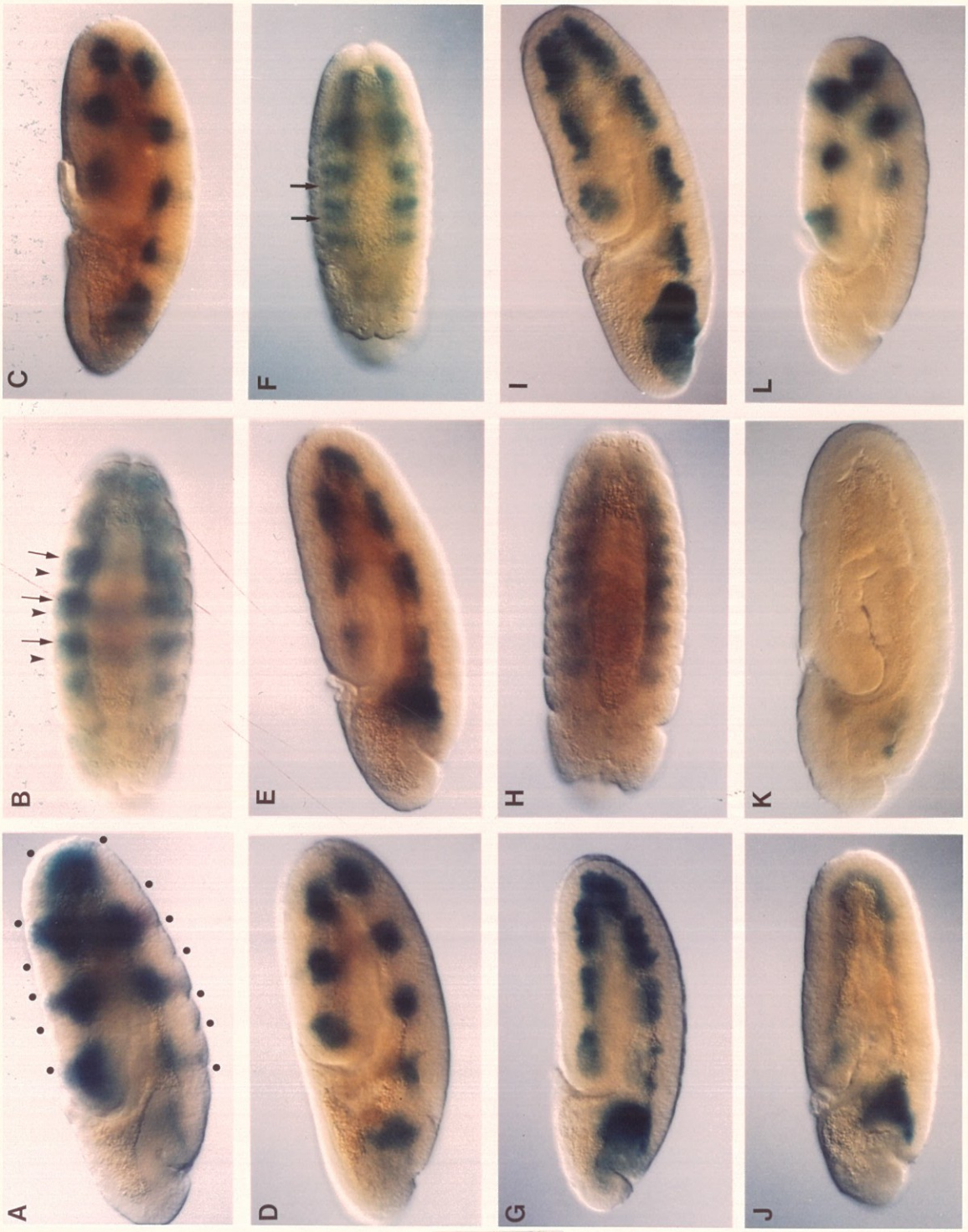


Figure 3.

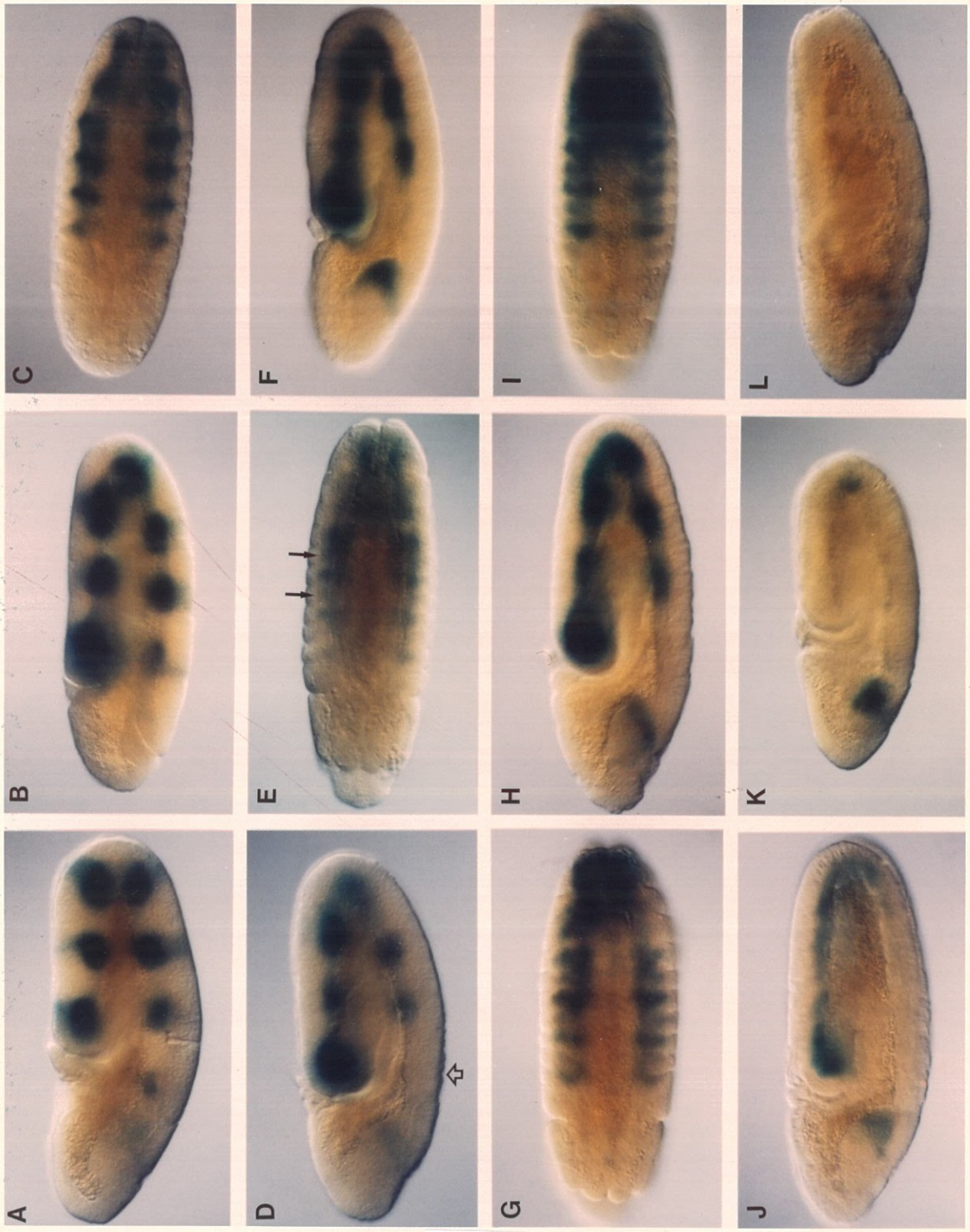
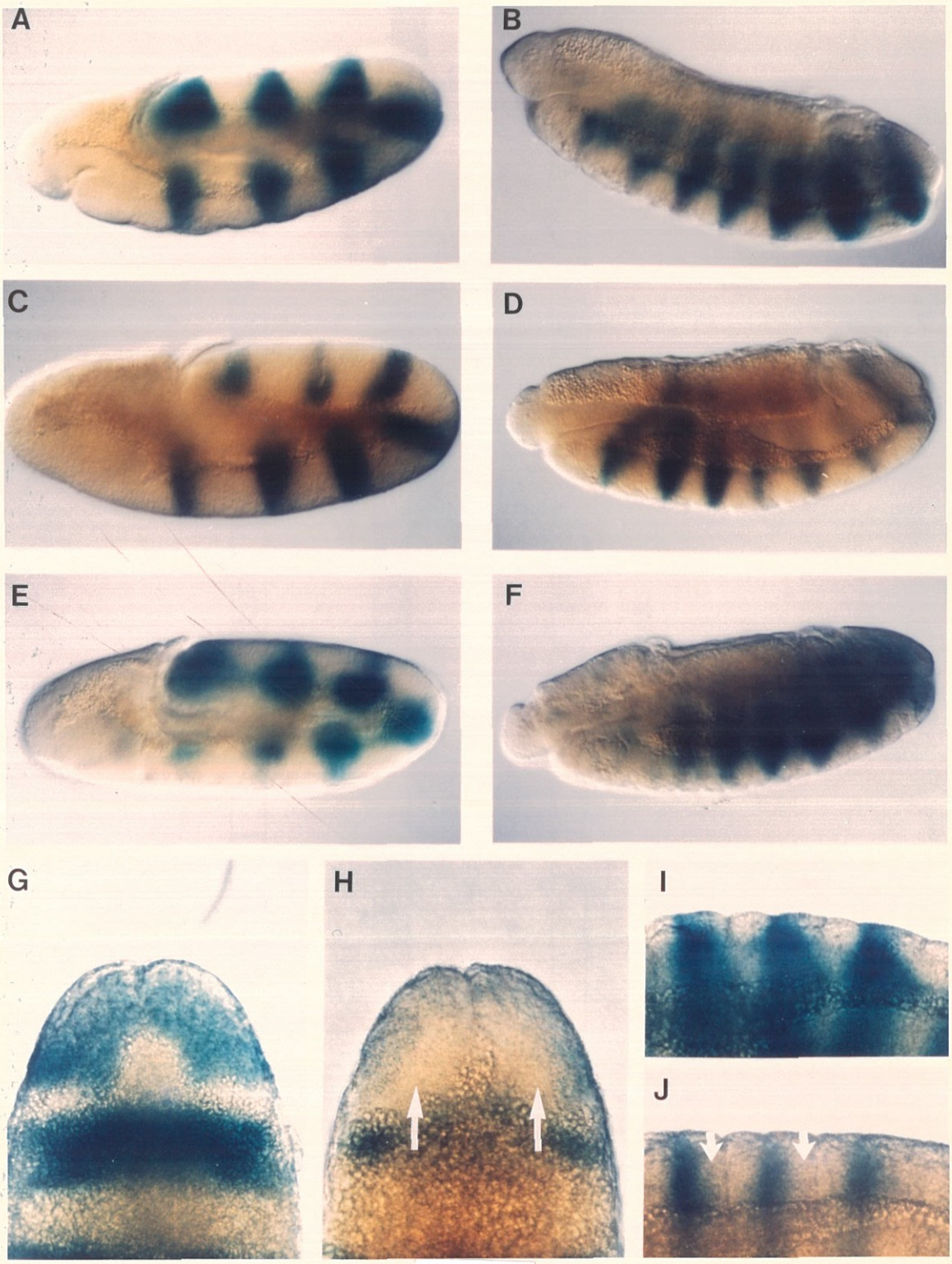


Figure 4.



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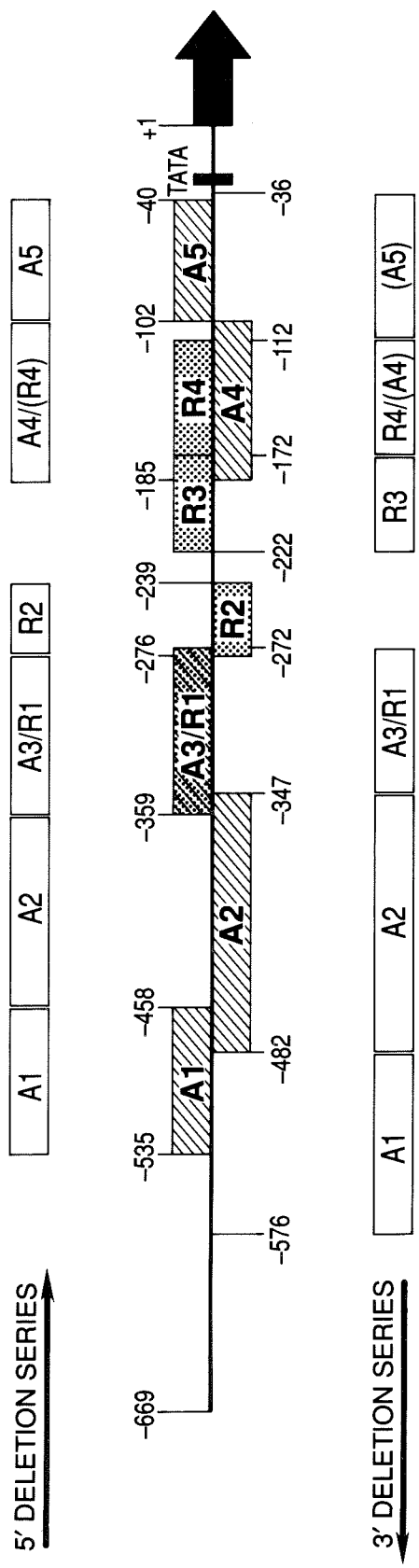
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A2
A3/R1
R2
A4/(R4)
A5
-669
-576
-535
-482
-458
-359
-347
-276
-272
-239
-222
-185
-172
-112
-102
-40
-36
+1
TATA
A1
A2
A3/R1
R3
R4/(A4)
(A5)
3′ DELETION SERIES

Figure 6.

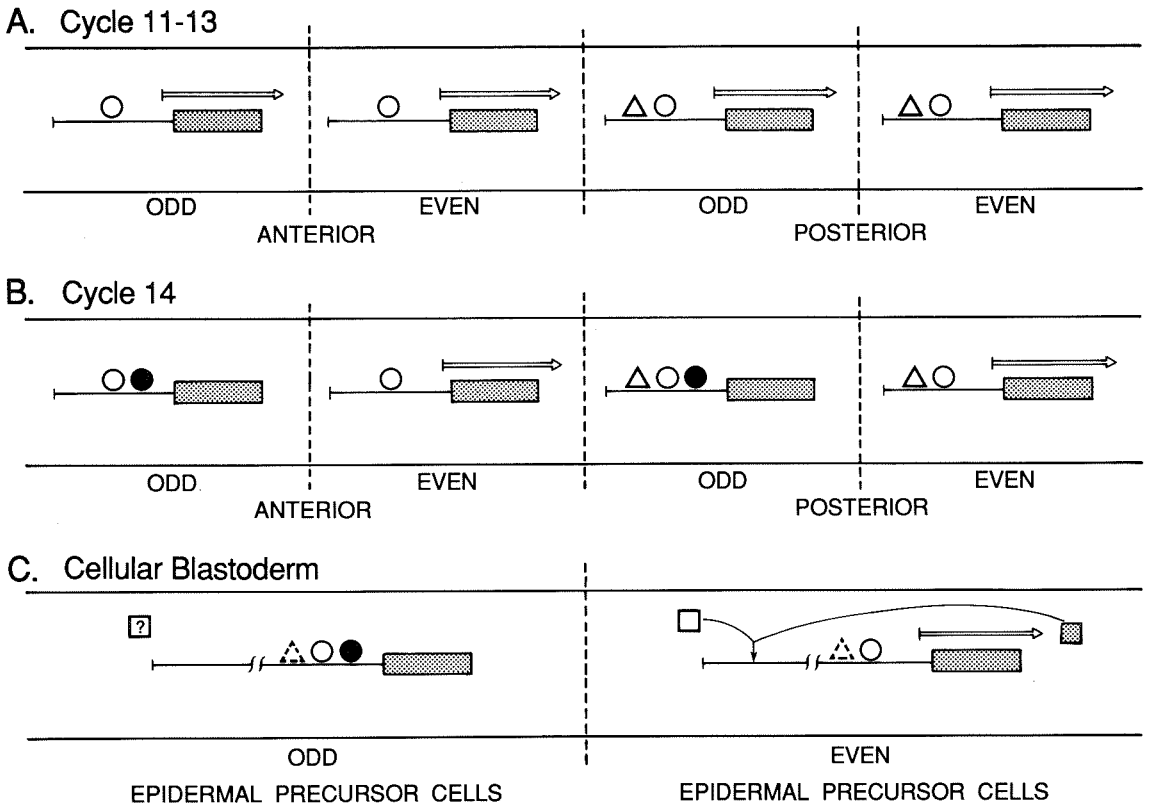
A. Cycle 11-13
ODD
EVEN
ODD
EVEN
ANTERIOR
POSTERIOR
B. Cycle 14
ODD
EVEN
ODD
EVEN
ANTERIOR
POSTERIOR
C. Cellular Blastoderm
?
ODD
EPIDERMAL PRECURSOR CELLS
EVEN
EPIDERMAL PRECURSOR CELLS

Figure 7.

Figure Legends

Figure 1. Determination of the Transcription Start Site of the *ftz* Gene

ftz RNA was primer extended, and the reaction products electrophoresed on a 6% acrylamide, 8M urea gel. The primer was a ^{32}P-end-labelled synthetic oligonucleotide complementary to the *ftz* transcript between positions +105 to +124 bp. (Lane 1) Reverse transcription of 10 μg of poly A$^+$ RNA from 0-12 hr embryos. (Lane 2) Reverse transcription of RNA synthesized *in vitro* from the *ftz* template with a 0-12 hr embryonic nuclear extract. (Lanes 3 and 4) The corresponding *ftz* DNA sequence; chemical cleavage (Maxam and Gilbert, 1980) of *ftz* genomic DNA labelled at the Rsa I site at +124 bp, specific for pyrimidines (Lane 3) and for purines (Lane 4). The reverse transcriptase-generated band is elevated 1 1/2 bp relative to the sequencing standards because of the difference in migration between these fragments (Sollner-Webb and Reeder, 1979).

Figure 2. *ftz/lacZ* Fusion Genes Used for Transformation

The location of promoter elements necessary for *ftz* expression in its seven stripe pattern is indicated at the top. The TATA homology is at -26 bp, and the start point of translation is at +70 bp. (A-D) The *ftz* constructs, inserted into Carnegie 20, used for transformation. Solid horizontal lines represent the included *ftz* sequences. The constructs are named such that the number following the Δ sign indicates the deletion endpoint from the start point of transcription. (A) 5' deletion series of *ftz/lacZ*. 5'Δ-669 contains the complete zebra stripe element of Hiromi et al. (1985). (B) 3' deletion series. *ftz* promoter fragments were ligated to 5'Δ-40. All 3' deletions have a 5'

endpoint at -669 bp. (C) *hsp70*LΔ-36. The deletion fragment from 3'Δ-36 was inserted upstream of the *hsp 70* basal promoter. (D) Enhancer series. 5'Δ-669 and 5'Δ-40 were inserted 3' to the 2.7 kb upstream enhancer element.

Figure 3. *ftz/lacZ* Expression of 5' Zebra Stripe Deletion Constructs and of *hsp70*LΔ-36

Localization of β-galactosidase expression in whole-mount transformant embryos. (B, F, and H) show a ventral view of embryos following germ band retraction (stage 13 of Campos-Ortega and Hartenstein, 1985). All other embryos shown were examined during germ band extension (stages 8-11). A more detailed description of the expression patterns is given in the text. (A-B) 5'Δ-669 embryos. Expression is strongest in the mesoderm, although stain is present in the anterior portion of parasegments in the ectoderm. The transient parasegmental grooves are indicated by closed circles. Following germ band retraction (B), the ectodermal stain (arrowheads) is anterior to that in the mesoderm (arrows). (C) 5'Δ-458 embryo. (D) 5'Δ-359 embryo. (E-F) 5'Δ-276 embryos. Ectopic expression is seen as broadened bands during germ band extension (E), and as additional stripes following germ band retraction (F) (examples of ectopic expression are indicated by arrows). (G-H) 5'Δ-239 embryos. (I) 5'Δ-185 embryo. (J) 5'Δ-102 embryo. (K) 5'Δ-40 embryo. (L) *hsp70*Ld-36 embryo.

Figure 4. *ftz/lacZ* Expression of 3' Zebra Stripe Deletion Constructs

Localization of β-galactosidase expression in whole-mount transformant embryos. (C, E, G, and I) show embryos following germ band retraction, whereas all other embryos are shown during germ band extension.

(A) 3'Δ-36 embryo. (B-C) 3'Δ-112 embryos. (D-E) 3'Δ-172 embryos. In (D), note the absence of stain in parasegment 2 (open arrow) and the strong stain in the posterior parasegments. In (E), examples of ectopic expression are indicated by arrows. (F-G) 3'Δ-222 embryos. (H-I) 3'Δ-272 embryos. (J) 3'Δ-347 embryo. (K) 3'Δ-482 embryo. (L) 3'Δ-576 embryo.

Figure 5. Effects of the Upstream Promoter Element on *ftz/lacZ* Expression

Whole-mount transformant embryos were stained for β-galactosidase activity during germ band extension (A, C, E, G-H) or following germ band retraction (B, D, F, I-J). (A-B) EΔ-669 embryos, which contain both the upstream enhancer element and zebra stripe element fused to *lac*Z, stain well in both the ectoderm and mesoderm. (C-D) EΔ-40 embryos, which contain only the upstream element fused to *lac*Z, do not stain in the mesoderm. (G-J) Higher magnification of EΔ-669 embryos (G, I) and EΔ-40 embryos (H, J). Note the absence of mesodermal stain in (H) and (J) (arrows). (E-F) Included for comparison are 5'Δ-669 embryos, which contain only the zebra stripe element fused to *lac*Z. Stain is strongest in the mesoderm, but is present in lower levels in the ectoderm.

Figure 6. Location of *ftz* Activator and Repressor Elements in the Zebra Stripe Promoter

The deletion endpoints which remove activator elements (A1-A5) and repressor elements (R1-R4) are shown. The 5' deletion series endpoints and the elements they remove are positioned on top, and those of the 3' deletion series on bottom. An activator element is designated between the endpoints of those constructs in which the level of *ftz/lacZ* expression noticeably

decreases when comparing stained embryos by light microscopy. A repressor element is designated when ectopic expression appears or increases in odd numbered parasegments. The middle figure integrates the results of both the 5' and 3' deletion series. The location of activator elements is shown as slanted lines, that of repressor elements as dots. The effects of deleting elements A4 and A5 in the 3' direction and element R4 in the 5' direction are partially masked, and therefore indicated in parentheses (discussed further in text).

Figure 7. Transcriptional Regulation of *ftz* Zebra Stripe Expression

A model for the establishment of the *ftz* zebra stripe transcription pattern is given. (A) By cycle 11, *ftz* is transcribed (horizontal arrows) in peripheral nuclei throughout the region of the presumptive germ band. General transcriptional activators (open circles), and posterior-specific activators, possibly *cad* proteins (open triangles) are present as indicated. (B) During cycle 14, *ftz* transcription is eliminated in nuclei of the presumptive odd numbered parasegments by repressors, possibly *h* and *eve* proteins (solid circles). (C) By the cellular blastoderm stage, *ftz* proteins (stippled squares) are translated, and in conjunction with other factors (open squares) enhance *ftz* transcription through the upstream promoter element in epidermal precursor cells of the even numbered parasegments. It is not known whether these additional factors are also present in other cells. The dashed triangle indicates that the posterior-specific activators are present only in a subset of presumptive epidermal cells.

APPENDICES to CHAPTER 2

Appendix 2a: The *caudal* gene product is a Direct Activator of *fushi tarazu* Transcription During *Drosophila* Embryogenesis §

Charles R. Dearolf, Joanne Topol and Carl S. Parker

Appendix 2b: Transcriptional Regulation of the *Drosophila* Segmentation Gene *fushi tarazu* (*ftz*) ‡

Charles R. Dearolf, Joanne Topol and Carl S. Parker

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‡ Published in *BioEssays*, **12**:1-5, 1990.

Appendix 2a

The *caudal* gene product is a Direct Activator of *fushi tarazu* Transcription During *Drosophila* Embryogenesis

Introduction

A *Drosophila* pair-rule segmentation gene, *fushi tarazu* (*ftz*), encodes a protein which is expressed in a characteristic seven stripe pattern (1). The promoter sequences that are sufficient for generating this spatially restricted pattern of expression are located within 669 base pairs upstream of the transcription start site (2). Multiple transcriptional activators and repressors interact with this "zebra stripe" promoter unit to bring about the positional specificity of *ftz* transcription (3). Here we report that the homeodomain-containing protein encoded by *caudal* (*cad*) is one such regulator. The *cad* gene product can increases the level of *ftz* transcription in the posterior half of the embryo by interacting with multiple copies of a TTTATG consensus sequence located in the zebra-stripe unit. This result demonstrates one pathway by which the product of a maternally expressed segmentation gene, expressed in an antero-posterior concentration gradient, can directly regulate the expression of a pair-rule gene.

Results and Discussion

An analysis of sequential deletions of the zebra stripe promoter unit demonstrated that sequences between -482 and -386 base pairs (bp) from the start point of transcription can direct the expression of *ftz/lacZ* fusion constructs preferentially in the posterior parasegments of germ-line transformed embryos (Figure 1). The expression of β-galactosidase was

observed in a continuous band rather than in discrete stripes, because the constructs did not contain the repressor elements that would normally prevent transcription in the cells which comprise the interband regions (3). Staining was consistently seen between the parasegments that would correspond to *ftz* stripes 4 through 7, with the stripe 7 staining most strongly. Weak staining was also detected in most embryos in the parasegments just anterior to the region corresponding to stripe 4.

The posterior-specific expression of these *ftz* promoter fusions is similar to the pattern of expression observed for the *cad* protein during early embryogenesis (4, 5). Because posterior *ftz* expression is drastically reduced in *cad*$^-$ mutant embryos (4), and because the *cad* protein contains a homeodomain, which indicates that the protein has a DNA binding capability, (6), we examined whether the *cad* gene product could function as a direct regulator of *ftz* transcription. To determine if the *ftz* promoter region responsible for posterior-specific expression contained a *cad* DNA recognition element (CDRE), we performed DNA binding experiments with bacterially overproduced *cad* protein. We found that this promoter fragment contains two elements that are bound *in vitro* by *cad* protein (Figure 2A). Both of these elements contain two copies of a motif whose consensus is TTTATG; the distal element has an inverted repeat of the motif separated by 4 bp, whereas the proximal element has a direct repeat of the sequence separated by 2 bp (Figure 3A). This *cad* protein DNA recognition sequence is different from that reported for other *Drosophila* homeodomain proteins (see reference 7 for a review), which is consistent with the diverged nature of the *cad* homeodomain (6). Additionally, at least two homeoproteins which recognize a TCAATTAAAT homeodomain-binding consensus, the *even-*

skipped and *engrailed* proteins, do not bind *in vitro* to the TTTATG sequence (C.R.D., unpublished observations). These observations support the idea that the CDRE is specific for the *cad* protein.

Both of the elements to which *cad* protein binds are required for posterior expression of *ftz/lacZ* fusion constructs in germ-line transformed embryos. Point mutations in which the first and fourth base pairs of the consensus hexamer are changed to G and to T, respectively, eliminated the binding of *cad* protein *in vitro* (Figures 2B-C; Figure 3). Mutations in either the two distal consensus sequences or in the two proximal consensus sequences also eliminate the posterior pattern of expression. The expression of β-galactosidase in embryos that were transformed with either of these mutant fusion genes resembles that of embryos transformed with gene fusions that completely lacked both the distal and proximal elements (Figure 1). In addition to the negative effects that the mutations had on transcription, point mutations in one of the repeats seemed to reduce the level of binding of *cad* protein at the remaining binding site approximately 100 bp away (Figure 2B-C). This indicates that some form of cooperative binding may occur with *cad* protein It has been suggested that homeoproteins recognizing the TCAATTAAAT consensus sequence also bind cooperatively (8, 9).

To demonstrate that *cad* protein can increase transcription of the *ftz* gene by acting through the CDRE, we performed transient co-transfection assays in *Drosophila* Schneider 2 cells (10). When *cad* protein was overexpressed in these cells, the increase in the transcription of a fusion construct that contained six copies of the TTTATG sequence was 12-fold more than the increase in the transcription of a construct containing six copies of a mutated hexamer (Figure 4A). Furthermore, the transcription of intact *ftz*

promoter sequences which contained the CDREs was increased more than six times over that of a similar construct from which the binding sites had been deleted, and point mutations in either of the CDRE repeats reduced this transcriptional increase by 42-48% (Figure 4B). This effect of CDRE point mutations on transcription differs from that found in germ-line transformants, in which mutations in either of the consensus repeat sites eliminated all detectable posterior-specific expression of the fusion constructs. This difference is probably due to the nature of the transient expression assay, in which artificially high levels of *cad* protein are present within the cell.

We suggest that *cad protein* activates *ftz* transcription in both ectodermal and mesodermal cells, even though primarily mesodermal *ftz/lacZ* expression was observed in the embryos reported here. The -6.1 to -3.4 kb upstream promoter region is necessary for full *ftz* expression in the ectoderm (2, 3, 11); it is possible that these sequences are also necessary for high levels of ectodermal *cad*-driven transcription. It is also possible that *cad* protein activates *ftz/lacZ* expression in low but relatively similar amounts in both ectoderm and mesoderm, and that a mesodermal enhancer element within the wild-type *rosy* gene of the Carnegie 20 injection vector may increase the mesodermal expression to readily detectable levels (12).

The work reported here demonstrates that the *ftz* promoter is activated by a regulatory factor expressed with positional specificity in the embryo, and illustrates the usefulness of detailed promoter dissections for elucidating the complicated regulation of *Drosophila* segmentation genes; indeed, the direct interaction between the *cad* protein and the *ftz* zebra stripe promoter unit would have been difficult to predict from a comparison of the overall expression pattern of the *cad* and *ftz* proteins. It should be noted that other

more general transcription factors also seem to contribute to activating *ftz* transcription through the zebra stripe unit, even in the posterior half of the embryo (reference 3, and our unpublished observations). Therefore it is possible that the extreme changes in *ftz* expression in *cad*$^-$ mutant embryos (4) are due to both direct and indirect effects of the mutation.

It has been postulated that the gap genes may act as intermediates between maternal and pair-rule segmentation genes (13). Indeed, the maternally expressed *bicoid* gene product can activate transcription of the gap gene *hunchback* (14, 15). Furthermore, *cis*-acting elements that allow transcription in only one or a pair of stripes, and may respond to gap gene products, have been reported for the pair-rule loci *hairy* and *even-skipped* (16-18). By contrast, the evidence so far indicates only an indirect regulation of *ftz* expression by the products of the gap genes; in addition, the data presented here demonstrate that the product of a maternally expressed gene, present in a concentration gradient along the antero-posterior axis, can interact directly with a pair-rule segmentation gene. Therefore, it appears that several types of hierarchical networks are used in the regulation of pair-rule gene transcription.

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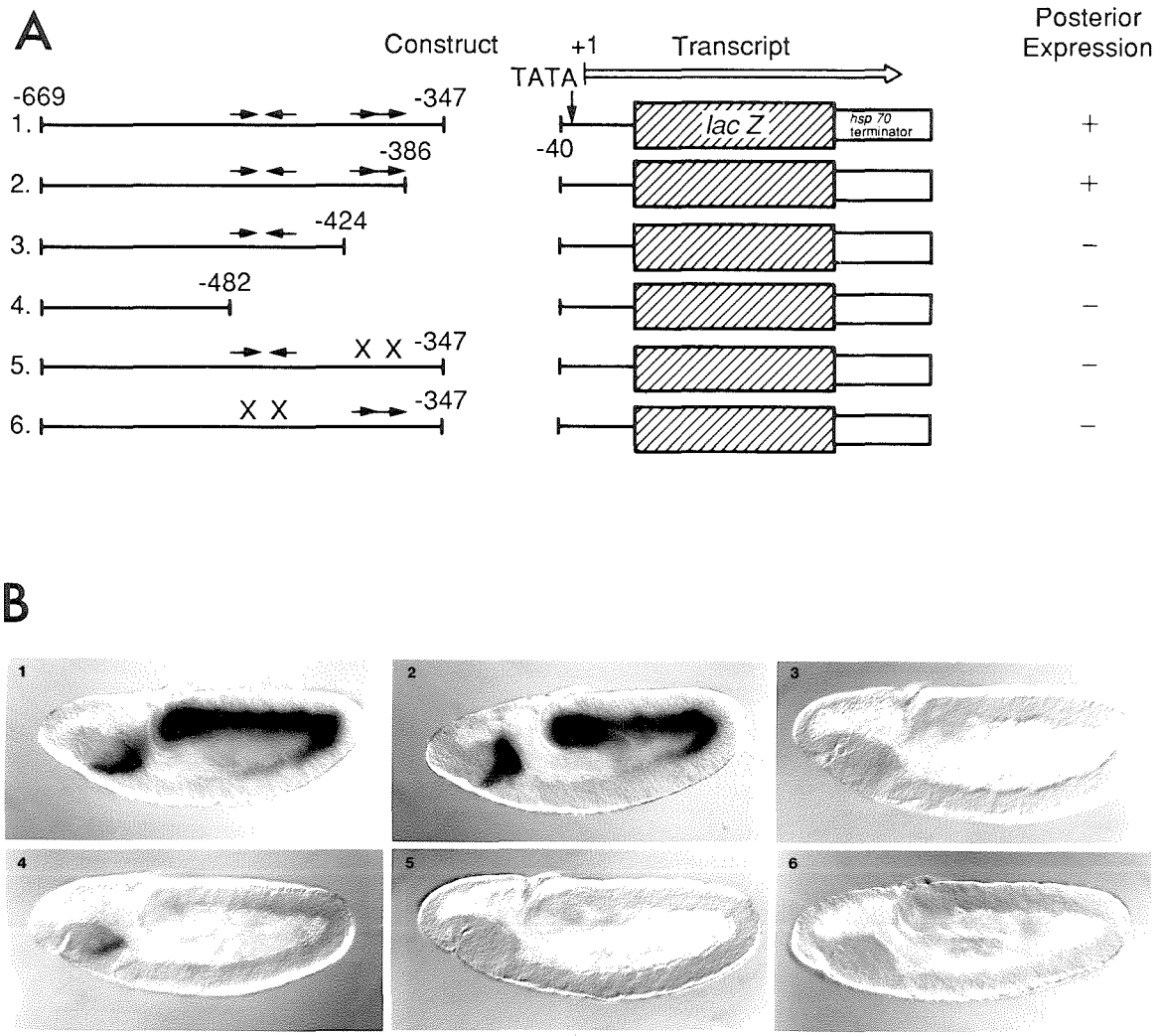
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4.
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5.
-347
X X
6.
-347
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lac Z
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+
+
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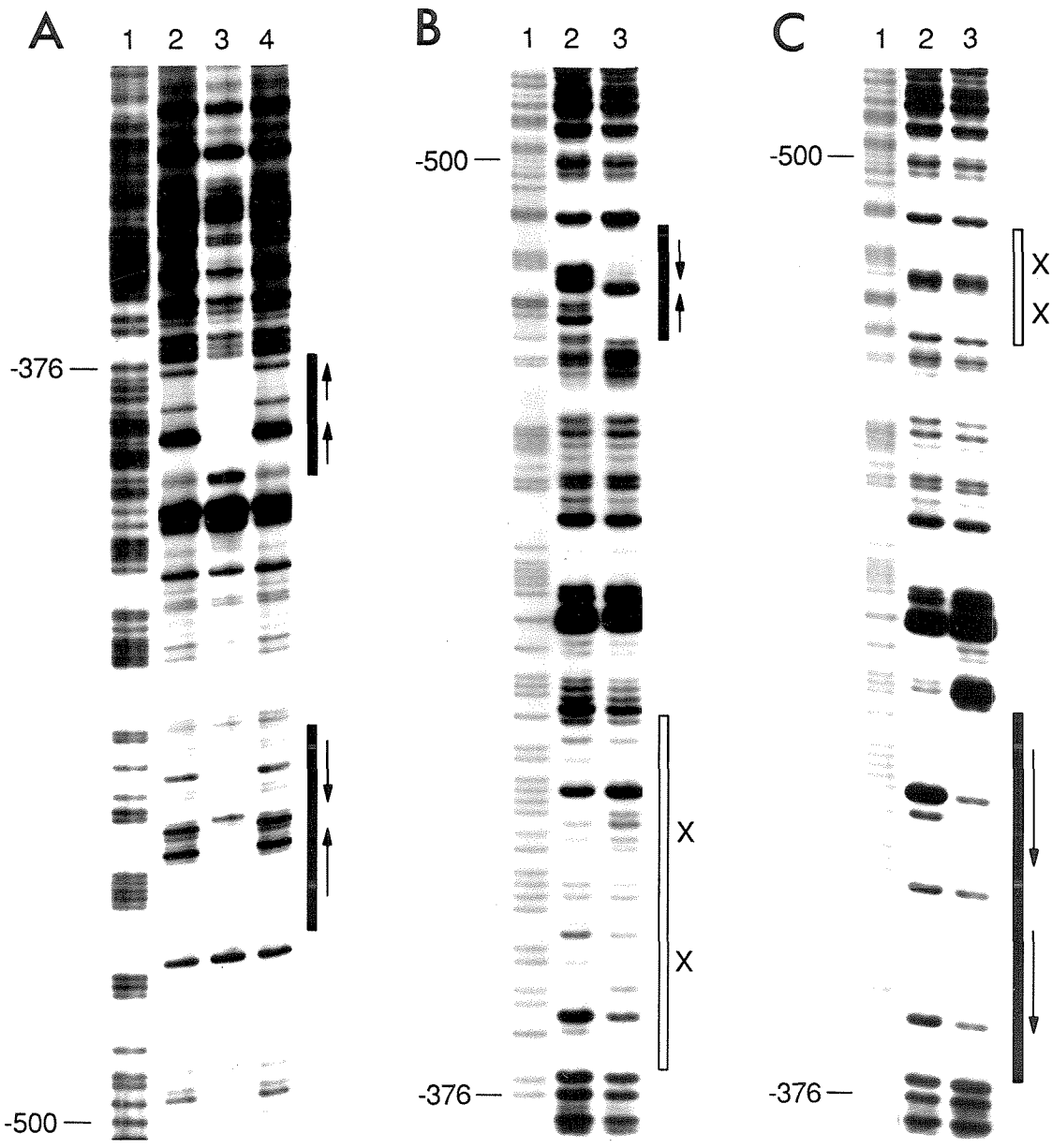
Figure 1.



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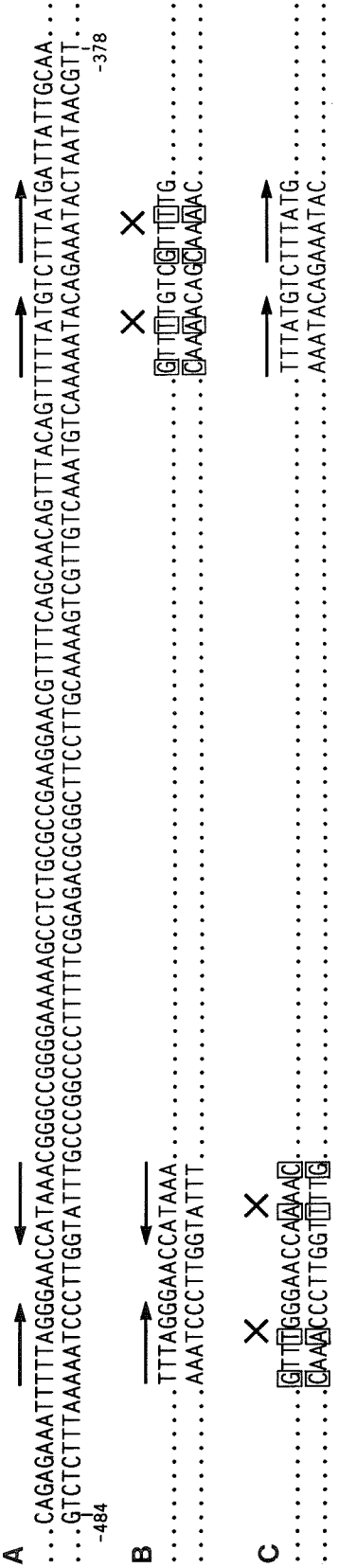
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-484
-378
B
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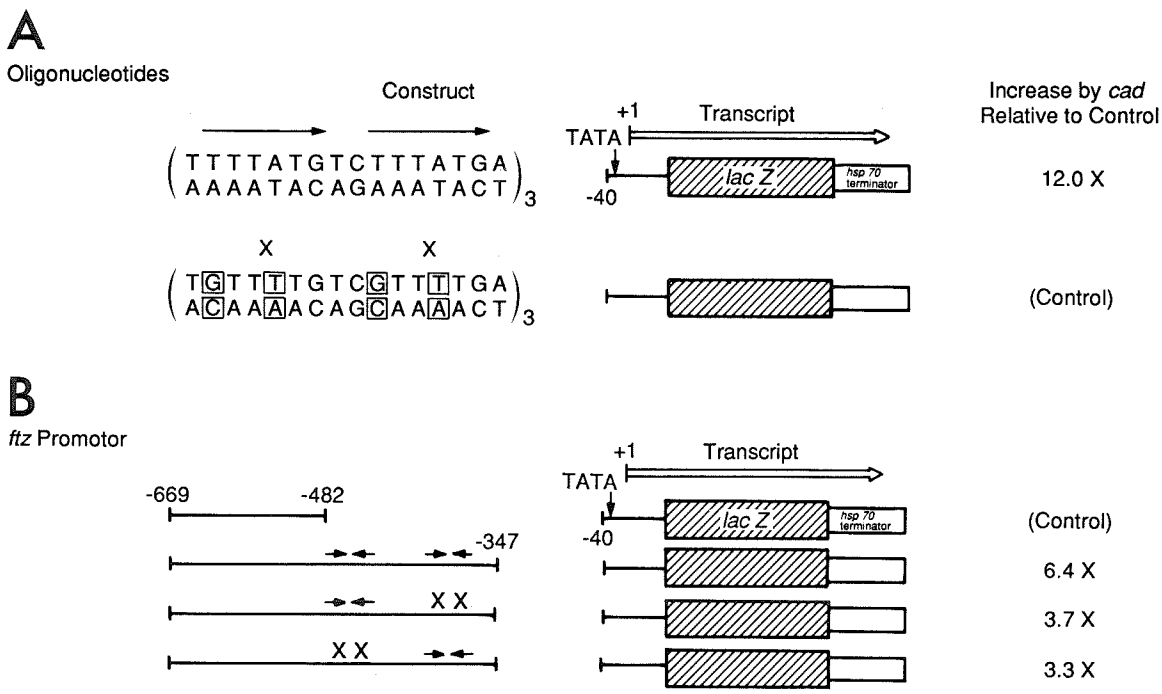
Figure 3.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Relative to Control
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(Control)
B
ftz Promotor
-669
-482
-347
X X
X X
(Control)
6.4 X
3.7 X
3.3 X

Figure 4.

Figure Legends

Figure 1. Embryonic Expression of Germ-Line Transformed *ftz/lacZ* Fusion Constructs

(A) A diagram of the *ftz/lacZ* portion of the constructs injected, and a summary of β-galactosidase expression observed in the germ band of transformed embryos. The numbers given are base pairs from the start point of transcription. Each arrow represents a single copy of the *cad* DNA recognition element (CDRE), while each X represents a mutated CDRE to which *cad* protein does not bind. (B) A representative embryo from each of the transformed stocks summarized above, stained for β-galactosidase activity using X-Gal as substrate. Because these constructs lack the *ftz* upstream enhancer element and most of the zebra stripe element, the expression levels are greatly reduced (3). Therefore, embryos were examined during germ band extension, as this is the earliest stage in which β-galactosidase activity (or antigen) can be readily detected. Constructs 1 and 2, which contain two intact copies of the CDRE repeats, express β-galactosidase in a posterior-specific pattern (described in text). Constructs 3-6, in which one or both of the CDRE repeats is deleted or mutated, do not express β-galactosidase in the germ band. The cephalic staining observed is variable, and is most probably due to elements in the Carnegie 20 injection vector used in these studies (2, 3, 12).

Methods *ftz* promoter fragments were ligated to the 5Δ-40 construct (3), in which *ftz* sequences from -40 bp to +73 from the cap site are fused to the *E. coli lac Z* coding sequences. The construction of *ftz/lacZ* fusion genes, generation of germ-line transformants, X-Gal staining of embryos, and photography were as described previously (3). Point mutations (see Figure 3 for actual base pairs

altered) were made with the Amersham oligonucleotide-directed *in vitro* mutagenesis system, version 2, according to the instructions of the manufacturer. At least five independent transformed lines were examined for each construct described. Embryos stained for longer periods of time than those presented show slight amounts of β-galactosidase activity in the germ band, apparently due to the presence of a general activator element located between -482 and -535 bp (3). Constructs 1 and 4 were previously described in (3), as 3'Δ-347 and 3'Δ-482, respectively.

Figure 2. DNAse I Protection of the *ftz* Promoter by *cad* Protein

Numbers given are base pairs from the start point of transcription. Sequences protected from DNAse I digestion are indicated by solid rectangles, while sequences not protected because of the generation of point mutations are indicated by open rectangles. The approximate locations of the TTTATG consensus motif are shown with arrows, while the locations of the mutated consensus with an X. (A) Intact fragment of the *ftz* zebra stripe promoter unit. (Lane 1) T and C nucleotides; (Lane 2) No extract added; (Lane 3) Extract from bacteria expressing *cad* added; (Lane 4) Crude extract from bacteria not expressing *cad* added. Two DNAse I footprint regions are seen, specific for digestion reactions in which the *cad* extract has been added. (B) *ftz* zebra stripe promoter fragment containing mutated consensus binding sites at the proximal protection region. (Lane 1) T and C nucleotides; (Lane 2) No extract added; (Lane 3) *cad* extract added. (C) *ftz* zebra stripe promoter fragment containing mutated consensus binding sites at the distal protection region. (Lane 1) T and C nucleotides; (Lane 2) No extract added; (Lane 3) *cad* extract added. Point mutations in the TTTATG consensus motif eliminate protection from DNAse I digestion around those sequences, while reducing

the level of protection at the intact footprinting region.

Methods *cad* protein was overexpressed in BL21 (DE3) bacteria using the system of Studier and Moffatt (19). p*cad*316, a T7/*cad* vector used to overproduce *cad* protein, was a gift of P. Macdonald. Induction of *cad* protein and production of crude extract were performed as described in Hoey et al. (20), with the exception that only the supernatant from the lysate centrifugation was used for all further steps. Crude *cad* extract gave reasonably strong DNAse I footprints (data not shown); but to optimize footprinting activity, *cad* extract was chromatographed on a heparin agarose column. The flow-through was collected and the column washed in Z buffer (100 mM KCl, 25 mM HEPES, pH 7.8, 12.5 mM $MgCl_2$, 1 mM Dithiothreitol [DTT], 0.1% Nonidet P-40 [NP-40], 20% glycerol and protease inhibitors 1 mM phenylmethylsulfonyl flouride [PMSF], 2 mM benzamidine), then fractions were step-eluted by the sequential addition of Z buffer with 200 mM NaCl, 400 mM NaCl, and finally 700 mM NaCl. The majority of *cad* protein eluted in the 400 mM NaCl fractions, as determined by anti-*cad* antibodies (gift of P. Macdonald); these fractions were pooled, dialyzed against Z buffer, and frozen at -70°C until use. 15 µl of the heparin agarose eluate was used for each footprinting reaction, where indicated. Crude extract from bacteria not expressing *cad* protein was made from DE3 cells not transformed with p*cad*316, but otherwise treated in the same manner. 15 µl of this material was used for each footprinting reaction, where indicated. DNAse I digestions were performed as described previously (21), using ^{32}P end-labelled DNA. Each reaction mix was electrophoresed on a 6% acrylamide gel, and the DNA fragments were detected by autoradiography.

Figure 3. Sequence of the *ftz* Zebra Stripe Promoter Unit Containing the *cad* Recognition Sites

(A) Portion of the intact *ftz* promoter. Two repeats of the consensus motif are located between -474 and -388 bp from the startpoint of transcription. Three of the CDREs contain a TTTATG hexanucleotide, while the fourth CDRE has a TTTAGG sequence. Each copy of the motif is indicated by an arrow. (B) Portion of the *ftz* promoter in which point mutations have been introduced into the proximal CDREs, and (C) into the distal CDREs. The mutated base pairs are boxed.

Figure 4. Expression of CDRE Fusion Constructs in Schneider 2 Cells Overproducing *cad* Protein

Fusion constructs were co-transfected with either the plasmid pPAc (10), indicated as *-cad*, or the *cad* expression plasmid pPAc*cad*325 (gift of P. Macdonald), indicated as *+cad*. The level of β-galactosidase activity in the cells was determined spectrophotometrically, and is given as arbitrary units. For each fusion construct, the increase in β-galactosidase expression due to overexpression of *cad* was determined by subtracting the activity in cells co-transfected with pPAc from that in the cells co-transfected with pPAc*cad*325. The calculated increases in expression levels were then compared, as indicated. (A) Effects of *cad* overexpression on β-galactosidase activity of a fusion construct containing three copies of the proximal CDRE repeat. The control construct contains three copies of a CDRE repeat to which *cad* protein does not bind. Mutated base pairs are boxed. (B) Effects of *cad* overexpression on expression of constructs containing intact or mutated *ftz* promoter fragments. Each intact CDRE repeat is indicated by arrows, each mutated

CDRE by an X. The control construct does not contain a CDRE repeat.

Methods The *cad* expression vector pPAc*cad*325 contains the *cad* coding region and alcohol dehydrogenase gene polyadenylation signal, inserted into pPAc, which contains the Actin 5C promoter. Fusion constructs, cloned into the Carnegie 20 vector, were co-transfected with either pPAc or pPAc*cad*325 according to the method of Krasnow et al. (10), with the following alterations. 10 μg of each plasmid and 5 ml of Schneider 2 cells were used for each individual experiment. After transfection, cells were grown for 48 hr at 24°C, harvested, resuspended in 270 μl assay buffer (50 mM potassium phosphate, 1 mM $MgCl_2$, [pH 8.0]), and frozen at -70°C until further use. β-galactosidase enzyme activity was determined spectrophotometrically by the method of Simon and Lis (22), using the substrate Chlorophenol red-β-D-galactopyranoside (CPRG). Three independent experiments were performed, with the results averaged, for each value obtained.

Appendix 2b

Transcriptional Regulation of the *Drosophila* Segmentation Gene *fushi tarazu* (*ftz*)

Abstract

ftz is one of the pair-rule segmentation genes of *Drosophila melanogaster*, and is an important component of the segmentation process in the fruit fly. We discuss the transcriptional mechanism which causes *ftz* to be expressed in a seven stripe pattern during embryogenesis.

Introduction

Regularly repeating segments are present in the fruit fly *Drosophila melanogaster* throughout all stages of development, first appearing by the middle of embryogenesis (1). This basic body plan is established in the first few hours after fertilization by the products of approximately fifty genes (2-4). These genes act to define parasegments, the fundamental units of antero-posterior pattern formation in the ectoderm and mesoderm (5), and to determine the regional identity of these parasegments. From this blueprint develops the visible segmentation pattern of the fly.

A large number of the genes which establish segmentation have been identified (2). These loci are conventionally grouped into several classes, based on mutant phenotypes and on the time of gene activity. The products of maternally expressed genes are necessary for the development of regions of the embryo. Subsets of these loci affect the anterior half, the posterior half, or the terminal embryonic regions (6). Of the zygotically expressed segmentation

genes, gap genes regulate broad embryonic domains consisting of multiple adjacent parasegments, whereas pair-rule genes are expressed in alternating parasegments, and segment polarity genes are expressed in parts of each parasegment. The homeotic genes, including the loci of the Bithorax and Antennapedia Complexes, determine and maintain the positional identity of each parasegment (3,4).

The segmentation genes and their products regulate one another to a large extent, both within a given gene class and among the various classes, in a hierarchy (or perhaps as aptly, a web) of interactions. Currently, a major emphasis in this field of research is to identify the specific interactions and regulatory mechanisms involved. The pair-rule gene *fushi tarazu* (*ftz*) is useful for addressing these questions.

ftz is one of the best characterized segmentation genes, and is interesting for several reasons. First, the *ftz* locus is an important component of the process which establishes the embryonic antero-posterior axis. *ftz* product is expressed during early embryogenesis in a characteristic seven stripe pattern (7; Figure 1). The relevance of this expression to normal development is seen in *ftz* mutants. These mutants die as unhatched larvae lacking every other segment, with the defective regions derived from the cells normally expressing *ftz* (8). In addition, *ftz* is relatively downstream in the segmentation process. *ftz* expression is regulated in part by other pair-rule genes (9,10), and it is likely that *ftz* protein regulates the expression of homeotic genes (11,12). As the *ftz* gene contains a homeobox, a 180 bp sequence encoding a polypeptide domain capable of binding to DNA (13), it is probable that *ftz* protein functions as a transcription factor. Further, the *ftz*

locus serves as a model for studying the transcriptional control of segmentation gene expression. Even though the regulation of *ftz* expression is quite complex, the promoter of *ftz* is possibly one of the simpler and more experimentally tractable of the *Drosophila* segmentation genes.

In this review, we discuss what is currently known about how *ftz* transcripts and protein become expressed in the seven striped pattern. Although the picture is still incomplete, a fair amount of progress has been made in recent years to elucidate the molecular mechanisms regulating this expression.

Promoter Elements Responsible for *ftz* Expression

ftz transcripts are initially generated in a continuous band of nuclei (14,15), in the region of the embryo which will for the most part develop into the germ band (the portion of the embryo which is subdivided into metameric units) (1). At the time of cellularization, *ftz* transcripts become restricted to a seven stripe pattern. During this period *ftz* protein is transiently expressed in broader domains, but by the end of cellularization is also limited to a similar "zebra stripe" pattern (7,16). For the most part, both the stripes and interband regions are approximately the same number of cells in width, although the seventh, most posterior stripe is slightly wider than the others. This striped expression persists throughout gastrulation (Figure 1) and until the germ band is almost fully extended, at which time the striped expression fades. *ftz* then becomes expressed primarily in neural cells. Still later in embryogenesis, *ftz* antigen is also detected in the hindgut (17).

The *ftz* upstream promoter sequences responsible for these expression patterns are located within 6.1 kb from the start of the transcription unit. Germ-line transformants containing *ftz/lacZ* fusion genes demonstrated that the *ftz* promoter can be divided into three functional subunits (18; Figure 2). Sequences between -6.1 kb to -3.4 kb contain an enhancer element through which the *ftz* protein elevates transcription of its own gene (19). This upstream promoter subunit contains a large number of *in vitro* protein binding sites (20), although it is not clear whether this promoter region directs *ftz* expression other than through the self-enhancement activity. Sequences between -2.5 kb to -669 bp are required for the later neural expression. This neurogenic element contains binding sites for the NTF-1/Elf-1 protein, which is expressed in neural cells and may also transcriptionally regulate the *Drosophila Ultrabithorax* and *dopa decarboxylase* genes (21,22).

Sequences between -669 bp to the start point of transcription are sufficient for the generation of the seven stripe pattern. This zebra stripe subunit is a mosaic of individual activator and repressor elements (23-25). First, several types of general activator elements are present. These elements allow *ftz/lacZ* constructs to be expressed in transformed embryos in a continuous band of cells throughout the germ band, although the level of this expression is not completely uniform. Second, multiple copies of a TTTATG consensus element provide additional activation in a posterior to anterior concentration gradient in the posterior half of the embryo. This posterior-specific activation is strongest in the cells which comprise the seventh *ftz* stripe. Third, several different repressor elements are present in the zebra stripe promoter subunit. These pair-rule repressor elements allow

ftz transcription to be inhibited in the nuclei and cells which comprise the interband regions. Hence, the successive deletion of these repressor elements from *ftz/lacZ* constructs causes each of the stripes to become wider, and ultimately to become one long continuous band.

Specific Regulators of the Zebra Stripe Promoter Subunit

A combination of approaches has been used to determine the direct activators and repressors responsible for the striped expression pattern of *ftz* transcription. At this time, only a few of these *trans*-acting factors are known, although additional regulatory proteins, including some not yet identified by genetic methods, will probably be found.

Repressors

The product of the *hairy* (*h*) gene probably interacts with a subset of the pair-rule repressor elements located in the zebra stripe promoter subunit. The *h* product is expressed in a striped pattern during early embryogenesis (26). For the most part, *h* protein is detected in nuclei and cells out of phase with those expressing *ftz*, although cells expressing both *h* and *ftz* protein are found at some times. In *h* mutant embryos, the stripes of *ftz* transcription (27), and of *ftz* protein (9), are much wider than in wild type embryos. Further, *ftz* expression is greatly reduced in embryos in which *h* protein is ectopically expressed in the cells which normally comprise the *ftz* stripes (28). The *h* gene has recently been sequenced, and found to encode a protein containing the helix-loop-helix domain similar to that found in a number of regulatory proteins, including N-*myc* (29). This homology suggests that *h* protein could bind directly to *ftz* DNA elements, perhaps as a homodimer or

heterodimer. Nevertheless, the exact mechanism by which *h* represses *ftz* transcription remains to be determined.

The product of the *even-skipped* (*eve*) gene is a possible candidate for another pair-rule repressor of *ftz*. Indeed, *eve* protein binds at several sites to the *ftz* zebra stripe subunit (C.R.D., unpublished observations). However, it remains to be seen whether the *eve* product has a direct or indirect effect on *ftz* expression. Both the *eve* protein expression pattern (30) and the pattern of *ftz* expression in *eve* mutants (9) is more complicated than in the case with *h*. In addition, it is not yet clear what function (if any) is provided by the *eve* protein binding sites in the zebra stripe subunit.

Activators

The identities of the generally expressed activators are not yet known. However, the product of the *caudal* (*cad*) gene, which contains a homeodomain, has been shown to be the posterior-specific activator of *ftz* transcription. *cad* protein increases the level of *ftz* transcription in the posterior half of the embryo above that caused by the general activators.

Multiple lines of evidence indicate that the *cad* protein interacts with the posterior-specific activator elements (24). Fusion constructs which contain *ftz* promoter sequences between -480 bp to -375 bp from the transcription start site are expressed in a posterior to anterior concentration gradient in germ-line transformed embryos. This expression is continuous, rather than striped, because this promoter fragment apparently lacks elements recognized by repressors. This expression pattern is consistent with that of the *cad* protein (31). Bacterially overproduced *cad* protein binds *in vitro* to

multiple copies of a TTTATG sequence located within this promoter fragment (Figure 3). *cad* is the only reported segmentation gene to recognize this DNA binding motif. In transient co-transfection experiments the overexpression of *cad* activates transcription of fusion constructs containing copies of this sequence. Point mutations which disrupt the *in vitro* binding of *cad* protein to its recognition site also eliminate the posterior-specific expression in transformed embryos, and reduce the level of activation in co-transfection assays. Finally, *ftz* expression in *cad* mutant embryos is eliminated or greatly reduced in the posterior three stripes (31).

It is interesting that the evidence to date, while not definitive, suggests that *ftz* is not extensively regulated by other region-specific factors. This situation is different than with the pair-rule genes *h* (32) and *eve* (33,34), which apparently respond to products of the gap genes. *cis*-acting promoter elements which generate one or a pair of stripes have not been seen in the *ftz* promoter (18,23), as in the *h* and *eve* promoters. Although *ftz* expression is altered in embryos mutant for the gap segmentation genes, this alteration can be explained as the effects of the mutations on *h* expression, which in turn alters *ftz* expression (35). Further, there is no conclusive evidence at this time for an anterior-specific activator of *ftz* transcription.

Despite the posterior activation by *cad* protein and the apparent lack of other region-specific activators, the concentration of *ftz* protein in the various *ftz* stripes is similar (7). It is possible that the *ftz* self-enhancement activity may be responsible for maintaining a comparable level of expression in each of the stripes, in spite of differences in initial levels. In any event, it is not clear how the increase in *ftz* transcription due to *cad* protein serves the

embryo. Perhaps this extra activation is responsible for the greater width of the seventh *ftz* stripe. In addition, it might play a role in the expression of *ftz* late in embryogenesis.

The Generation of *ftz* Striped Expression

In this section, we describe a mechanism by which the *ftz* striped expression pattern may be established (Table 1). While some of the details remain to be documented, this model is supported by several independent lines of evidence, including an analysis of the *ftz* transcription and protein patterns prior to cellularization (14-16), the use of injected cyclohexamide to block production of later-acting genes (10), and the expression of *ftz/lacZ* constructs in germ-line transformants (18,19,23,24). In addition, a mathematical basis for the refinement of pair-rule gene stripes, consistent with the model described here, has been recently proposed (36).

ftz transcripts first appear during early embryogenesis in a continuous band of nuclei (Table 1, step 1). For the most part, this activation is driven by several transcription factors which appear to be expressed in nuclei throughout much, if not all, of the length of the embryo. At this stage of development a generalized transcriptional activation of a large number of *Drosophila* genes occurs (37,38). Therefore, it is suggestive that at least some of these activators also drive the expression of other segmentation and non-segmentation genes, as occurs with the GAGAG sequence recognition factor (39,40). In addition to these more general factors, *ftz* transcription is also activated in the posterior half of the embryo by the *cad* protein. Strong *ftz* expression is not seen in the polar regions of the embryo, even though at least

some activators are present in these regions. Edgar, et al. (10) postulated that "polar" repressors block *ftz* expression in these nuclei. However, individual *ftz* promoter elements responsive to such "polar" repressors have not yet been localized (23). This suggests that these repressors might interact directly with the activator proteins or their genes, rather than with the *ftz* promoter itself.

ftz transcripts have a short half-life of approximately 6-8 minutes (10), so that new transcripts must be continually synthesized to maintain the overall expression pattern. Following the appearance of *ftz* RNA, the activators continue to function, and the expression pattern remains the same for several nuclear division cycles. During the final cycle prior to cellularization, however, the effects of recently synthesized pair-rule repressors, including *h* protein, are observed (Table 1, step 2). These repressors function in a striped pattern out of phase with the final *ftz* stripes; they act in a dominant manner to prevent *ftz* transcription in the nuclei (and later cells) which form the regions between the bands of *ftz* expression. As *ftz* transcripts continue to decay, they are replaced only in the regions lacking the pair-rule repressors, causing the seven stripe pattern to resolve. *ftz* protein is translated at this stage, primarily in a similar seven stripe pattern, and enhances transcription of its own gene (Table 1, steps 3-4). Whereas the regulatory factors which initiate and then refine the *ftz* transcription pattern interact with DNA sequences in the zebra stripe promoter subunit, *ftz* protein interacts with the upstream enhancer promoter element. It is not yet known whether the zebra stripe factors become superfluous to *ftz* expression at this

time, or whether these factors remain vital, in addition to the upstream enhancer activity.

ftz protein also has a short half-life (41), and like its transcript must be continually replaced to maintain the expression pattern. Presumably, the self-enhancement of *ftz* plays an important role in this maintenance process. Yet despite the enhancement activity, *ftz* expression in the striped pattern fades and then disappears during germ band elongation (Table 1, step 5). It is not clear why this progressive loss of expression occurs, although there are several possibilities. First, the zebra stripe activators might cease to function (if indeed *ftz* expression continues to require this activity following the appearance of *ftz* protein), so that in their absence the *ftz* enhancer activity might no longer maintain high levels of expression. Second, the *ftz* protein itself could be modified so as to lose its self-enhancement function. For example, *ftz* protein has multiple phosphorylated forms during embryogenesis (17), some of which might be functionally inactive. Third, yet another repressor could become expressed in the same cells expressing *ftz* protein, acting in a dominant manner to eliminate all transcription of the *ftz* gene.

Concluding remarks

Clearly, much work remains to be done on the regulation of *ftz* expression, as well on the elucidation of other genetic pathways underlying *Drosophila* segmentation. This is a challenging assignment, as the regulation of these genes is extremely complex. Nevertheless, it appears that a combination of genetic, molecular, and biochemical approaches will

successfully lead to an understanding of the mechanisms involved. The data obtained from such studies will provide a great deal of information about how regulatory factors function at the molecular level, and should provide a framework for understanding segmentation in a wide range of eukaryotes, including humans.

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Table 1. *A model to explain the transcriptional regulation of* ftz *striped expression*

Step	Expression of Regulatory Factors	Expression of *ftz*
1. Activation (pre-cellularization)	General activators – in continuous band in all parasegments *caudal* activator – in continuous posterior to anterior gradient	In all parasegments
2. Repression (pre-cellularization	General activators – same as in step 1 *caudal* activator – same as in step 1 'pair rule' repressors (e.g., *hairy*) – in odd numbered parasegments	In even-numbered parasegments
3. Enhancement (cellular blastoderm)	General activators – same as in step 1 *caudal* activator – in single stripe in posterior of embryo 'pair rule' repressors – same as in step 2 *ftz* self-enhancement – in even numbered parasegments	In even-numbered parasegments
4. Maintenance (gastrulation through germ band extension)	General activators – ? *caudal* activator – same as in step 3 'pair rule' repressors – same as in step 2 *ftz* self-enhancement – same as in step 3	In even-numbered parasegments
5. Loss of expression (germ band fully extended)	? ? ?	No 'zebra stripe' expression

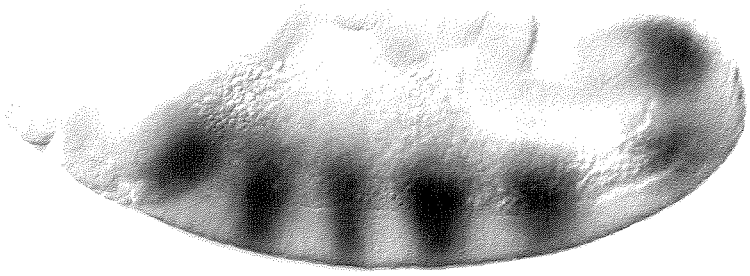

Figure 1.

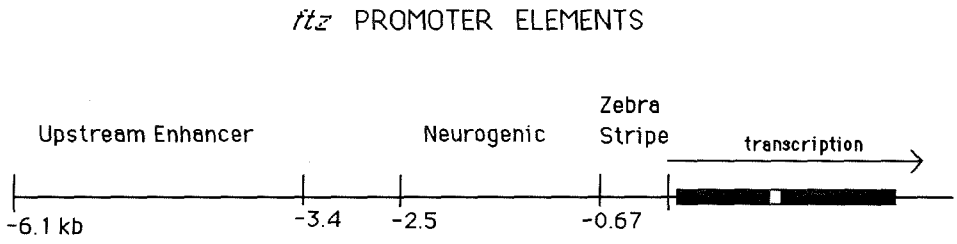

Figure 2.

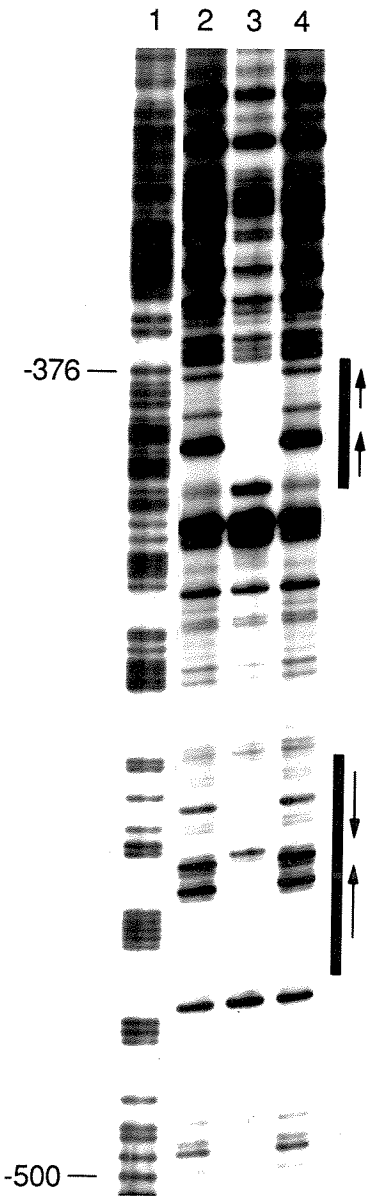

Figure 3.

Figure legends

Figure 1. The Seven Stripe Pattern of *ftz* Expression in an Embryo about to Undergo Germ Band Extension

This embryo has been transformed with a *ftz/lacZ* fusion construct containing the upstream enhancer and zebra stripe subunits of the *ftz* promoter (see Figure 2), and was stained for β-galactosidase activity. Expression is seen in the even numbered parasegments.

Figure 2. The *ftz* Promoter Subunits

The zebra stripe subunit allows *ftz* to be expressed in its seven stripe pattern; the neurogenic subunit allows expression in the nervous system; and the upstream subunit contains enhancer elements through which *ftz* protein increases transcription of its own gene. The bp given are from the start point of transcription.

Figure 3. Binding of *cad* Protein to *ftz* Zebra Stripe Promoter Sequences

Each arrow represents a TTTATG consensus motif recognized by *cad* protein. (Lane 1), T and C nucleotide sequence; (Lanes 2-4), DNAse I partial digestion of the *ftz* promoter in the presence of: (Lane 2) no extract; (Lane 3) bacterial extract containing overproduced *cad* protein; and (Lane 4) bacterial extract lacking *cad* protein.

CHAPTER 3

Synthetic Oligonucleotides Recreate *fushi tarazu* Zebra Stripe Expression

Joanne Topol, Charles R. Dearolf, Kulkarni Prakash, and Carl S. Parker

Abstract

fushi tarazu (*ftz*) "zebra stripe" expression is regulated by multiple activator and repressor sites located within the 670 base pairs proximal to the *ftz* transcriptional start site. By reconstructing promoters with synthetic oligonucleotides containing *cis*-regulators of *ftz* expression, we show that these elements can function as independent units to control position-specific transcription in the *Drosophila* embryo.

Introduction

The molecular processes governing cell fate along the longitudinal axis of the *Drosophila* embryo are controlled by a set of genes that are expressed in a spatially restricted manner early in embryogenesis (reviewed in Akam 1987; Ingham 1988). The result of such position-specific gene expression is the specification of discrete spatial domains within the developing embryo that ultimately define the body plan of the adult fly. It is known that the spatially restricted expression patterns of these early-acting genes are generated by a network of genetic interactions, and that, in many cases, these interactions take place at the transcriptional level. However, the precise molecular mechanisms responsible for forming these restricted patterns of gene expression remain undefined.

The pair-rule segmentation gene *fushi tarazu* (*ftz*) is a member of the regulatory network controlling pattern formation in the *Drosophila* embryo; it has a restricted pattern of transcription that evolves gradually during early embryogenesis (Hafen, et al. 1984; Kuroiwa, et al. 1984; Weiner, et al. 1984). *ftz* transcripts are first detected after the ninth nuclear division, throughout most

of the embryo. By the time the cellular blastoderm has formed, the RNA expression pattern has resolved into seven transverse stripes. Because the promoter sequences sufficient to direct the *ftz* stripe pattern are within a region whose size is extremely amenable to fine-tuned dissection (Hiromi, et al. 1985), the *ftz* gene provides an excellent system in which to study the molecular mechanisms governing position-specific transcription during embryogenesis.

Spatially restricted transcription can be established by localized transcriptional activation or, alternatively, by a combination of general activation and localized transcriptional repression (reviewed in Carroll 1990). It is likely that both these schemes are being utilized by *Drosophila* segmentation genes. In the case of *ftz*, it appears that the combination of general activation and localized repression is the primary mechanism mediating stripe formation (Dearolf, et al. 1989b; Edgar, et al. 1986). This model, initially proposed from the results of studies using inhibitors of protein synthesis (Edgar, et al. 1986), was strongly supported by our data from detailed *ftz* promoter deletion studies (Dearolf, et al. 1989b). Using P element-mediated transformation with *ftz/lacZ* fusion genes, we demonstrated that a pattern of general activation, i.e., a continuous band of gene expression, can be generated when repressor recognition sites are deleted from the *ftz/lacZ* fusion gene promoter and activator sites are retained. This result suggests that repressors of *ftz* transcription are necessary to prevent *ftz* expression in the interband regions. In addition, the result demonstrates that, in the context of stripe formation, transcriptional repressor molecules can act through *cis*-regulatory elements that are distinct from the recognition sites of activator proteins.

In the following article we provide molecular details that further elucidate the means by which both repression and activation are encoded in the *ftz* promoter and confirm the proposed model for the generation of *ftz* stripes. We demonstrate that relatively small protein recognition sites within the zebra element possess repressor function, and that these repressor sites can act independently to generate stripes from a continuous pattern of gene expression. We also identify a protein binding site that can act as a general activator of *ftz* transcription.

Results

Determination of Protein Recognition Site Function by Deletion Studies

In previous studies on the regulation of *ftz* transcription, we found that the promoter region sufficient to generate the characteristic seven stripes (defined by Hiromi, et al. (1985) as the zebra element) contains multiple activator and repressor elements (Dearolf, et al. 1989a; Dearolf, et al. 1989b; Dearolf, et al. 1990; Topol, et al. 1987). The complexity of this promoter region with regard to the number and variety of its protein recognition sites can be seen in the schematic drawing in Figure 1. DNA binding proteins present in embryonic nuclear extracts specifically recognize at least 15 sites within the zebra stripe regulatory region (Topol, et al. 1987 and Figure 2). In fact, most of the regulatory elements described in our previous study contain more than one sequence-specific protein binding site (compare Figure 6, Chapter 2 to Figure 1, this chapter). Due to this complexity, we chose to analyze the mechanisms mediating the position-specific transcription of *ftz* by focusing further studies on a small region within the zebra stripe promoter that clearly possesses both the repressor and activator functions necessary to generate *ftz*

stripes. That region was previously defined as A3-R1 (A refers to its activator function and R to its repressor function; see Chapter 2 for details on the A3-R1 region).

DNase I footprinting assays reveal that there are three protein binding sites located within the A3-R1 region (Figures 1 and 2; Note the protein binding sites located between -359 bp and -272 bp.). By assaying additional *ftz/lacZ* fusion genes using P element-mediated transformation, we have correlated these three protein binding sites with the *cis*-acting regulatory functions of the A3-R1 region. The three sites are referred to as *ftz* zebra element (*fz*) site A3a, R1a and R1b-A3b depending on whether the *cis*-regulator functions as an activating region (A), repressing region (R) or both (R-A). The deletion constructs designed to provide preliminary functional assignments for these protein recognition elements are diagrammed in Figure 3A. They consist of progressive 5' deletions of the *ftz* zebra element, progressive 3' deletions ligated to the *ftz* TATA homology and untranslated leader, and internal deletions constructed by ligating together appropriate 5' and 3' deletions.

The deletion of the fzA3a site from *ftz/lacZ* fusion genes, in the 5' to 3' direction, does not result in a change in the stripe pattern, i.e., no loss of repression is observed (Chapter 2 and Figure 3B). However, the deletion of the fzA3a site does result in a significant loss of ß-galactosidase expression (Appendix 3a); in embryos transformed with 3' deletion constructs this is evidenced by the loss of staining in the region where the anterior-most stripes are normally found (compare Figure 4J, Chapter 2 with Figure 3B, 3'Δ-318, this chapter). Within the fzA3a protein binding site there are two copies of the GAGAG or CTCTC DNA sequence motifs found in a number of

Drosophila genes (Biggin and Tjian 1988; Gilmour, et al. 1989; Soeller, et al. 1988). A protein that recognizes this motif has been purified in other laboratories and shown in cell-free systems to be a transcriptional activator of the *engrailed* (*en*) (Soeller, et al. 1988) and *Ultrabithorax* (*Ubx*) (Biggin and Tjian 1988) genes. We demonstrate that purified "GAGA" protein also binds to the fzA3a site within the *ftz* promoter (Figure 2), suggesting that the fzA3a site is indeed a *cis*-activator of *ftz* transcription.

When the fzR1a site is deleted from the 5' direction, a derepressed pattern of expression can be observed in a subset of the embryos stained; that is, in all the transformant lines analyzed, the stripes appear broadened in some of the embryos and not in others (Figure 3B; compare embryo transformed with 5'Δ-322 to those with 5'Δ-297). This result suggests that fzR1a has some repressor function. Deleting the fzR1a site from the 3' direction does not result in an additional loss of repression since the completely derepressed pattern is observed when fzR1a is still present (Figure 3B, 3'Δ-298). Consequently, a single repressor site (in this case fzR1a) is insufficient to mediate detectable repression in our assay (also see Appendix 3b).

When the binding site adjacent to fzR1a (fzR1b-A3b) is deleted in the 5' deletion series, all embryos display broadened stripes (Figure 3B, 5'Δ-276). This change from a variable phenotype to a uniform one suggests that the fzR1b-A3b site also recognizes a repressor protein. Deleting this site from the 3' direction supports this notion (Figure 3B, 3'Δ-272 to 3'Δ-298).

In order to determine the effects of eliminating the repressor recognition sites without also removing the sequences found upstream or

downstream, internal promoter deletions were constructed and assayed for ß-galactosidase expression (Figure 3A). Deleting either of the two repressor sites singly (ΔfzR1a or ΔfzR1b-A3b) results in a variable phenotype in which only a small subset of embryos display a derepressed pattern (data not shown); this variability is similar to that seen when only the fzR1a repressor site is removed in the 5' direction (Figure 3B, 5'Δ-297). When the fzR1b-A3b site is deleted, and the proper spacing of the promoter is maintained, the overall level of ß-galactosidase expression decreases relative to the control (Table 1, ΔfzR1b-A3b vs. 5'Δ-669; also note 5'Δ-297 vs. 5'Δ-276). In addition, unlike the control (Chapter 2, 5'Δ-669), there is no expression in the ectoderm of embryos transformed with ΔfzR1b-A3b (data not shown). Consequently, we believe that fzR1b-A3a functions as an activator site as well as a repressor site. Finally, as expected, when both repressor sites are removed (ΔfzR1a/fzR1b-A3b), a consistent derepressed pattern of expression is observed (data not shown); similar to the expression of the 5' deletion construct removing both repressor sites (Figure 3B, 5'Δ-276), the stripes appear broadened in all the transformed embryos.

Activity of Synthetic Regulatory Sites

To directly assess the functional roles of the A3-R1 protein recognition sites, we designed *ftz/lacZ* fusion genes in which protein binding sites are added to (rather than deleted from) the *ftz* promoter (Figure 4A). Synthetic oligonucleotides containing protein recognition sites were ligated together and inserted into *ftz/lacZ* deletion constructs. To assay for the ability of recognition sites to selectively repress transcription, oligonucleotides were inserted into a 3' deletion construct which expresses ß-galactosidase in a continuous band throughout the mesoderm (Figure 3B, 3'Δ-298); the

insertions were made between the more distal activator sites and the TATA homology. Conversely, to assay for the ability of recognition sites to activate transcription, oligonucleotides were inserted into a 5' deletion construct that is incapable of promoting *ftz* transcription in the germ band (Chapter 2, 5'Δ-40); this construct lacks all *ftz* promoter sequences upstream of the TATA homology. Finally, we have ligated repressor sites upstream of synthetic activator sites to determine whether position-specific expression can be generated entirely by oligonucleotides in tandem. (See Figure 4A for diagrams of reconstructed promoters.)

The most striking result in the selective repression assay is seen in embryos transformed with the 3'fzR1b-A3b construct (Figure 4B). Four copies of the fzR1b-A3b repressor site are able to convert a continuous band of expression (directed by the sequences upstream of -298 bp) into a highly resolved seven stripe pattern characteristic of *ftz* expression. The fact that no ß-galactosidase expression is seen between the stripes indicates that the fzR1b-A3b site completely represses expression in the cells that normally comprise the *ftz* interband region. This result can be compared to that of the 3' construct in which only one copy of fzR1b-A3b is present downstream of -298 bp (Figure 3B, 3'Δ-272). Although *ftz* stripes are detectable in the ß-galactosidase expression pattern of 3'Δ-272 transformed embryos, strong staining is seen in the interbands. Thus, four copies of the R1b-A3b site can mediate repression significantly better than one copy.

When four copies of fzR1a are inserted into the basal construct for the repression assay, a striped pattern is also recreated; in this case, however, multiple repressor elements are unable to completely repress transcription in the interbands (Figure 4B, note expression in the interband region in both

germ band extended and shortened embryos). In fact, immediately upstream of -298 bp (at -299 to -313 bp) is a fifth copy of fzR1a; alone, this recognition element is incapable of repressing transcription (See Figure 3B, 3'Δ-298).

The 3'fzR3 construct contains oligonucleotides with a protein binding site found downstream of the A3-R1 region (Figures 1 and 2). Progressive 5' and 3' deletion studies described in Appendix 3b strongly suggest that this domain is also an interband repressor binding site in the endogenous *ftz* promoter; nonetheless, the fzR3 element is unable to repress transcription when placed in triplicate within the basal construct for the repression assay (Figure 4B). This result demonstrates that the ability of a *cis*-regulator to selectively inhibit expression of *ftz/lacZ* fusion genes is specific for the site and not merely due to the presence of protein recognition sequences between the activator elements and the TATA homology. As an additional control, we synthesized an oligonucleotide, X, that lacks the ability to bind proteins present in embryonic extracts (data not shown). The ß-galactosidase expression in embryos transformed with 3' Oligo X display no signs of selective repression, although the overall level of expression is slightly lower than that of the basal construct for the repression assay (Figure 4B).

Two distinct recognition elements were tested in the activation assay: the fzA3a site and the fzR1b-A3b site (Figure 4A). Footprinting data with purified GAGA protein and our results from *ftz/lacZ* fusion genes (see Appendix 3a) suggested that these protein binding sites may be capable of independently activating transcription in the embryo, when placed upstream of the *ftz* TATA box. This is indeed the case when three copies of the fzA3a site are ligated upstream of -40 bp (Figure 4B and Table 1). ß-galactosidase expression is seen throughout most of the presumptive mesoderm in

embryos transformed with 5'fzA3a. This continuous band of staining, however, is not uniform in strength; instead, expression appears less intense in the region where the first and sixth stripes are normally found. Interestingly, ß-galactosidase expression in the first and sixth stripes are also lower than the neighboring stripes when the entire zebra element is used to drive *lacZ* expression (Chapter 2, 5'Δ-669).

Embryos transformed with 5'fzR1b-A3b display very low levels of ß-galactosidase expression, despite the fact that four copies of the recognition element are ligated upstream of -40 bp (Figure 4B). The pattern generated by this construct, however, is interesting in light of the dual function of the fzR1b-A3b site. Faint stripes, (barely detectable in a photograph), can be observed in transformed embryos, suggesting that the fzR1b-A3b site can function as a weak activator in the stripe region and a repressor in the interband region.

Four copies of the oligonucleotide X inserted into 5'Δ-40 are not able to promote transcription of *ftz/lacZ* fusion genes (Figure 4B); this result is consistent with the lack of binding sites within the oligonucleotide X. Finally, we were unable to generate stripes when multiple fzR1b-A3b sites were ligated upstream of fzA3a activator recognition sites; instead, we observed the completely derepressed pattern of ß-galactosidase expression seen with the 5'fzA3a construct (Figure 4B). This result may reflect a requirement for the fzR1b-A3b element to be downstream of the fzA3a activator element in order for repression to take place in the interband cells, or it may be due to inappropriate spacing between repressor sites and the sequences mediating activation.

The *ftz* zebra element activator and repressor sites defined in this thesis are summarized in Figure 1. They include two *caudal* DNA recognition elements (CDRE): fzA2a and fzA2b (Appendix 2a), the GAGAG consensus activator site fzA3a, a cluster of five repressor sites and an activator region proximal to the TATA homology. Within the repressor sites are several recurring DNA sequence motifs: an inverted repeat of the GCNGTAA motif in FZR1a, the CAAGGNC motif in fzR1b-A3b and fzR4-A4 and direct repeats of the CGGATAA motif in both fzR2 and fzR3. It is likely that multiple, distinct proteins recognize many of these regulatory sites. (This has been demonstrated *in vitro* for the fzR4-A4 regulatory site; Harrison and Travers 1990; Ueda, et al. 1990) In addition, sites that share motifs are likely to recognize at least some of the same regulatory proteins. (This has also been shown *in vitro* for the fzR1b-A3b and the fzR4-A4 sites; Ueda, et al. 1990.) Although we have identified all the zebra element *cis*-regulators detectable by our assay, we have not excluded the possibility that additional regulatory sites, not detectable by these methods, may be present within the *ftz* zebra element.

Discussion

We have shown that oligonucleotides containing *cis*-regulators of *ftz* expression can act as independent units to direct position-specific transcription in the *Drosophila* embryo. More specifically, we have demonstrated that synthetic sites ~25-30 bp in length can retain repressor function when placed in a novel context within the zebra stripe promoter, and that a ~50 bp oligonucleotide can function as a general activator of transcription, without the aid of sequences upstream of the TATA homology.

Regarding *ftz* repressor elements, when multiple copies of individual repressor recognition sites (fzR1a or fzR1b-A3b) are ligated to a portion of the *ftz* promoter, they transform the continuous pattern of gene expression generated by a group of endogenous *cis*-activators into the seven stripe pattern characteristic of *ftz* expression. Thus, an individual repressor element is able to function in all the *ftz* interband regions. This result supports the notion that the selective repression of *ftz* is primarily controlled by other members of the pair-rule class of segmentation genes, particularily those genes which function in periodic patterns out of phase with the *ftz* pattern.

We found that multiple copies of either of the repressor sites mediate repression better than a single copy. In the case of fzR1a, one copy is insufficient to mediate repression whereas five copies generate stripes; similarly, one copy of fzR1b-A3b can barely repress transcription whereas four copies completely eliminate transcription in the interband regions. Perhaps the presence of multiple repressor elements ensures that the proper protein-protein interactions will take place between the activator and the repressor molecules.

Several points can also be made about *cis*-activators within the *ftz* zebra stripe promoter. The most noteworthy is that an activator element capable of functioning in only one or several stripes has not been identified in our studies. Instead, the activator elements characterized thus far mediate *ftz* transcription in both the odd and even numbered parasegments and throughout most of the germ band. The first *ftz* activator site to be defined was the *caudal* (*cad*) DNA recognition element (CDRE) (Appendix 2a); the *cad* gene product interacts with the CDRE to increase the level of *ftz* transcription

in the posterior half of the embryo. In this study we characterize an additional *cis*-activator of *ftz* transcription, the fzA3a site. Since the fzA3a site is able to direct expression throughout the presumptive mesoderm, we believe it is involved in generating the uniform distribution of *ftz* transcripts found in syncytial blastoderm embryos prior to stripe formation. FzA3a possesses two copies of the GAGAG or CTCTC consensus motif found within the activator sites of other *Drosophila* promoters (Biggin and Tjian 1988; Soeller, et al. 1988). The fact that fzA3a serves as a recognition element *in vitro* for the protein that binds to those other *cis*-activators is consistent with its *in vivo* role as an activator of *ftz* transcription.

Finally, we find that the fzR1b-A3b element is only able to mediate low levels of transcriptional activation when multiple copies are placed upstream of 5'Δ-40. The quantitation data (Table 1), nonetheless, strongly suggests the fzR1b-A3b site possesses activator function in the context of the endogenous *ftz* promoter. Perhaps, in order to promote transcription, this *cis*-activator must form protein-protein interactions with *ftz* regulators bound to sequences located upstream of the TATA homology. The fzR1b-A3b site has been reported by other to promote transcription preferentially in stripes 1, 2, 3 and 6 (Ueda, et al. 1990). This hypothesis is not supported by the expression pattern of our internal promoter deletion in which fzR1b-A3b has been singly remove or by our data with the reconstructed promoters containing multiple copies of fzR1b-A3b; instead, we believe that fzR1b-A3b also functions as a general activator of *ftz* transcription. Regarding the dual function of this site, it remains unclear whether it is due to the activity of multiple proteins competing for the same (or overlapping) site(s), conformational changes in a

single protein, different protein complexes in different cell types, or different post-translational modifications of the same protein in different cell types.

We have also made several observations regarding the structure of the zebra stripe promoter. Most notable is the clustering of repressor sites within less than 200 bp of the zebra element (Figure 1); this promoter organization may be a requirement for effective inactivation of transcription factors located both upstream and downstream of the repressor site cluster. In addition, there appears to be redundancy in the zebra element with regard to interband repression. Some embryos in our deletion study display a fully repressed transcription pattern even when a repressor site is lost (Figure 3, 5'Δ-297, ΔfzR1a and ΔfzR1b). This result suggests that all the repressor elements within the cluster need not be occupied with regulatory molecules to fully repress *ftz* transcription. Perhaps the presence of the entire cluster simply increases the probability that the necessary number of repressor sites will be occupied in a particular cell type, at the critical time in embryogenesis. Conversely, a single repressor recognition element is insufficient to mediate repression (as is the case with 3'Δ-298, Figure 3B). Perhaps multiple sites act synergistically and are therefore optimally functional when clustered or ligated in tandem.

In addition, there appear to be different classes of repressor recognition sites within the zebra element that contain distinct DNA sequence motifs. It is possible that the repressor proteins that recognize these distinct *cis*-elements function in different cells in the interstripe regions; unfortunately, the resolution of the ß-galactosidase staining technique is not sufficient to directly address this question, and other detection techniques with higher resolution are not adequately sensitive for our purposes.

We find that the different classes of repressor sites are functionally non-equivalent in our assay. For instance, when placed in the basal construct for the repression assay, fzR1b-A3b is a more effective repressor site than fzR1a, and fzR1a is more effective than fzR3. However, this may simply reflect differences in the ability of the different classes of repressor sites to act independently rather than differences in their relative strength. For example, the inability of fzR3 to function in the repression assay may indicate that all the sequences required for mediating fzR3 repressor function are not present in the reconstructed promoter. Alternatively, there may be a particular spacing requirement for the fzR3 site relative to the upstream activator sites that is not maintained in the reconstructed promoter. Nonetheless, it is worth noting that multiple copies of fzR1b-A3b can mediate the complete inhibition of transcription in the interband cells, in the absence of one class of repressor site (the fzR3 class).

The model put forth by this group (Dearolf, et al. 1989b; Dearolf, et al. 1990) and others (Edgar, et al. 1986) regarding the generation of *ftz* striped expression is strongly supported by the data presented here. General activators, such as the fzA3a "GAGA" factor, promote transcription in a continuous band of nuclei prior to the generation of stripes. During blastoderm cell formation, pair-rule repressors act through *cis*-regulatory sites such as fzR1a and fzR1b-A3b, to establish the highly resolved pattern of *ftz* expression. These repressors must function in a periodic pattern out of phase with the endogenous *ftz* stripes and in a dominant manner relative to the general activator molecules.

We have been able to recreate both the early unresolved and the highly resolved stripe pattern of *ftz* expression using synthetic oligonucleotides of protein binding sites. As is the case with any reconstructed system, we are now in the position to make controlled changes in our system and determine the effects of these changes on the reconstructed zebra stripe expression. For instance, we can place our reconstructed promoter fusion genes into mutant backgrounds to see whether the patterns generated by the oligonucleotides are lost in the mutant embryos. In this manner, *trans*-regulators acting through particular protein recognition sites can be identified. In addition, *cis*-regulators can be inserted in various orientations, positions and combinations within the fusion genes and the effects of such controlled changes can be determined *in vivo*. Through these additional experiments we hope to gain further insight into the mechanisms governing position specific transcription in organismal development.

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Table 1. Expression of *ftz/lac Z* Constructs

Construct	Number of Transformant Lines Quantitated	Units of β-Galactosidase Activity[1]	
		Mean ± S.D.	Range
5'Δ -669	5	914 ± 157	744 - 1131
ΔfzR1b - A3b	4	531 ± 128	379 - 689
5'Δ -322	3	303 ± 109	178 - 373
5'Δ -297	3	283 ± 67	241 - 364
5'Δ -276	3	112 ± 19	91 - 127
5'fzA3a	4	197 ± 125	98 - 358
5'Δ Oligo X	3	60 ± 14	45 - 73

[1]Each unit of β-galactosidase activity is equivalent to one $O.D._{574}$ unit per mg protein x 10^2. The background value for untransformed *ry* embryos was subtracted from each value. The value for each transformant line was obtained from measurement of two separate egg collections.

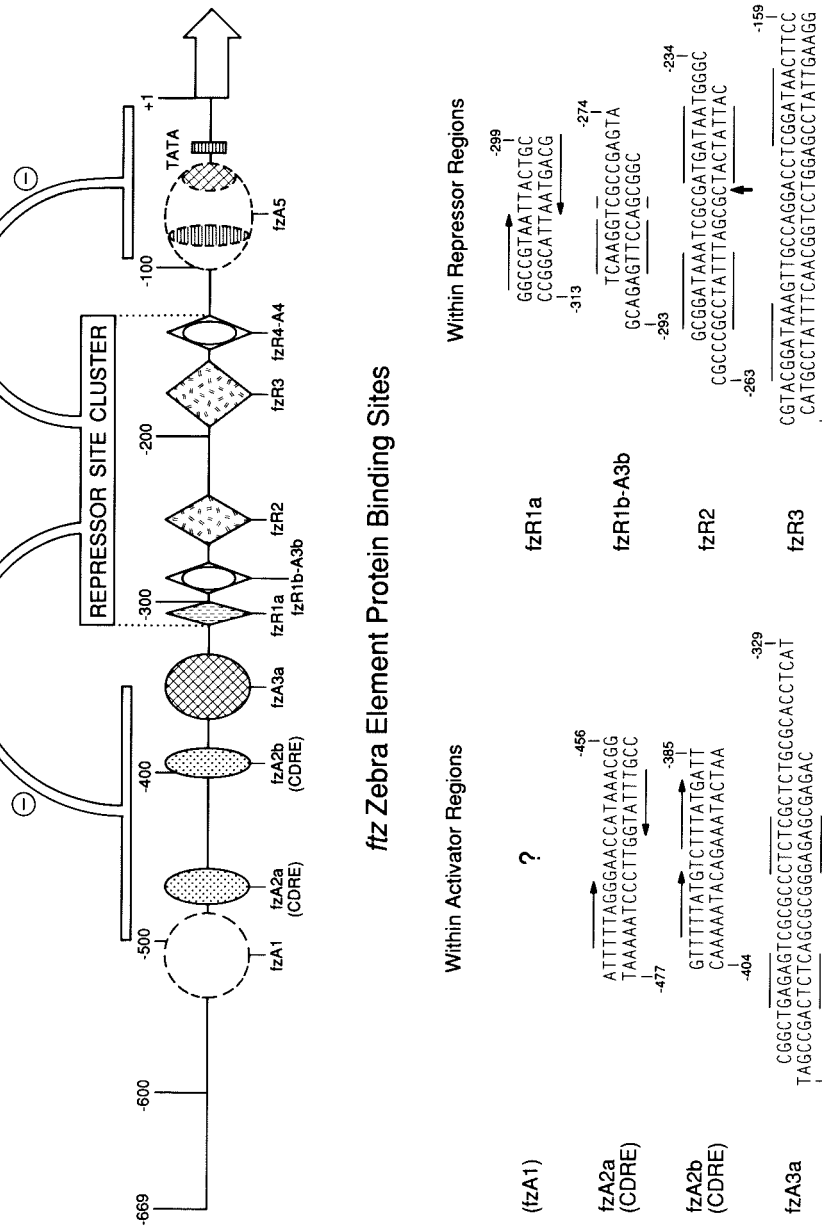

ftz Zebra Element Protein Binding Sites
REPRESSOR SITE CLUSTER
-669
-600
-500
-400
-300
-200
-100
+1
TATA
fzA1
fzA2a (CDRE)
fzA2b (CDRE)
fzA3a
fzR1a
fzR1b-A3b
fzR2
fzR3
fzR4-A4
fzA5
Within Activator Regions
Within Repressor Regions
(fzA1)
?
fzR1a
fzA2a (CDRE)
fzR1b-A3b
fzA2b (CDRE)
fzR2
fzA3a
fzR3
fzR1b-A3b
fzR4-A4
fzR4-A4
(fzA5)

Figure 1.

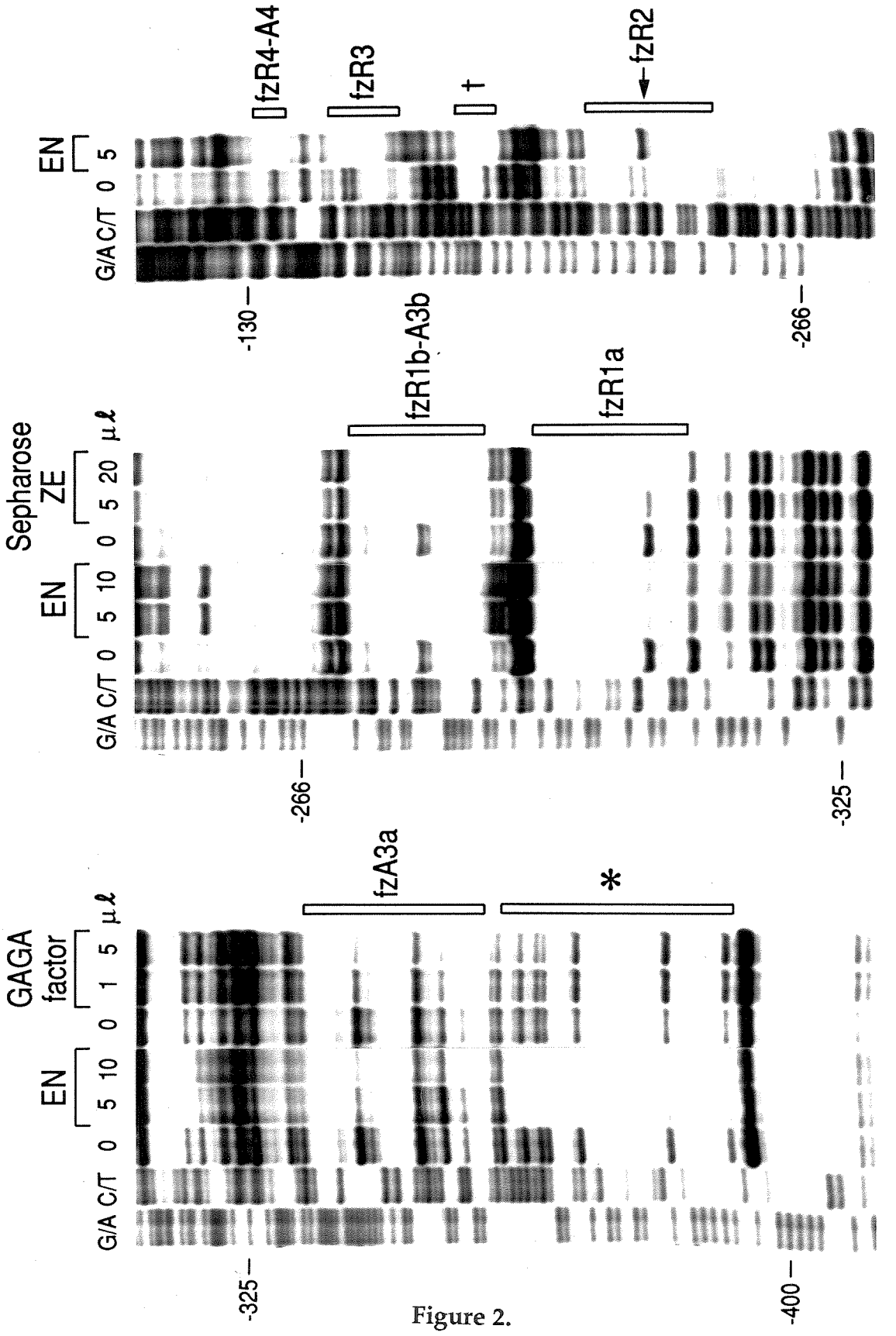

GAGA factor
EN
G/A C/T 0 5 10 0 1 5 µl
-325
fzA3a
*
-400
Sepharose ZE
EN
G/A C/T 0 5 10 0 5 20 µl
-266
fzR1b-A3b
fzR1a
-325
EN
G/A C/T 0 5
-130
fzR4-A4
fzR3
†
fzR2
-266

Figure 2.

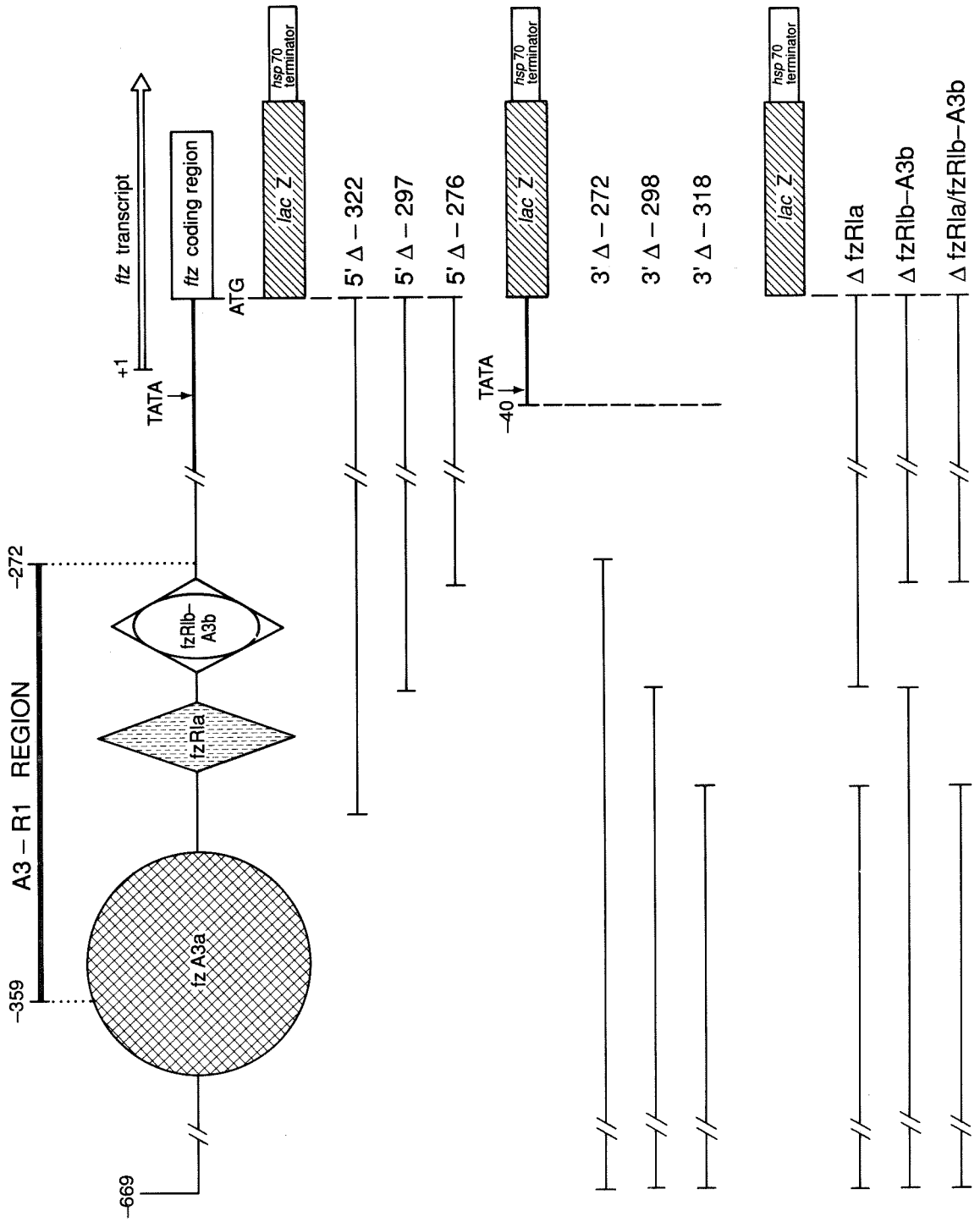

A3 – R1 REGION
–272
–359
–669
fz A3a
fzRla
fzRlb–A3b
+1
ftz transcript
ftz coding region
ATG
TATA
hsp 70 terminator
lac Z
5' Δ – 322
5' Δ – 297
5' Δ – 276
TATA
–40
3' Δ – 272
3' Δ – 298
3' Δ – 318
Δ fzRla
Δ fzRlb–A3b
Δ fzRla/fzRlb–A3b

Figure 3A

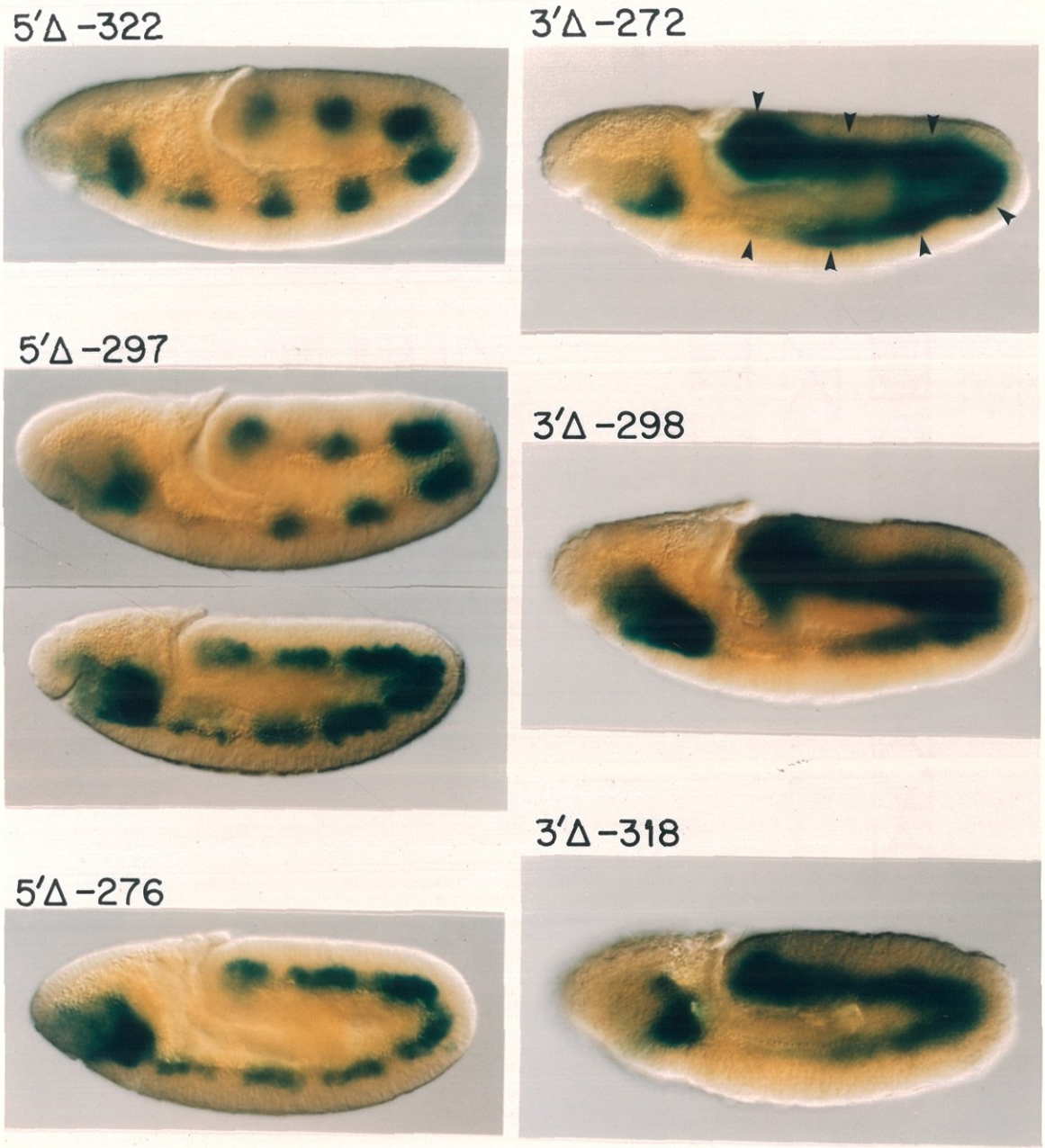


Figure 3B

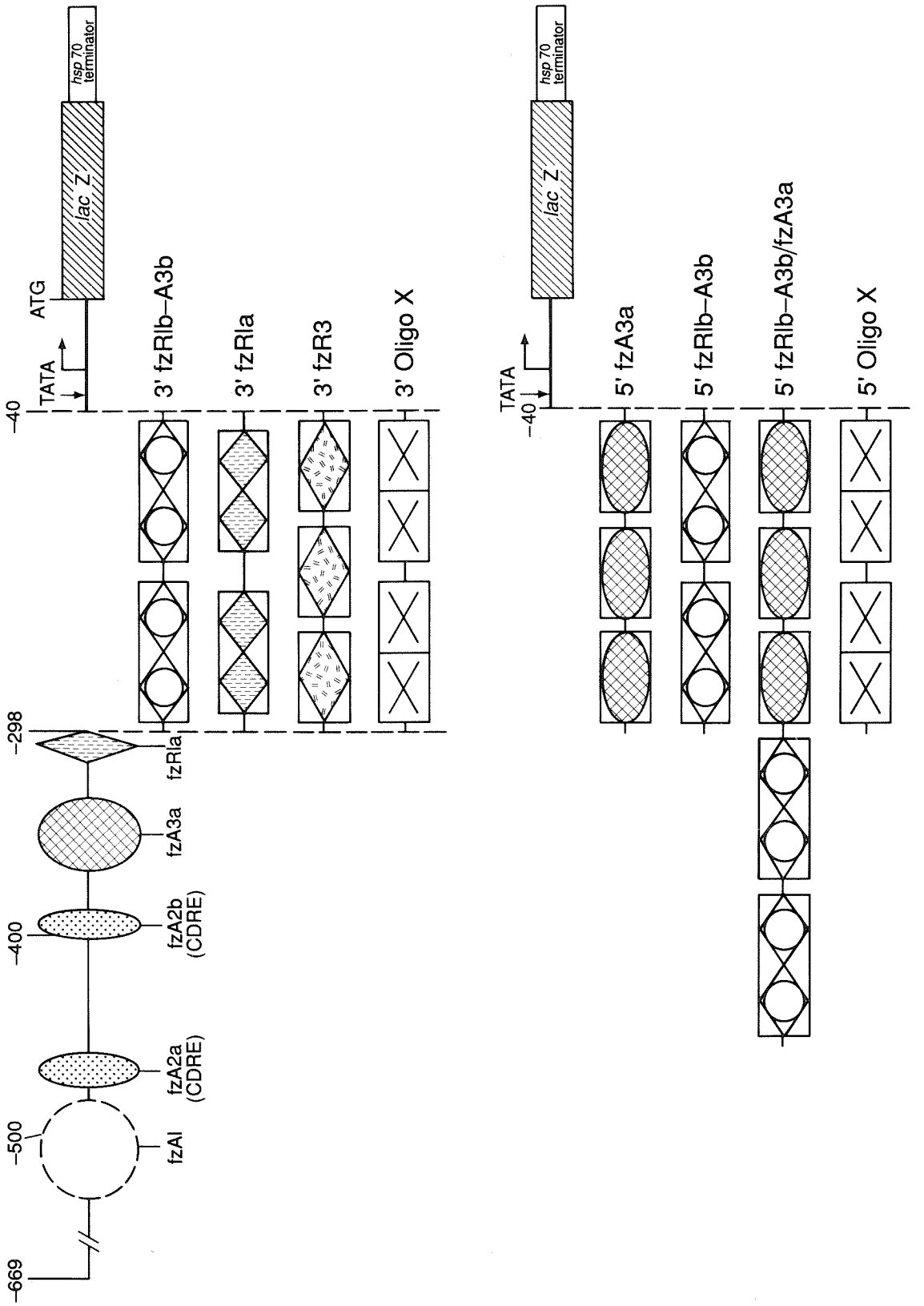

hsp 70 terminator
lac Z
ATG
TATA
-40
3' fzRlb–A3b
3' fzRla
3' fzR3
3' Oligo X
-298
fzRla
fzA3a
-400
fzA2b (CDRE)
fzA2a (CDRE)
-500
fzAl
-669
hsp 70 terminator
lac Z
TATA
-40
5' fzA3a
5' fzRlb–A3b
5' fzRlb–A3b/fzA3a
5' Oligo X

Figure 4A.

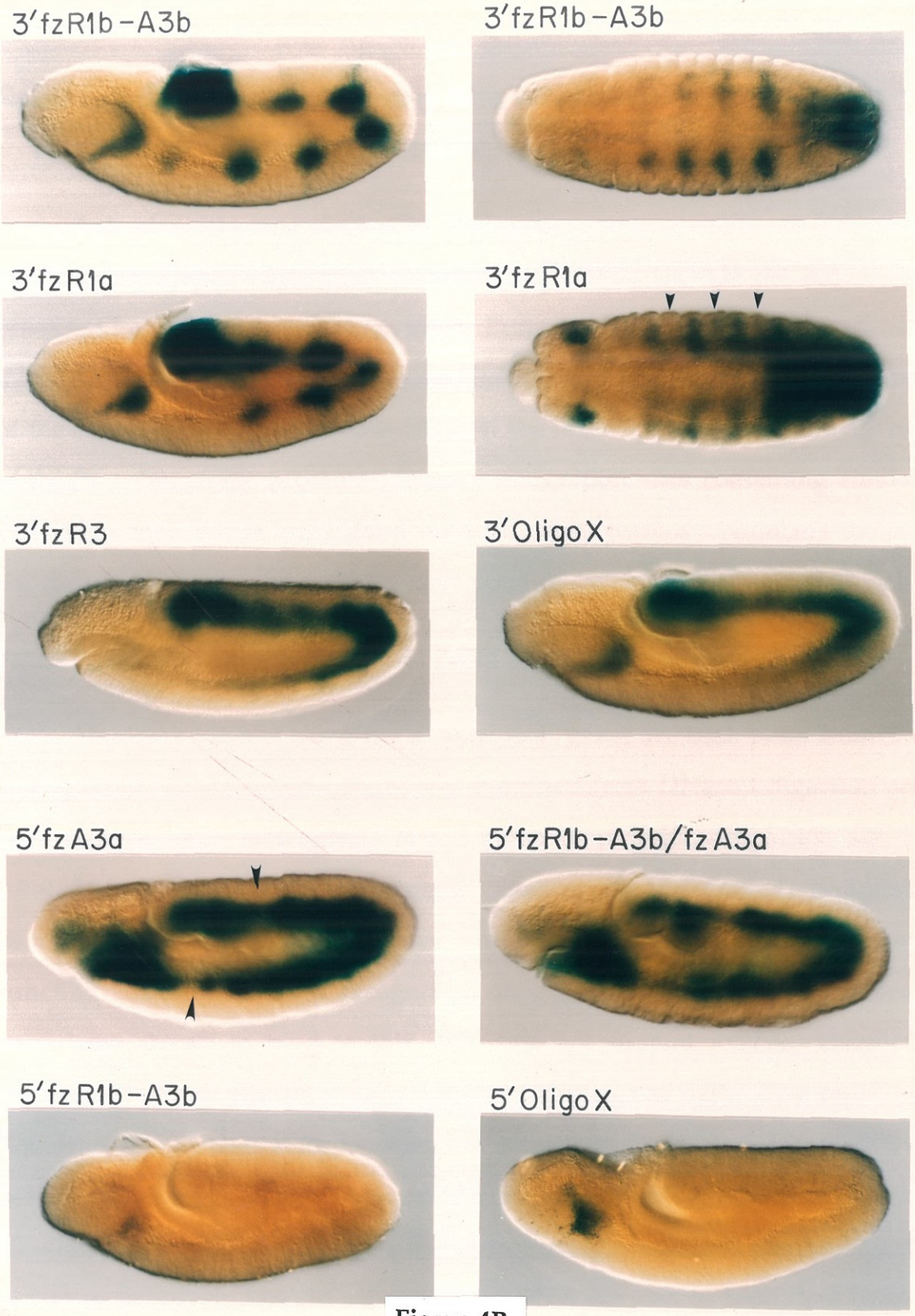

3'fzR1b-A3b
3'fzR1b-A3b
3'fzR1a
3'fzR1a
3'fzR3
3'OligoX
5'fzA3a
5'fzR1b-A3b/fzA3a
5'fzR1b-A3b
5'OligoX

Figure 4B.

Figure Legends

Figure 1. Location of Protein Binding Sites with Activator and/or Repressor Function in the *ftz* Zebra Stripe Promoter

Activator sites are indicated by oval shapes; repressor sites are indicated by diamond shapes. The protein binding sites listed include the sequences protected by DNase I footprint reactions. Protected regions on both strands are included. Lines and arrows above and below sequences indicate the location of the conserved DNA motifs described in the text. In the schematic diagram of the *ftz* zebra stripe promoter, protein recognition sites containing similar DNA sequence motifs are shaded with the same pattern. Numbers denote the distance in base pairs from the start point of transcription.

Figure 2. DNase I Protection of the *ftz* Zebra Stripe Promoter

Numbers located along the side of each panel are base pairs from the start point of transcription. Sequences protected from DNase I digestion are indicated by open rectangles. Name given to protein recognition site is located on the right side of rectangle. Asterisk(*) and cross(†) designate protein recognition sites that have not been correlated with regulatory function. *Homeodomain recognition site; *ftz*, *eve* and *engrailed* proteins have been shown to bind to this site (data not shown). G/A: chemical cleavage at purine residues. C/T: chemical cleavage at pyrimidine residues. EN refers to 0-12 hour embryonic nuclear extract used in DNase I footprinting reactions. GAGA factor refers to the purified "GAGA" binding protein (Soeller, et al. 1988). Sepharose ZE refers to protein that was purified by its ability to selectively bind to zebra element DNA coupled to Sepharose beads.

Numbers above each lane refer to the amount of protein added to the footprint reactions, given in microliters. Arrow within the fzR2 site indicates location of a DNase I hypersensitive site.

Methods Embryonic nuclear extracts were made as described in Chapter 2. Footprint reactions were performed as described in Appendix 1. Promoter fragments used for footprint reactions were derived from deletion constructs described in Chapter 2 and this paper. First Panel: 5'Δ-458; Second Panel: 5'Δ-359; Third Panel: 5'Δ-322. The plasmid DNA was labeled by DNA polymerase I (Klenow fragment) filling, at the Hind III linker site which was ligated to the deletion endpoint. The DNA was subsequently cut at the Eco RI site located near the *ftz* promoter/*lacZ* fusion site. The labeled fragment was purified following agarose gel electrophoreses. The zebra element-Sepharose material was prepared as described in Wiederrecht, et al. (1987). The zebra element DNA was derived from 5'Δ-535 DNA cut with Hind III and Eco RI. One milligram of fragment was gel purified following agarose gel electrophoresis and coupled to Sepharose beads. Protein concentration of EN: 20 mg/ml. Protein concentration of Sepharose ZE: 1 mg/ml (20-fold purification of *ftz* DNA binding proteins). Protein concentration of "GAGA" factor: less than 10 μg/ml.

Figure 3A. *ftz/lac Z* Deletion Constructs Used for Transformation

(TOP) Schematic of A3-R1 regulatory region described in Chapter 2. The TATA homology is at -26 bp, and the start point of translation is at +70 bp. The constructs are inserted into Carnegie 20, and named such that the number following the Δ indicates the deletion endpoint from the start point of transcription.

Methods The procedure for generating 5' and 3' deletion constructs is described in Chapter 2. Internal deletion constructs were generated by ligating XbaI-Hind III fragments from the 3' deletion series into the Xba I and Hind III sites in the polylinker of the 5' deletion series. Polylinker DNA was inserted between the 5' and 3' endpoints in the ΔfzR1b-A3b construct to restore the spacing to that of the endogenous promoter. Only 4 out of 21 base pairs in that region were conserved relative to the endogenous protein recognition site.

Figure 3B. *ftz/lacZ* Expression of Zebra Stripe Deletion Constructs

Localization of ß-galactosidase expression is displayed in whole-mount transformant embryos that have developed to the germ-band extension stage (stages 8-11). Located above each embryo is the name of the injected construct. A detailed description of the expression patterns is given in the text. Arrows on the 3'Δ-272 embryo indicate the location of the seven stripes which are superimposed on continuous band of ß-galactosidase expression.

Methods P element-mediated transformation procedure, X-Gal staining of embryos, and photography were performed as described in Chapter 2. To avoid the difficulties that may arise due to P element insertional position effects (i.e., ectopic expression or low levels of activity) eight independent transformant lines were established for each construct. The expression pattern of each transformant line was determined by ß-galactosidase staining of embryos. Since ß-galactosidase activity is more stable than *ftz* transcripts and protein, the expression patterns generated by fusion genes in cellular blastoderm embryos can be observed most easily after the ß-galactosidase

protein has accumulated in the embryo, at the germ band extension stage and later.

Figure 4A. *ftz/lacZ* Oligonucleotide Constructs Used for Transformation

Oligonucleotides were synthesized that contain either one (fzR3, fzA3a) or two (fzR1a, fzR1b-A3b, fzR3) copies of protein recognition sequences found in the endogenous *ftz* promoter. Several base pairs located outside the protected region were included on each end of the oligonucleotides. All the oligonucleotides in the reconstructed promoters were directionally inserted so that the recognition sites are in the orientation found in the endogenous *ftz* promoter. Two copies of the double sites and three copies of the single sites were inserted into either 3'Δ-298 (repression assay) or 5'Δ-40 (activation assay). Therefore, either three or four copies of each recognition element were placed in the constructs, depending on their size (three copies for sites over 35 bp, four copies for all others). The designation for promoter constructs used in the repression assay include a "3'" before the synthetic site name; constructs used in the activation assay include a "5'" before the synthetic site name.

Methods In order to directionally insert oligonucleotides into deletion constructs, a Hind III site was placed on the 5' end of each oligonucleotide, and a Hind III overhang was placed on the 3' end; ligations were conducted in the presence of excess amount of Hind III. All oligonucleotide constructs were sequenced to confirm that the proper recognition sites were inserted into the truncated *ftz* promoter regions. The oligonucleotides used have the following sequences:

Synthetic Oligonucleotides

fzA3a

```
5'-AGCTTGAGATCGGCTGAGAGTCGCGCCCTCTCGCTCTGCGCACCTCATAGGTAGGC
       ACTCTAGCCGACTCTCAGCGCGGGAGAGCGAGACGCGTGGAGTATCCATCCGTCGA
```

fzR1a

```
5'-AGCTTCATGGCCGTAATTACTGCAGCACCTCATGGCCGTAATTACTGCAGCACC
       AGTACCGGCATTAATGACGTCGTGGAGTACCGGCATTAATGACGTCGTGGTCGA
```

fzR1b-A3b

```
5'-ACGTTGCACCGTCTCAAGGTCGCCGAGTAGGAGAAGCGCACCGTCTCAAG
       ACGTGGCAGAGTTCCAGCGGCTCATCCTCTTCGCGTGGCAGAGTTC

                        GTCGCCGAGTAGGAGAAGC
                        CAGCGGCTCATCCTCTTCGTCGA
```

fzR3

```
5'-AGCTTGCACCGTACGGATAAAGTTGCCAGGACCTCGGATAACTTCCCCTCTCC
       ACGTGGCATGCCTATTTCAACGGTCCTGGAGCCTATTGAAGGGGAGAGGTCGA
```

Oligo X

```
5'-AGCTTGCACCCTCAACCCTAACGGTAGTAGGAGAAGCGCACCCTCAACCC
       ACGTGGGAGTTGGGATTGCCATCATCCTCCTCGCGTGGGAGTTGGG

                        TAACGGTAGTAGGAGAAGC
                        ATTGCCATCATCCTCTTCGTCGA
```

Figure 4B. *ftz/lacZ* Expression of Oligonucleotide Constructs

The localization of ß-galactosidase expression in whole-mount transformant embryos is displayed. Both germ band extended (left) and shortened (right) embryos are included for the 3'fzR1b-A3b and the 3'fzR1a constructs. Located above each embryo is the name of the injected construct. A detailed description of expression patterns is given in the text. Arrows on the 3'fzR1a germ band shortened embryo indicate the expression of ß-galactosidase in the interstripe regions; arrows on the 5'fzA3a embryo indicate regions where there are relatively low levels of ß-galactosidase activity.

Methods P element-mediated transformation, X-Gal staining of embryos, and photography were performed as described in Chapter 2.

APPENDICES TO CHAPTER 3

Appendix 3a: Table of ß-galactosidase Quantitation Data

Appendix 3b: Results from Additional 5' and 3' Deletion Constructs

Expression of *ftz/lac Z* Constructs

Construct	Number of Transformant Lines Quantitated	Units of β-Galactosidase Activity[1]		Level of Derepression
		Mean ± S.D.	Range	
5'Δ -669	5	914 ± 157	744 - 1131	None
5'Δ -535	5	824 ± 255	576 - 1193	None
5'Δ -458	4	544 ± 219	343 - 847	None
5'Δ -359	5	225 ± 66	139 - 315	None
5'Δ -322	3	303 ± 109	178 - 373	None
5'Δ -297	3	283 ± 67	241 - 364	Very Slight
5'Δ -276	3	112 ± 19	91 - 127	Mild
5'Δ -239	5	215 ± 42	174 - 263	Moderate
5'Δ -185	5	119 ± 26	86 - 145	Moderate
5'Δ -157	4	111 ± 36	70 - 158	Moderate/Strong
5'Δ -102	4	42 ± 35	8 - 87	Strong
5'Δ -56	4	21 ± 11	7 - 34	N.A.[2]
5'Δ -40	5	8 ± 9	2 - 18	N.A.[2]
3'Δ -36	4	520 ± 90	457 - 650	None
3'Δ -112	3	449 ± 109	323 - 519	None
3'Δ -163	4	371 ± 132	180 - 483	Very Slight

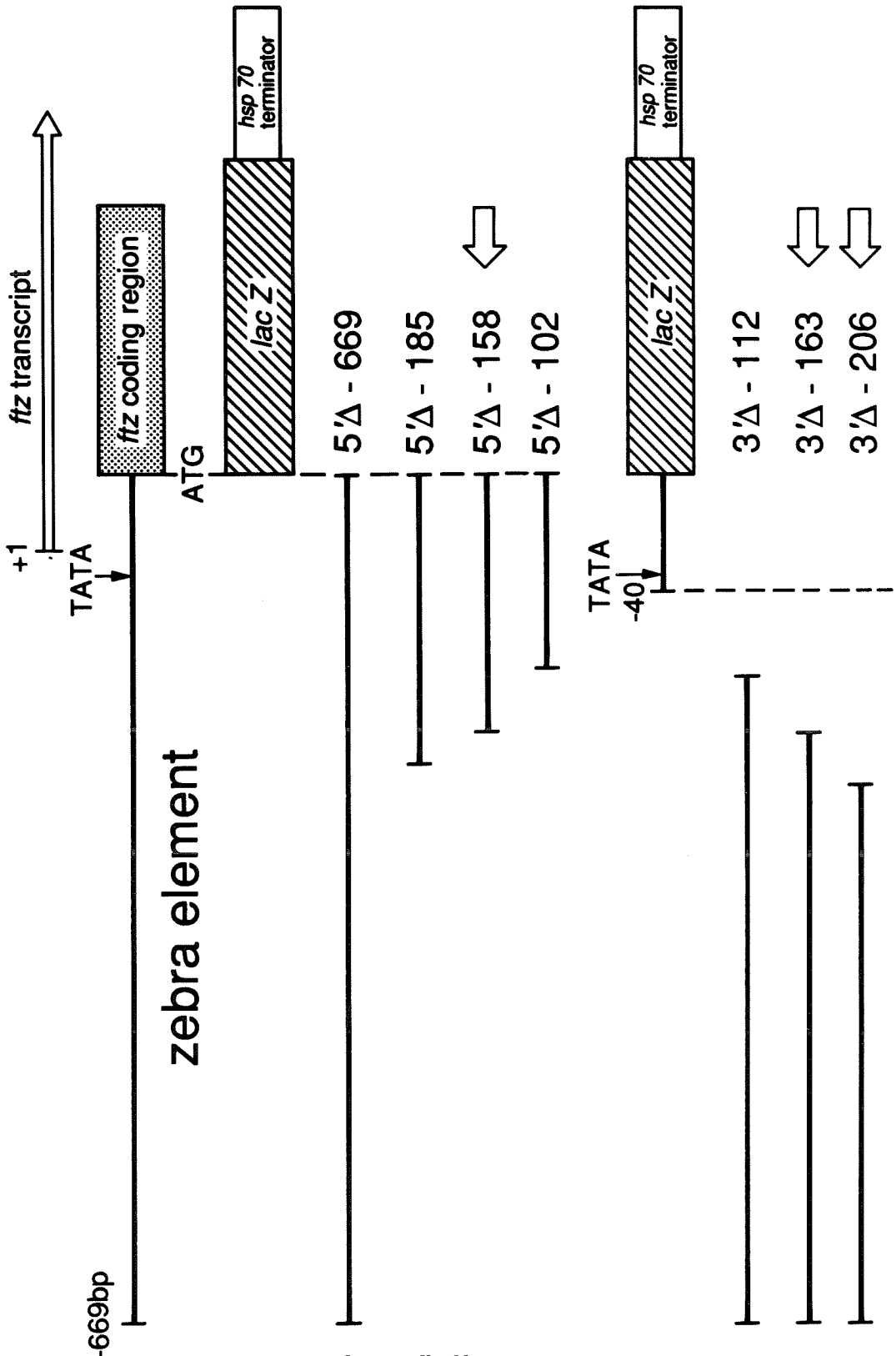
Expression of *ftz/lac Z* Constructs

Construct	Number of Transformant Lines Quantitated	Units of β-Galactosidase Activity[1]		Level of Derepression
		Mean ± S.D.	Range	
3'Δ -172	5	412 ± 52	337 - 463	Mild
3'Δ -206	5	248 ± 89	167 - 398	Moderate
3'Δ -222	5	264 ± 112	141 - 415	Moderate
3'Δ -272	5	391 ± 242	119 - 635	Moderate
3'Δ -298	4	256 ± 100	133 - 342	Strong
3'Δ -318	3	226 ± 157	87 - 397	Strong
3'Δ -347	5	97 ± 46	31 - 158	Strong
3'Δ -386	4	111 ± 64	30 - 184	Strong
3'Δ -424	4	66 ± 22	37 - 91	N.A.[2]
3'Δ -482	5	46 ± 16	25 - 67	N.A.[2]
3'Δ -576	3	5 ± 9	0 - 16	N.A.[2]
*hsp 70*L Δ -36	4	176 ± 25	142 - 204	None
EΔ -669	3	1566 ± 347	1174 - 1831	---
EΔ -40	5	110 ± 49	43 - 160	---

Expression of *ftz/lac Z* Constructs

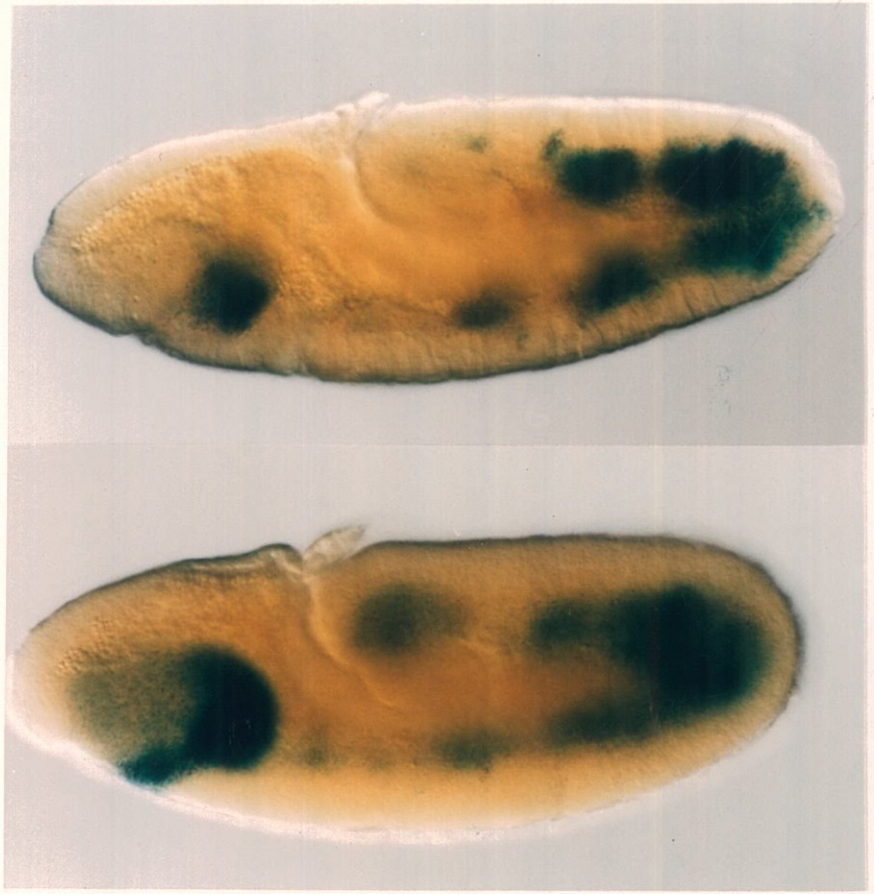
Construct	Number of Transformant Lines Quantitated	Units of β-Galactosidase Activity[1]		Level of Derepression
		Mean ± S.D.	Range	
ΔfzR1b - A3b	4	531 ± 128	379 - 689	---
5'ΔfzA3a	4	197 ± 125	98 - 358	---
5'Δ Oligo X	3	60 ± 14	45 - 73	---

[1]Each unit of β-galactosidase activity is equivalent to one $O.D._{574}$ unit per mg protein x 10^2. The background value for untransformed *ry* embryos was subtracted from each value. The value for each transformant line was obtained from measurement of two separate egg collections.
[2]N.A. indicates that derepression could not be assayed in these transformant lines due to the low levels of β-galactosidase activity.

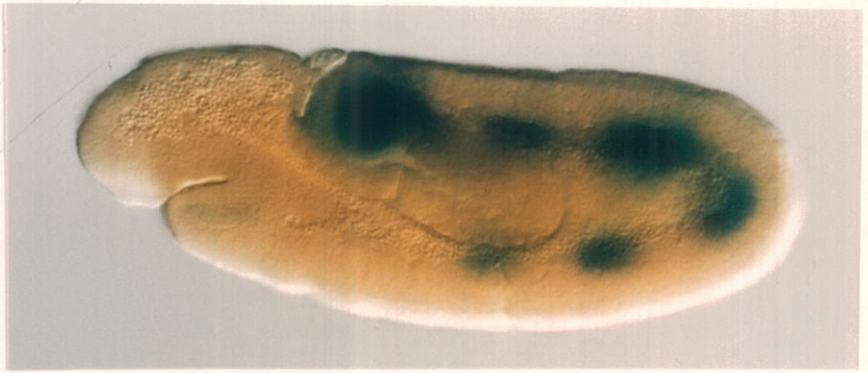

ftz transcript
+1
TATA
ftz coding region
-669bp
zebra element
ATG
hsp 70 terminator
lac Z
5'Δ - 669
5'Δ - 185
5'Δ - 158
5'Δ - 102
hsp 70 terminator
lac Z
TATA
-40
3'Δ - 112
3'Δ - 163
3'Δ - 206

Appendix 3b

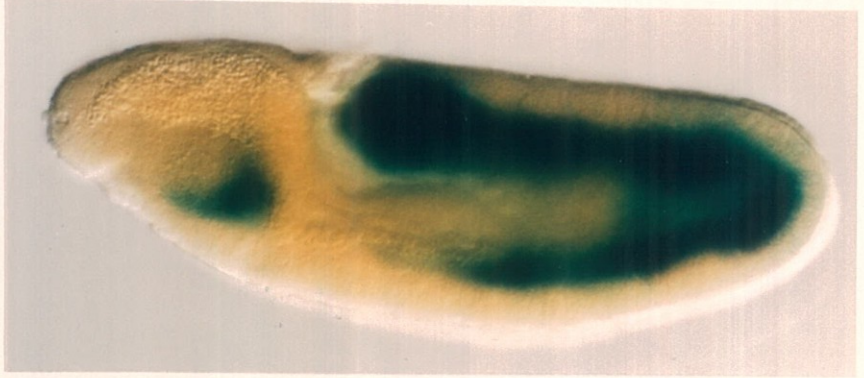
5′Δ −158



3′Δ −163



3′Δ −206



Appendix 3b

Figure Legend for Appendix 3b

The effects of removing fzR3 from the 5' direction can be seen when embryos transformed with the 5'Δ-158 construct (Appendix 3b) are compared to those transformed with 5'Δ-185 (Chapter 2, Figure 3I). Although varying amounts of repression can be observed in the ß-galactosidase pattern of embryos transformed with the 5'Δ-158 construct (which lacks the fzR3 site but retains the fzR4-A4 repressor site), the stripes are clearly less resolved than those generated by the 5'Δ-185 construct (which retains fzR3). This result indicates that fzR3 may function as a repressor binding site.

This notion is more clearly illustrated with the 3' deletion constructs. The expression pattern in embryos transformed with the 3' deletion construct which retains the fzR3 site but has lost the fzR4-A4 repressor site (3'Δ-163), display some loss of repression; however, a clear increase in staining throughout the presumptive mesoderm (most notably in the interbands) can be seen in the embryos transformed with the construct that has lost the fzR3 site in addition to fzA4-R4 (3'Δ-206). This observation strongly suggests that fzR3 functions as a repressor site in the endogenous *ftz* promoter. (These results also support the notion that the fzR4-A4 site is a *cis*-acting repressor.)

Methods Construction of the deletion fusion genes, the transformation procedure, X-Gal staining procedure and photography as described in Chapter 2.