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MOLECULAR MECHANISMS UNDERLYING THE REGULATION OF THE *DROSOPHILA E93* GENE BY ECDYSONE AND βFTZ-F1

by

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under the direction of
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for twelve credits

To,
Mamu and Daddy

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ABSTRACT

Steroid hormones play a critical role in the regulation of physiological and developmental processes in eukaryotes. *Drosophila melanogaster* is an ideal system for the study of steroid hormone action because it has only one known physiologically active steroid hormone: 20-hydroxyecdysone. During metamorphosis, ecdysone directs morphogenesis as well as programmed cell death. It regulates the expression of a set of genes called the early genes that, in turn, regulate the transcription of other genes important in metamorphosis called the late genes. In larval salivary glands, βFTZ-F1, a nuclear hormone receptor, provides *E93*, an early gene, with the competence to respond to the high ecdysone titer in the late prepupal stage. Preliminary studies have indicated that βFTZ-F1 binds to the 3'-end of the first intron of *E93*.

This project delves into the molecular mechanisms by which *βFTZ-F1* and ecdysone regulate the expression of the *E93* gene in *Drosophila*. It aims to map βFTZ-F1 binding site(s) in *E93*. In order to achieve this goal, the *βFTZ-F1* open reading frame was cloned into a pET42a vector. The recombinant plasmid was used to transform competent bacterial expression cells. Protein expression was successfully induced in these bacterial cells and a purer form of βFTZ-F1 was obtained. Gel-shift assays with βFTZ-F1 and a consensus βFTZ-F1 binding sequence was carried out. Gel-shift assays with βFTZ-F1 and *E93* are to be conducted in the near future to determine the exact DNA sequence of the βFTZ-F1 binding site(s) in *E93*.

INTRODUCTION

The Study of Steroid Hormones and the Model Organism

Steroid hormones regulate vital developmental and physiological processes in higher organisms, including humans. The life processes directed by steroid hormones include development of secondary sex characteristics, reproduction, metabolism, metamorphosis and homeostasis. The complex machinery involved in the regulation of steroid hormone action has been studied for decades. This project aims to provide a better understanding of the molecular mechanisms of steroid hormone action during animal development.

Drosophila melanogaster is an ideal system for the study of steroid hormone action. It has only one physiologically active steroid hormone known as 20-hydroxyecdysone (hereafter referred to as ecdysone). The short life cycle of the fruitfly and its well-characterized genome, which enables relatively easy genetic manipulations, work to the advantage of researchers using this model organism.

The life cycle of the fruitfly is about 10 to 12 days (Figure 1). The cycle starts in the embryonic stage. The embryo develops into a feeding larva in a day. The animal molts twice in the larval stage as it increases in size from the first instar to the second and finally to the third. It then undergoes puparium formation, when the larva transforms into a stationary prepupa. The prepupa then develops into a pupa and finally emerges as an adult fly.

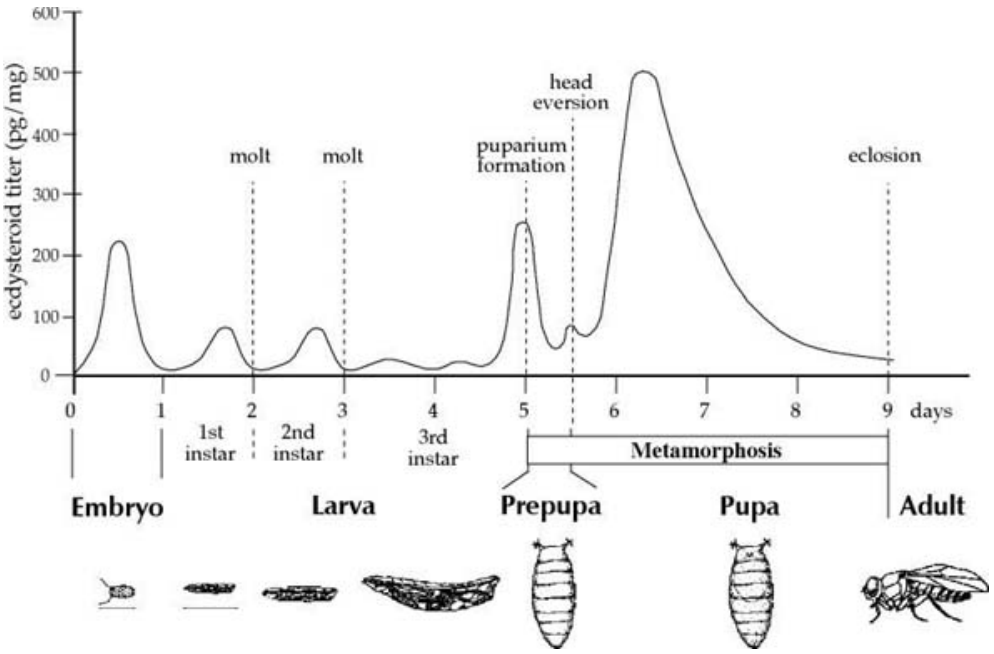

Figure 1: Ecdysone levels during the life cycle of *Drosophila melanogaster*. The morphologically distinct stages in the life cycle, including the embryo, larva, prepupa, pupa and adult, are shown in the horizontal axis in number of days from embryogenesis to the respective stages. The vertical axis shows the ecdysone titer in pg/mg. The ecdysone pulses, or high titers, during the life cycle are shown in the graph. Various landmarks during the development process, including larval molts, puparium formation, head eversion, eclosion and metamorphosis, are labeled. The two ecdysone pulses at days 5 and 5.5 are focused in this project. (Thummel, 2001a)

Puparium formation marks the beginning of metamorphosis, which is defined as the transformation from the larva to the adult. Metamorphosis, which is directed by ecdysone, involves the death of larval tissues and simultaneous differentiation and morphogenesis of adult tissues from imaginal discs.

Steroid Hormone Action

Steroid hormones are characterized by a typical sterol nucleus based on cholesterol (Figure 2a) (Mangelsdorf *et al.,* 1995). They are hydrophobic molecules made up of three six-membered rings and a five-membered ring (Considine, 1984). According to the conventional theory of steroid hormone action, the lipophilic nature of steroid hormones enables them to diffuse through the phospholipid bilayer and bind to intracellular receptors (Yamamoto, 1985). Ligand-binding changes the conformation of the receptors thus activating them. The activated steroid hormone receptors (SHRs) consequently bind to palindromic DNA sequences called Hormone Response Elements (HREs). They interact with transcription initiation factors and alter the transcriptional level of the target genes (Aranda and Pascaul, 2001; Beato *et al.,* 1995).

The SHRs make up a group of receptors that falls under the Nuclear Receptor superfamily. The nuclear receptors (NRs) are made up of five domains, which alternate between variable and conserved (Figure 2b). The amino-terminal variable region, (domain A/B) consists of the ligand-independent transactivation factor (AF-1). This domain is followed by a conserved 66-amino acid-long DNA-

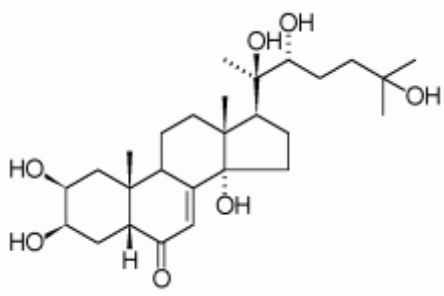

Figure 2a: Structure of 20-hydroxyecdysone. The characteristic sterol nucleus made up of three six-membered rings and one five-membered ring is shown along with substituents that make up the ecdysone molecule.

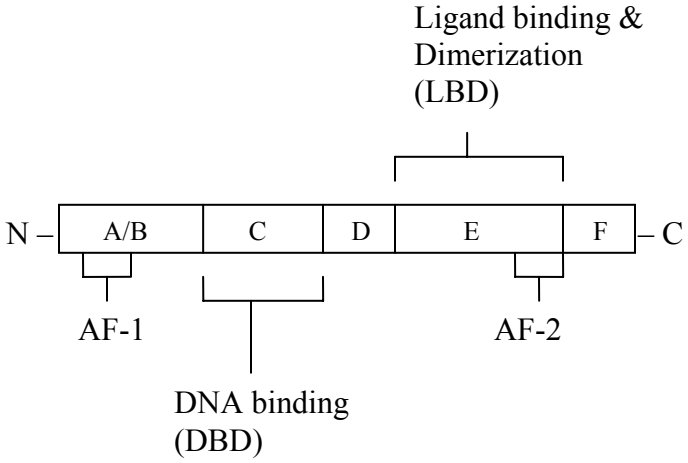

Figure 2b: Structural representation of a nuclear hormone receptor. Domains A/B, D and F are variable while domains C and E are conserved. The variable linker region D connects the C domain, which binds to DNA, with domain E, which contains the dimerization surface and the ligand-binding domain. The N-terminal A/B region contains the ligand-independent transactivation domain AF-1 while the ligand-dependent transactivation domain AF-2 lies in the carboxy-end of the conserved E domain.

binding domain (DBD, domain C). The DBD of the NRs is made up of two zinc fingers, which distinguish NRs from other DNA-binding proteins. The DBD binds to the core consensus sequence, AGGTCA, of the HREs (Horner *et al.,* 1995; Mangelsdorf *et al.,* 1995). The sequence of the HREs determines the specificity of the NR towards its target gene. The second conserved domain of the NRs (domain E) contains a 225-amino acid-long ligand-binding domain (LBD), which determines the specificity of the receptor towards the ligand (Mangelsdorf and Evans, 1995; Mangelsdorf *et al.,* 1995). The DBD and the LBD are separated by a variable linker domain (domain D). A highly conserved ligand-dependent transactivation factor (AF-2) is present in the carboxy-terminal of the LBD. The carboxy-terminal of the NRs contains the third and last variable region (domain F).

The categorization of the nuclear receptor superfamily into subfamilies remains controversial. Chowla *et al.* (2001) classified the nuclear hormone receptors into two distinct divisions based on the pathways involved in ligand availability (Figure 3). The first group includes classic nuclear steroid hormone receptors (SHRs), such as the glucocorticoid (GR), mineralocorticoid (MR), estrogen (ER), androgen (AR), and progesterone (PR) receptors. These receptors bind to their ligands in the cytoplasm and form homodimers that pass through the nuclear membrane into the nucleus in order to bind to the HREs (Figure 3a). The second group constitutes adopted orphan nuclear receptors, which are receptors that were previously characterized as orphan, but several have then been shown

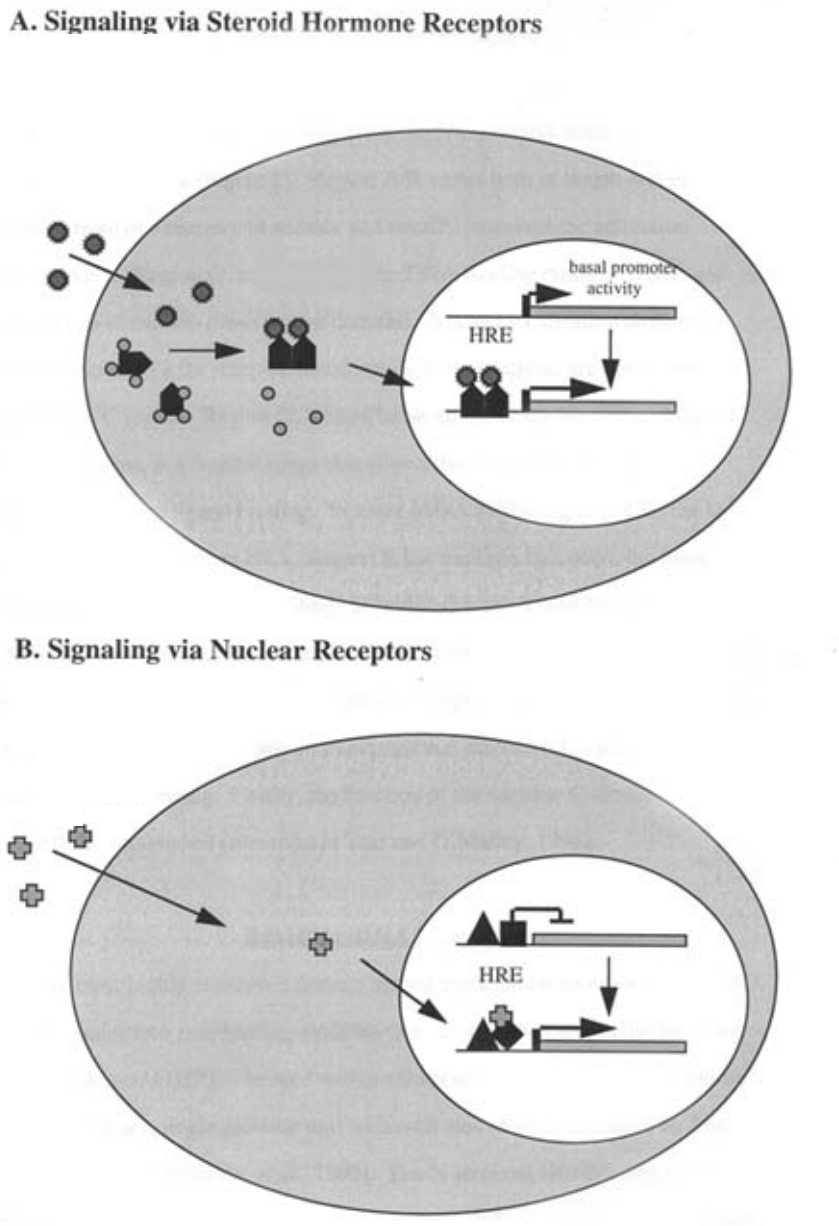

Figure 3: Signaling via Steroid Hormone Receptors (SHRs) and Nuclear Receptors (NRs). The receptor dimers bind to Hormone Response Elements (HREs) in the nucleus and activate transcription of target genes. A). SHRs are present in the cytoplasm and only enter the nucleus as homodimers after binding to their ligands. B). NRs are found as heterodimers bound to HREs. They repress transcription in the absence of their ligands while ligand-binding activates them.

to bind to physiological ligands. The members of this group form functional heterodimers with retinoid X receptors (RXRs). The group includes receptors for fatty acids (PPARs), oxysterols (LXRs), bile acids (FXRs) and xenobiotics [steroid xenobiotic receptor/pregnane X receptor (SXR/PXR) and constitutive androstane receptor (CAR)]. These receptors behave as lipid sensors in maintaining homeostasis by feedforward or feedback cascades. Additionally, four other members of this group of nuclear receptors, the thyroid hormone (TR), retinoic acid (RAR), vitamin D (VDR) and ecdysone (EcR) receptors, heterodimerize with RXRs but do not behave as lipid sensors (Chowla *et al.,* 2001).

The RXRs are localized in the nucleus and form heterodimers that bind to DNA to regulate transcription of target genes (Figure 3b). The dimerization of receptors occurs in two steps. The two receptors first bind to each other with their LBDs and then bind to the DNA using their DBDs (Mangelsdorf and Evans, 1995). The heterodimeric RXRs do not always need ligands for their activation however, for several of the RXR heterodimers ligand-binding is essential for activation (Aranda and Pascaul, 2001).

Ecdysone acts as a ligand for a heterodimer of two nuclear receptors: the ecdysone receptor (EcR), and the insect RXR homolog, Ultraspiracle (USP). The EcR-USP complex binds to the ecdysone-response element, Hsp27-EcRE, to activate a hierarchical transcriptional cascade for the regulation of various life processes in insects (Horner *et al.,* 1995; Koelle *et al.,* 1991; Riddiford, 1993;

Yao *et al.,* 1993). Ecdysone acts as an allosteric effector for the physical association of the two receptors. Conversely, heterodimerization of EcR-USP is required for high-affinity binding of ecdysone (Yao *et al.,* 1993).

A 2.24Å structure of the EcR-USP DBD bound to its substrate shows that the receptor complex binds to the minor groove of the DNA (Devarakonda *et al.,* 2003). The heterodimer binding site is highly degenerate and pseudo-palindromic, containing inverted repeats of 5'-AGGTCA-3' separated by 1 bp. A 2.60Å structure of the EcR-RXR, the mammalian homolog of USP, showed the same DNA-binding interface as that of EcR-USP (Devarakonda *et al.,* 2003).

The ecdysone receptor (EcR) has three isoforms in *Drosophila*. They are EcR-A, EcR-B1 and EcR-B2. (Talbot *et al.,* 1993; Yao *et al.,* 1993). The isoforms are highly homologous in the DNA- and ligand-binding domains but differ in the amino-terminal transactivation domain (AF-1). EcR-A is highly expressed in adult tissues that differentiate in response to ecdysone, whereas tissues that undergo programmed cell death in response to the steroid hormone predominantly express EcR-B1 (Thummel, 1995). The expression of the third isoform, EcR-B2, is understood less well. Nevertheless, Cherbas *et al.,* (2003) show that the isoform is expressed in the larval fat bodies and epidermis. Additionally, EcR-B2, in association with EcR-B1, plays important roles in larval molting as well as the remodeling of the nervous system during metamorphosis (Schubinger *et al.,* 1998). All three isoforms can weakly bind to ecdysone as monomers and

heterodimerize with USP. However, ligand-binding in all three cases is greatly stimulated by receptor heterodimerization.

The Ashburner Model: Hierarchical Gene Regulation by Ecdysone

Steroid signaling has been extensively studied in *Drosophila* salivary glands with the use of larval salivary gland giant polytene chromosomes. When treated with ecdysone, the polytene chromosomes undergo chromatin decondensation to form puffs, which indicate active transcription. The model designed by Ashburner (1974) shows a wave of puff formation when treated with ecdysone. Thus, it demonstrates a transcriptional hierarchy induced by ecdysone (Figure 4) (Ashburner, 1972; Clever, 1964; Richards, 1976a; Richards, 1976b).

Ashburner (1967) was first to observe two time points at which puffing activity peaks in response to ecdysone. One peak occurs in the late larval stage while the other occurs in the late prepupal stage. Each puffing peak consists of two groups of puffs. The first group comprises about six puffs that readily occur in response to ecdysone. These puffs occur at positions 74EF, 74B and 2B5, which correspond to the *E74*, *E75* and the *Broad Complex* (*BR-C*) genes (Ashburner *et al.,* 1974; Burtis *et al.,* 1990; Segraves and Hogness, 1990). These genes, which correspond to the puffing loci that are directly induced by ecdysone, are called the 'early' genes. The second group of puffs during a puffing peak consists of about a hundred puffs that correspond to 'late' genes. These puffs are formed a few hours after a high ecdysone titer and are induced by the protein

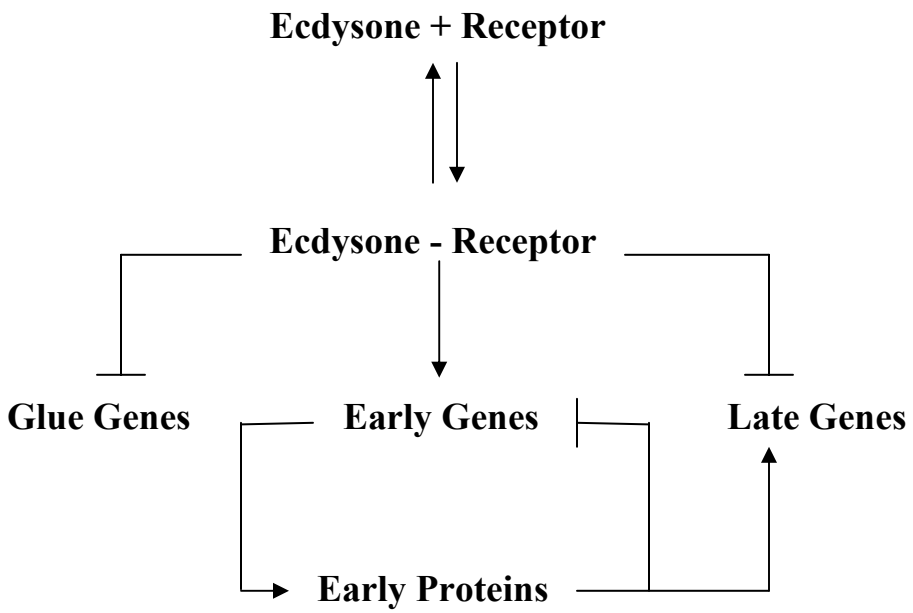

Figure 4: Transcriptional hierarchy induced by ecdysone. Ecdysone binds to its receptor forming a complex that is responsible for the activation of early genes and the repression of the late and the glue genes. The protein products of the glue genes are secreted by the larval salivary glands in order to affix the animal to a solid surface for puparium formation. The protein products of the early genes activate the late genes while repressing their own expression (Ashburner, 1974).

products of the early genes (Ashburner *et al.,* 1974). Inhibition of protein synthesis after early puff formation not only increases the duration of the puffs at the early gene loci, but also results in the absence of the late puffs. These observations indicate that the protein products of the early genes repress their own expression and induce the expression of late genes (Figure 4) (Ashburner, 1974; Walker and Ashburner, 1981). Furthermore, the removal of ecdysone causes the early puffs to regress prematurely (Ashburner *et al.,* 1974). The regression of the early puffs is due to low levels of the ecdysone receptor. The expression of the ecdysone receptor is induced by ecdysone, in the absence of which, the heterodimeric receptor complex dissociates due to a decrease in stability (Yao *et al.,* 1993). The removal of ecdysone also causes premature induction of late gene puffs, which indicates that the presence of ecdysone represses the late genes to inhibit their premature expression during the high ecdysone titer (Ashburner and Richards, 1976).

In the larval salivary glands the late larval as well as the late prepupal pulse of ecdysone induce the same set of early puffs, with one exception. The 93F early puff is stage- and tissue-specific and is induced in the larval salivary glands only by the late prepupal ecdysone pulse (Richards, 1976b). The puff at the 93F locus is one of the four largest during the 10-12 hour prepupal stage (Ashburner, 1967) (Figure 5).

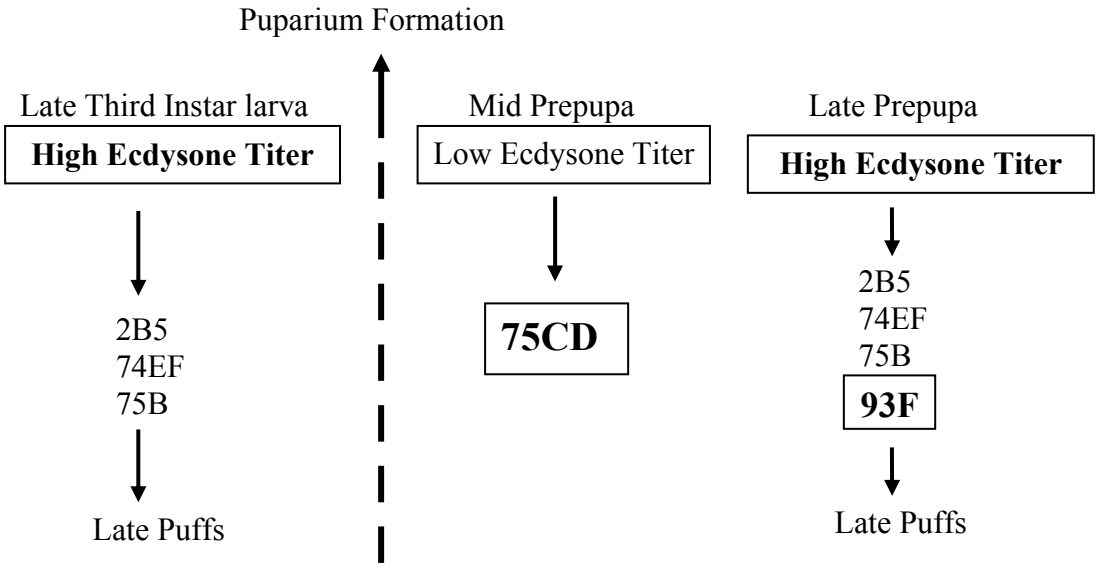

Figure 5: Puff formation in response to ecdysone in *Drosophila* salivary gland polytene chromosomes. In early metamorphosis, high ecdysone titers give rise to two puffing peaks at late larval and late prepupal stages. At each peak two groups of puffs are observed. The first group comprises of early puffs: 2B5, 74EF and 75B that correspond to the early genes *BR-C*, *E74A* and *E75A* respectively. The second group of puffs corresponds to the late genes. During the second puffing peak, a puff at 93F is observed in addition to the same group of early puffs that were present in the late larval stage. In between the two puffing peaks, a new set of puffs is observed. The 75CD locus, which corresponds to *βFTZ-F1*, shows a puff in the mid-prepupal stage at the time of low ecdysone titer.

Six to eight hours after puparium formation, or during the mid-prepupal stage that occurs between the two ecdysone pulses, a distinct set of puffs are observed. During this period of low ecdysone titer, the 75CD puff locus, which corresponds to *βFTZ-F1*, is induced (Ashburner, 1967; Richards, 1976a). Hence, *βFTZ-F1* is expressed in the larval salivary glands between the late larval and late prepupal pulses of ecdysone. Furthermore, βFTZ-F1 has been shown to bind to the 75CD locus by immunohistochemistry, suggesting that *βFTZ-F1* autoregulates its expression during the short expression period (Lavorgna *et al.,* 1993).

Gene Regulation by Ecdysone during *Drosophila* Metamorphosis

Pulses of ecdysone regulate important transitions in the development of *Drosophila*, including molting, cell death and differentiation. In support of Ashburner's model, molecular studies have shown that a high titer of ecdysone starts a cascade of hierarchical activation of genes that result in stage- and tissue-specific responses (Andres and Thummel, 1992; Huet *et al.,* 1993; Huet *et al.,* 1995; Li and White, 2003; Thummel 1996; Urness and Thummel, 1990; 1995).

Two ecdysone pulses are critical in early metamorphosis (Figure 6). The first of these high ecdysone pulses occurs at the end of the late third instar larval stage. This late larval pulse induces developmental events in the animal that mark the beginning of metamorphosis (Richards, 1981). The larval salivary glands are induced to secrete glue proteins. The animal shortens its body and the larval cuticle hardens to form a protective pupal case. Adult imaginal discs start

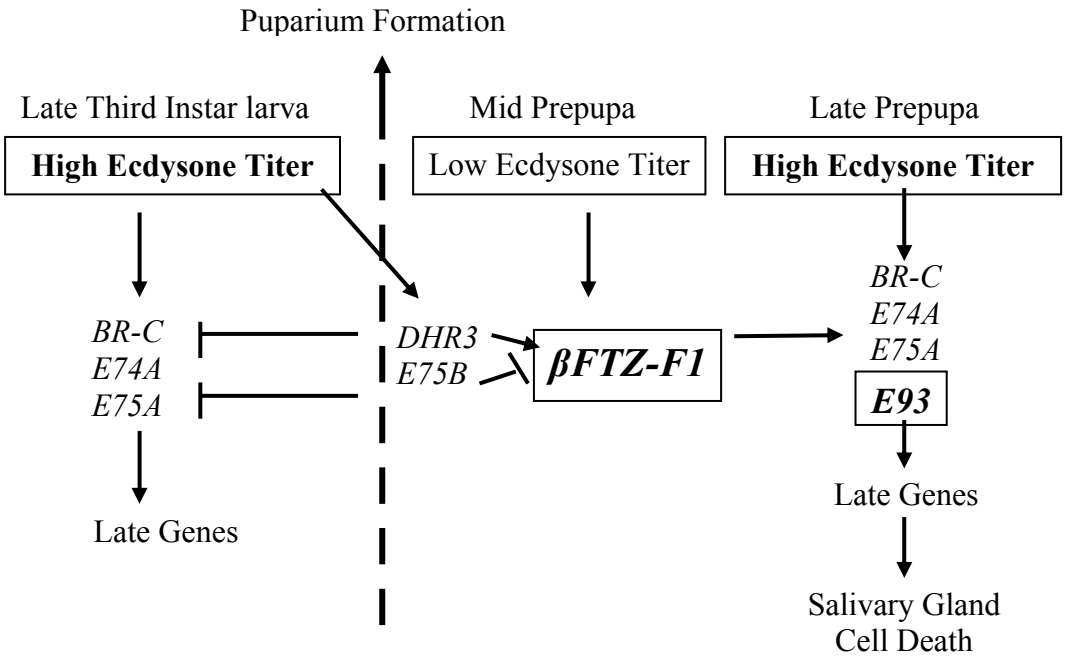

Figure 6: Gene regulation by ecdysone in larval salivary glands: The level of ecdysone is high in the late third instar larval stage and decreases to very low levels in the mid-prepupal stage. The titer increases again 10-12 hours APF at the late prepupal stage. The pulses of ecdysone activate the transcription of early genes such as *BR-C, E74A* and *E75A.* The early genes in turn activate the late genes. The expression of the early gene *E93* is stage- and tissue-specific. It is activated only by the late prepupal high ecdysone titer in the larval salivary glands. *E93* activates the cell death machinery and hence only the late prepupal ecdysone pulse initiates salivary gland destruction. During the low ecdysone titer *βFTZ-F1*, which is repressed by ecdysone, is expressed. The late-early genes, *DHR3* and *E75B* are directly regulated by ecdysone and in turn regulate the transcription of *βFTZ-F1*, which provides *E93* with the competence to respond the late prepupal ecdysone signal.

differentiating into legs and wings. The larval midgut undergoes programmed cell death while the adult midgut epithelium is formed. (Lee *et al.,* 2002a; Thummel, 2001a). The concentration of ecdysone drops to very low levels in the mid-prepupal stage, 6-8 hours after puparium formation (APF). *βFTZ-F1*, which is repressed by ecdysone, is activated in this period in the larval salivary glands. The ecdysone titer rises again at 10-12 hours APF in the late prepupal stage (Figure 6) (Handler, 1982; Sliter and Gilbert, 1992). The late-prepupal pulse of ecdysone directs head eversion and final leg and wing extension, marking prepupal-to-pupal transition. The larval salivary glands undergo programmed cell death in response to the second ecdysone pulse during metamorphosis (Figure 6).

A pulse of ecdysone directly activates a set of early genes. Ecdysone with its receptor, EcR-USP, regulates the expression of early genes such as *BR-C*, *E74*, *E75* and *E93*. *BR-C* constitutes a family of zinc-finger transcription factors, while *E74* encodes two isoforms of ETS (E twenty six)-like transcription factors. *E75* encodes three orphan nuclear hormone receptors and *E93* is responsible for the activation of the cell death machinery (Baehrecke, 2000; Burtis *et al.,* 1990). The early genes act as transcription factors to repress their own expression and regulate the transcription of a larger set of late genes that play a more direct role in metamorphosis including morphogenesis and programmed cell death (Fletcher and Thummel, 1995b; Urness and Thummel, 1995).

Studies have been conducted to determine the functions of the early and late genes, and hence their role in metamorphosis. *BR-C* acts as a transcription

factor by interacting with the *cis*-acting regulatory elements of the late genes (Crossgrove *et al.,* 1996). The isoforms of *BR-C* have been shown to have regulatory dependence on each other such that the balance of the isoforms determines the timing of the ecdysone pulse in fat bodies (Bayer *et al.,* 1997; Mugat *et al.,* 2000). *E74* loss-of-function mutants show that the ecdysone-induced early gene transcription is unaffected by the mutation. However, *E74* function is necessary for the regulation of late genes. *E74A* is required in the early prepupa whereas, *E74B,* in association with *BR-C,* is necessary during the mid third instar larval stage for the expression of glue genes (Fletcher and Thummel, 1995a). *E75A* mutants show reduced levels of ecdysone titer in the larval stages and delays and arrests in development. The mutants also show a heterochronic phenotype, where the second instar larva expresses genes specific to the third instar larva and undergo pupariation without molting (Bialecki et al, 2002).

Based on Ashburner's hierarchical model of gene regulation by ecdysone, Burtis *et al.* (1990) proposed 'the tissue coordination model'. The model states that groups of early genes activated due to ecdysone are responsible for the activation of late genes in specific patterns in order to carry out unique functions in each target tissue at definite developmental stages. According to this model, the regulatory hierarchy model is also activated from the mid-embryonic through pupal developmental stages in tissues other than the salivary glands and in other developmental stages that are distinguished by an ecdysone pulse.

E93 Directs Programmed Cell Death

E93, an early gene, consists of a 55 kb genomic DNA sequence that encodes a novel 146 kDa protein (Baehrecke and Thummel, 1995). *E93* corresponds to the 93F puff and was isolated and characterized by a chromosomal walk (Baehrecke and Thummel, 1995) (Figure 7). The *E93* sequence has no match in the sequence databases, but shows typical characteristics of *Drosophila* transcription factors. The E93 sequence consists of several acidic homopolymeric tracts. The glutamine residues that make up the *opa* repeats function as a transcription activation domain. The sequence also has two *pen* repeats of glycine and one alternating histidine repeat, all of which are characteristics of *Drosophila* transcription regulators. E93 also contains a potential nuclear localization signal sequence at position 761-768 (Arg-Pro-Lys-Arg-Gly-Lys-Tyr-Arg). Most importantly, E93 is localized in the nucleus, an important characteristic of transcription factors (Baehrecke and Thummel, 1995).

Studies conducted with *E93* mutants elucidate the role of *E93* in programmed cell death during metamorphosis. Animals mutant for the *E93* gene usually survive through embryogenesis and the larval stages, but die in the early stages of pupal development. The larval salivary glands and midgut tissues of these animals are unable to form autophagic vacuoles. As a result they fail to undergo steroid-triggered programmed cell death. Furthermore, these animals are unable to shorten their bodies at pupariation and die shortly after head eversion. The restoration of *E93* is sufficient to rescue the mutant and carry out cell death in

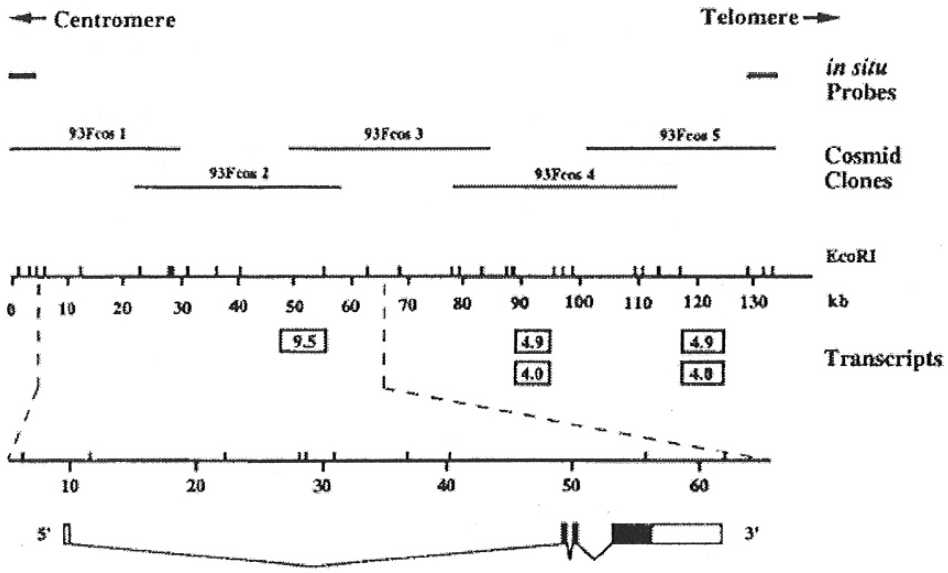

Figure 7: Structural map of the 93F puff locus. The 9.5 kb transcript of the *E93* gene is shown. The *E93* transcription unit exons and open reading frame are shown relative to the physical map of the 93F puff locus (Baehrecke and Thummel, 1995).

these tissues (Baehrecke, 2003; Lee *et al.,* 2000; Lee *et al.,* 2002a). Hence, *E93* is critical for the normal development of the animal.

E93 is a specific regulator of programmed cell death, although other early genes involved in programmed cell death, including *BR-C* and *E74A*, are more pleiotropic (Baehrecke, 2000; Broadus *et al.,* 1999; Fletcher and Thummel, 1995a; Lee and Baehrecke, 2001). Lee *et al.* (2000) show that *E93* expression foreshadows cell death and hence is expressed in dying tissues only. Larval tissues are destroyed after the ecdysone pulse that directs these tissues to induce the expression of *E93*, which activates the cell death machinery. Consequently, larval midgut cell death occurs after the late larval ecdysone pulse whereas the larval salivary glands are only destroyed after the late prepupal pulse of ecdysone. Furthermore, *E93* is not expressed in cells, such as the imaginal discs, which develop to form adult tissues. Ectopic expression of *E93* in cells that form adult structures causes these tissues to undergo cell death (Lee *et al.,* 2000). Similarly, Lee and Baehrecke (2001) expressed *E93* ectopically in the embryo to show complete destruction of embryonic tissues. Therefore, *E93* is expressed stage- and tissue-specifically for the destruction of larval tissues, and is both necessary and sufficient for a death response (Baehrecke and Thummel, 1995; Lee *et al.,* 2000; Lee *et al.,* 2002b; Thummel, 2001a).

Programmed Cell Death: Apoptosis and Autophagy

Programmed cell death is an essential phenomenon that sculpts tissues and removes unwanted cells during development. During the metamorphosis of *Drosophila melanogaster*, ecdysone regulates programmed cell death to remove obsolete larval tissues. Among the three types of programmed cell death that have been observed, nonlysosomal cell death is the least common, while apoptosis and autophagy are the most common in developing animals (Adams 2003; Baehrecke, 2000; Baehrecke, 2002; Riddiford, 1993).

Apoptosis and autophagy are morphologically distinct. The condensation and margination of chromatin to the nuclear membrane, DNA fragmentation, blebbing, and formation of apoptotic bodies mark apoptosis. During apoptosis the phospholipid phosphatidylserine, normally hidden within the plasma membrane, is exposed to the surface. This exposed phosphatidylserine binds to receptors on phagocytes leading to the phagocytosis of the cell and degradation of cellular content in the lysosome of the phagocyte.

The key effectors of apoptosis are cysteine proteases of the caspase family. There are seven caspases in *Drosophila*: *dronc, drice, dcp-1, dredd, decay, damm* and *strica* (Cakouros *et al.,* 2002). Ecdysone regulates the transcription of *dronc* as well as its downstream effector caspase, *drICE* (Cakouros *et al.,* 2002; Cakouros *et al.,* 2004b; Kilpatrick *et al.,* 2005). CARMER, an arginine-histone methyltransferase is also critical for apoptosis. It binds to a steroid hormone coactivator to methylate histone H3 and modifies the

chromatin. It is unique to the cell death pathway directed by ecdysone because its removal does not affect ecdysone-independent apoptosis (Cakouros *et al.,* 2004a).

Autophagy shares several characteristics with apoptosis. Although DNA fragmentation and cellular blebbing are observed in tissues that undergo autophagic cell death, autophagy is marked by tissue death as opposed to individual cell deaths, and self-digestion of cytoplasmic components. Cells undergoing autophagic cell death internalize cytoplasmic components into membrane-bound structures called autophagic vacuoles and are targeted to fuse with the cell's own lysosomes for degradation (Thummel, 2001b).

The larval salivary glands of *Drosophila* undergo autophagic cell death triggered by the late prepupal high ecdysone titer 10-12 hours APF and are completely destroyed by 16 hours APF. The tissues show DNA fragmentation, blebbing and are degraded through the formation of autophagic vesicles (Jiang *et al.,* 1997; Martin and Baehrecke, 2004). An hour after DNA fragmentation, large vacuoles that break into smaller vacuoles appear and carry out tissue degradation. The absence of phagocytosis during the degradation of larval salivary glands also supports the idea that salivary gland cell death occurs via autophagy (Martin and Baehrecke, 2004).

The study of the fruitfly salivary gland cell death, however, shows considerable overlap between the mechanisms of apoptosis and autophagy. Several common genes are activated in both pathways (Lee and Baehrecke, 2001). On one hand, steroid activation of caspases plays a critical role in the

autophagic cell death of *Drosophila* salivary glands (Martin and Baehrecke, 2004). On the other hand, dying larval salivary glands also exhibit high level of acid phosphatase and an increase in the number and size of autophagic lysosomal vesicles, which are distinct markers of autophagy (Lee and Baehrecke, 2001; Thummel, 2001b).

Cellular changes occurring in the larval salivary glands during autophagic programmed cell death also include changes in the cytoskeleton that are brought about by caspase activation. Caspase inhibition by p35 and mutations in cell death genes bring about similar consequences. Both studies have shown that localization of the filamentous actin is caspase-independent, but localization of α-Tubulin and nuclear lamins, which are usually seen during cell death, are prevented (Martin and Baehrecke, 2004). The expression of the baculovirus caspase inhibitor, p35, not only changes the morphology of autophagic vacuoles, but also prevents the destruction of larval salivary glands. Therefore, the utilization of caspases in autophagy of the larval salivary glands supports the idea that the autophagic tissues express core apoptotic regulators (Jiang *et al.,* 1997; Lee *et al.,* 2002a).

E93, the early gene that initiates the cell death pathway in *Drosophila* metamorphosis, acts both directly and indirectly to regulate key players of programmed cell death. It binds to salivary gland polytene chromosomes at sites that contain the late genes involved in programmed cell death. E93 directly regulates the transcription of *dredd, crq, dcp-1* and *drICE* but does not bind to

regulatory sites of *BR-C, βFTZ-F1* or that of cell death inducers such as *reaper (rpr),* and *head involution defective* (*hid*) (Lee *et al.,* 2000). However, in the larval salivary glands of *E93* mutants, the mRNA levels of the cell death genes *rpr, hid, crq, ark* and *dronc* are severely reduced. Although *E93* mutation does not directly affect the transcription of *EcR* and *βFTZ-F1*, mutations in these genes affect the transcription of *BR-C* and *E74A*, the early genes responsible for the expression of late genes such as *rpr* and *hid* (Lee *et al.,* 2000; Baehrecke, 2000).

BR-C, E74A and *E75* play important roles in salivary gland programmed cell death (Broadus *et al.,* 1999; Jiang *et al.,* 2000; Lee *et al.,* 2000; Lee and Baehrecke, 2001; Lee *et al.,* 2002b). BR-C is required for the maximal expression of *reaper* (*rpr*), although it is weakly expressed directly as a result of the ecdysone high titer. *BR-C* is also required for the expression of *dronc*. *BR-C* and *E74A* are essential for *hid* expression (Cakouros *et al.,* 2002, Jiang *et al.,* 2000), while E75 represses the death inhibitor that precedes *rpr* and *hid* expression (Jiang *et al.,* 2000).

Up-regulation of cell death initiators and down-regulation of inhibitors is important for programmed cell death of tissues, such as salivary glands, during metamorphosis. Ecdysone balances the pro- and anti-death molecules during *Drosophila* development. It also regulates the stage-specific expression of the cell death genes *rpr* and *hid* immediately before the destruction of the larval tissues while *diap2*, the anti-cell death gene, is repressed by ecdysone under the same conditions (Jiang *et al.,* 1997).

βFTZ-F1, the Competence Factor

The βFTZ-F1 sequence consists of 816 amino acids that forms an 88 kDa protein localized in the nucleus (Lavorgna *et al.,* 1993). βFTZ-F1 is an orphan nuclear receptor encoded by the *FTZ-F1* gene, which maps to the 75CD mid-prepupal puff locus. *FTZ-F1* encodes two isoforms: *αFTZ-F1* and *βFTZ-F1*, which differ in their amino-terminals. *αFTZ-F1*, which is maternally expressed, is distributed evenly in the embryo and cooperates with Ftz, a homeodomain protein (Gulchet *et al.,* 1997; Yu *et al.,* 1997). *βFTZ-F1*, on the other hand, is expressed in the late embryonic and prepupal stages (Lavorgna *et al.,* 1993)

βFTZ-F1 expression follows the late larval ecdysone pulse and plays a major role in ecdysone-regulated gene expression. It is expressed in the animal before each larval ecdysis and pupation (Andres *et al.,* 1993; Lavorgna *et al.,* 1993; Woodard *et al.,* 1994; Yamada *et al.,* 2000). In the mid-prepupal stage the level of ecdysone falls, allowing the induction of *βFTZ-F1* expression, otherwise repressed by ecdysone.

DHR3 is expressed in direct response to ecdysone. However, the time of its expression shows a lag when compared to those of the other early genes. Hence, *DHR3* is a 'late-early' transcription factor that binds most strongly to the 75CD locus of the polytene chromosome. It binds to three adjacent sites in the *βFTZ-F1* promoter (White *et al.,* 1997). *DHR3* is both necessary and sufficient for the activation of *βFTZ-F1* (Horner *et al.,* 1995; Lam *et al.,* 1999). It binds as a monomer to the 5'-AGGTCA-3' core sequence of several other ecdysone-induced

puffs. It is required for the maximal expression of *EcR, E74B* and *βFTZ-F1* in the mid-prepupal stage. On the other hand, DHR3 has the ability to interact with EcR for the repression of early genes (White *et al.,* 1997). *DHR3* binds to the early genes in the prepupal salivary gland polytene chromosomes. It acts as a negative regulator of ecdysone-induced activation (Lam *et al.,* 1997). Although DHR3 is sufficient for the repression of early genes such as *BR-C, E74A* and *E75A*, it is not necessary for this response (Figure 6) (Kageyama *et al.,* 1997; Lam *et al.,* 1997; Lam *et al.,* 1999; Thummel, 2001a).

E75B, another late-early gene, lacks a complete DBD and forms a complex with DHR3 on the *βFTZ-F1* promoter. White *et al.* (1997) observed that the *βFTZ-F1* expression level rises, not immediately after the activation of *DHR3*, its activator, but only after the level of *E75B* plummets. Thus, the expression of *E75B* results in the repression of *βFTZ-F1* (Figure 6). Furthermore, βFTZ-F1 represses its own expression, ensuring that the competence it provides the early and late genes is of short duration. Competence is defined as the ability to respond to an inductive signal such as ecdysone (Thummel, 1995; Woodard, *et al.,* 1994). Monomeric βFTZ-F1 binds to a PyCAAGGPyCPu consensus sequence. The first three nucleotides of the sequence are recognized by the FTZ-F1 box, which is adjacent to the carboxy-terminus of the DBD (Mangelsdorf and Evans, 1995; Ueda *et al.,* 1992).

Fortier *et al.* (2003) examined the role of *βFTZ-F1* in morphogenesis of *Drosophila melanogaster*. *βFTZ-F1* directs muscle contractions that drive

morphogenetic processes during the prepupal-pupal transition. Animals mutant for *βFTZ-F1* are unable to contract their muscles and consequently are unable to extend their legs, wings and shorten their bodies during the prepupal-pupal transition. A drop in external pressure during the prepupal-pupal transition rescues these mutant phenotypes.

Ectopic expression of *βFTZ-F1* from a transgene in the late-third instar larval stage increases the expression of early genes such as *BR-C, E74A* and *E75A* and induces the premature expression of *E93* (Lee *et al.,* 2002b; Woodard *et al.,* 1994). This premature expression results in the ectopic transcription of *rpr, dronc* and *crq* and the leads to the premature cell death of larval salivary glands (Lee *et al.,* 2002b). Moreover, ectopic expression of *βFTZ-F1* during postembryonic development is lethal (Yamada *et al.,* 2000). Therefore, the expression of *βFTZ-F1* is directed by the ecdysone titer and is regulated in a temporally specific manner.

βFTZ-F1 functions as a competence factor for ecdysone. *βFTZ-F1* mutants are defective in the expression of the early genes such as *BR-C, E74A, E75A* and *E93* in response to the late prepupal ecdysone pulse. βFTZ-F1 is necessary for the re-induction of the early genes at the late prepupal high ecdysone titer. *βFTZ-F1* provides competence for the early genes *BR-C* and *E74* for their re-induction during the late prepupal stage. The levels of the ecdysone receptor, however, remain unchanged in the mutants, suggesting that the competence provided by *βFTZ-F1* is through direct regulation of early genes (Broadus *et al.,* 1999). More

importantly, *βFTZ-F1* has been shown to be sufficient for providing one early gene, *E93*, with the competence to respond to the late prepupal ecdysone pulse. *βFTZ-F1* expression in the mid-prepupal stage is critical for the stage-specific expression of *E93* in the late prepupal stage (Figure 8) (Broadus *et al.,* 1999; Woodard *et al.,* 1994).

βFTZ-F1 can provide competence to *E93* only if the two genes are members of the same pathway and if βFTZ-F1 functions upstream of *E93*. Lee *et al.* (2002b) ectopically expressed *βFTZ-F1* during the late third instar larval stage of *E93* mutants and in animals wild-type for *E93*. Unlike animals wild-type for *E93,* the larval salivary glands of the homozygous mutants did not undergo premature cell death. However, in both types of animals DNA fragmentation, which is induced by *βFTZ-F1*, was observed. These findings support the competence argument by showing that βFTZ-F1 acts upstream of *E93* in the same pathway (Lee *et al.,* 2002b).

Competence can be achieved in at least two different ways at the transcriptional level (Broadus *et al.,* 1999). The first mechanism is via the expression of appropriate receptors and cofactors that regulate receptor activity. The cells are unable to respond to a signal in the absence of the receptor and cofactors. The second mechanism is through chromatin modifications. Expression of histone H1 subtypes or the modification of the histone H1 amino-terminus decreases the repression due to higher order of chromatin folding, hence enhancing transcription. The histone amino-terminus extends from the

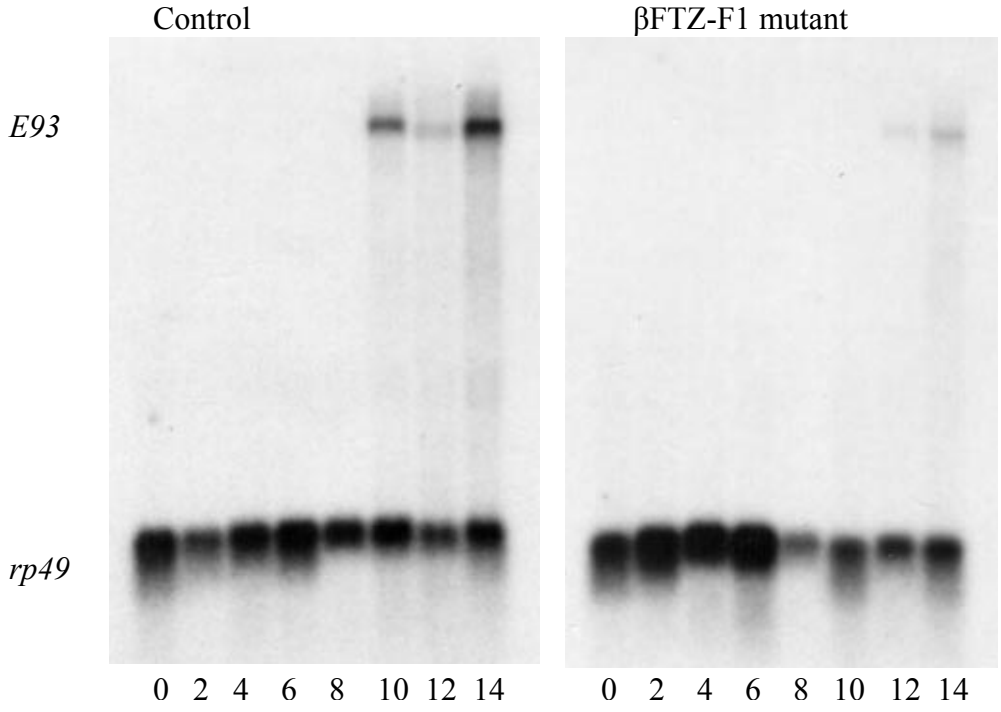

Figure 8: *βFTZ-F1* is required for *E93* to respond to the late prepupal pulse of ecdysone in larval salivary glands. The Northern blots show that in the presence of wild-type levels of *βFTZ-F1,* the control animals show a much higher level of *E93* from 10-14 hours APF, which coincides with the late prepupal ecdysone pulse. In βFTZ-F1 mutant animals however, the level of *E93* is low during the same period. *rp49* is a housekeeping gene, used here to ensure equal loading of the wells.

nucleosome core and can be modified by acetylation, methylation, ADP-ribosylation or phosphorylation (Aranda and Pascaul, 2001; Broadus *et al.,* 1999; Wolffe, 1997).

βFTZ-F1 also acts as a competence factor in the anautogenous mosquito, *Aedes aegypti* (Zhu *et al.,* 2003). In mosquitoes, ecdysone is responsible for the synthesis of the yolk protein precursor (YPP) gene *vitellogenin* (*Vg*). However, only in the presence of the juvenile hormone III (JH) does the animal produce the YPPs. Zhu *et al.* (2003) show that *βFTZ-F1* is activated only after exposure to JH. The female mosquitoes of this species require three days to become competent to activate vitellogenesis in response to a blood-meal initiated by an ecdysone titer. Although *βFTZ-F1* mRNA levels are abundant throughout the different stages, the protein is only available after three days. RNA interference of *βFTZ-F1* resulted in attenuated expression of early genes such as *EcR-B, E74B* and *E75A* as well as *Vg* in response to a blood-meal. Hence, βFTZ-F1 provides competence to the early genes and *Vg* to respond to ecdysone in *A. aegypti*.

A mammalian ortholog of βFTZ-F1, the orphan nuclear receptor Steroidogenic Factor-1 (SF-1), regulates the transcription of genes involved in gonadal and adrenal development (Achermann *et al.,* 2002). The function of βFTZ-F1 as a competence factor can be applied to SF-1 to help understand the mechanisms of SF-1 action. Additionally, another mammalian homolog of βFTZ-F1, LRH-1 (Liver receptor homolog-1), has been shown to function as a competence factor to enable the human *cholesterol ester transfer protein* (*CETP*),

in the presence of liver X receptor (LXR), to respond to the level of cholesterol (Luo *et al.,* 2001). LRH-1 enhances the LXR-mediated response of *CETP* to cholesterol by binding to the proximal promoter element of *CETP* as a monomer. This mechanism is independent of RXR ligands and the finding has been suggested to be a general property of competence factors in transcriptional responses (Luo *et al.,* 2001).

Aim

The aim of this project is to gain further insight into the molecular mechanisms by which the *Drosophila* steroid hormone, ecdysone, and its competence factor, βFTZ-F1, can bring about stage- and tissue-specific response of the *E93* gene. We hypothesize that βFTZ-F1 provides competence to *E93* through a mechanism that involves direct binding of βFTZ-F1 to the *E93* gene sequence. Preliminary studies have indicated that βFTZ-F1 binds to 93F DNA at the 3'-end of the first *E93* intron (Baehrecke, unpublished). Therefore, the binding of βFTZ-F1 to *E93* is likely to be a part of the mechanism by which βFTZ-F1 enables the activation of *E93* in response to the late prepupal ecdysone pulse.

The main goal of the project is to determine βFTZ-F1 binding site(s) on *E93*. A recombinant plasmid containing the *βFTZ-F1* open reading frame (ORF) was used to transform bacterial cells. Overexpression of *βFTZ-F1* followed by its purification was carried out to obtain the protein for gel-shift assays. Gel-shift assays conducted with a consensus sequence of βFTZ-F1 binding site and the

presence of that sequence in and upstream of the *E93* gene suggest that βFTZ-F1 may bind to *E93*. The project now aims to carry out competition reactions to determine the binding ability of the purified βFTZ-F1 protein to the consensus sequence. Then, gel-shift assays with radioactively-labeled fragments of the *E93* gene, can be conducted to further the goal of mapping the exact nucleotide sequence of βFTZ-F1 binding site(s) on *E93*. Such analyses will enable us to determine the interaction between the competence factor, βFTZ-F1, and *E93* and consequently provide a better understanding of the mechanisms of steroid hormone action.

MATERIALS AND METHODS

Bacterial Overexpression of *βFTZ-F1*

Transformation of Competent Bacterial Cells by pET42a- *βFTZ-F1*

A recombinant plasmid, pET42a-*βFTZ-F1,* was made by using a pET42a vector and inserting the *βFTZ-F1* open reading frame (ORF) as an *Eco*R I/*Hin*d III fragment (Mathur, 2003) (Figure 9a). The vector contains a T7 promoter, followed by a *lac* repressor, a Glutathione-S-transferase (GST)-tag, a HIS-tag made up of six consecutive histidine residues, and an S-tag. The kanamycin resistance gene acts as the selective marker for the vector. The *βFTZ-F1* ORF, inserted at the *Eco*R I site following the S-tag, contains a stop codon at the carboxy-terminus. Translation is initiated at the *Nde* I site (Figure 9b). The protein product contains an additional 867 bp upstream of the *βFTZ-F1* ORF hence adding 35 kDa to the expected molecular weight of βFTZ-F1.

The recombinant plasmid was used to transform competent B834(DE3) cells (Novagen). The competent cells were thawed at room temperature for 10 minutes and mixed for proper cell suspension. The cells were then placed on ice for another 10 minutes. One microliter of the recombinant plasmid was mixed with 100 µl of the competent cells in a microfuge tube. They were placed on ice for 20 minutes and mixed properly by tapping every few minutes. The mixture was heat shocked for 2 minutes at 37 °C. Eight hundred milliliters of Luria-Bertani (LB) broth was added to the microfuge tube, which was then incubated at 37 °C for 50 minutes. The LB-kanamycin plates, which contained 10 µl/ml

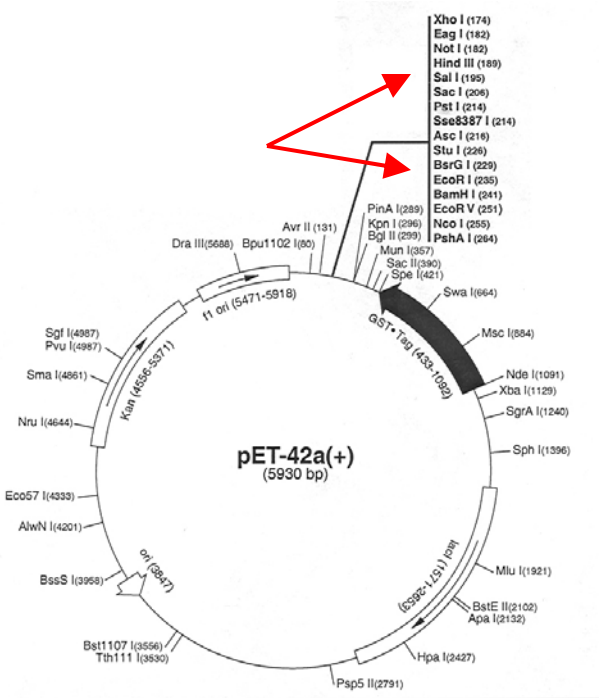

Figure 9a: Map of the pET42a vector (Novagen). The 5.9 kbp-long vector has a kanamycin resistance gene as the selective marker. It has a T7 promoter, a *lac* operon, which contains the *lac* repressor and the operator, a GST-tag and a multiple cloning site. The *Eco*R I and *Hin*d III sites (red arrows) are used to insert the *βFTZ-F1* ORF into the vector.

Figure 9b: pET42a cloning/expression regions (Novagen). The cloning/expression region sequence of the vector shows tags, including the GST (green box), the HIS (blue box) and the S (yellow box) tags. The methionine residue (green arrow) at the *Nde* I site is the translation initiation site. The *βFTZ-F1* ORF begins at the *Eco*R I (red box) site, which is 867 bp downstream of the translation initiation site. Hence, the βFTZ-F1 protein from the recombinant plasmid has an excess of 35 kDa at its amino-terminus.

kanamycin added from a 10 µg/µl stock solution (all kanamycin-containing buffers and solutions contain this concentration of kanamycin), were pre-warmed at 37 °C during this time. After the incubation, the cells were centrifuged at maximum speed in a table-top microcentrifuge for 1 minute. Almost all of the supernatant was poured-off and the pellet was resuspended in the remaining supernatant. The cells were spread, using a sterile glass spreader, on pre-warmed LB-kanamycin plates and incubated at 37 °C overnight (12-16 hours). The plates were stored at 4 °C up to 3 months.

pET42a-*βFTZ-F1* Miniprep (Qiagen)

A single colony of transformed B834(DE3) cells was picked from the LB-kanamycin plate to inoculate 2 ml of LB-kanamycin broth and was cultured in a shaker incubator overnight at 37 °C. The culture was transferred to a microfuge tube and centrifuged at 4000 rpm for 90 seconds. The supernatant was discarded and the pellet was resuspended in 250 µl of buffer P1 (see Appendix). Equal amount (250 µl) of buffer P2 (see Appendix) was added to the tube, which was carefully inverted 4-6 times. Immediately, 350 µl of buffer N3 (see Appendix) was added and the mixture was centrifuged for 10 minutes. The supernatant was transferred to a Qiagen column, which was centrifuged for 30-60 seconds at 15,000 rpm and the flow-through was discarded. Buffer P3 (see Appendix), 0.75 ml, was added to the column and centrifuged for 30-60 seconds. The flow-through was discarded and the column was again centrifuged for 1 minute and the flow-

through was discarded. The column was placed in a 1.5 ml microfuge tube and 50 µl of buffer EB (see Appendix) was added to it. It was allowed to stand for 1 minute, after which it was centrifuged for another minute. The flow-through was collected in the microfuge tube and was stored at -20 °C for future use.

Restriction Digests

The pET42a-*βFTZ-F1* plasmid miniprep was used for the restriction digest. A double digest using *Eco*R I and *Hin*d III was carried out to determine the presence of the pET42a-*βFTZ-F1* recombinant plasmid in the bacterial culture. Single digest with *Xho* I was also carried out to linearize the recombinant plasmid. The digests were prepared (see Appendix) and were incubated at 37 °C for 2 hours. The samples were mixed with 1µl loading buffer (see Appendix) and 20 µl of sample was loaded per well. A 1 kbp DNA ladder was used for size reference. The samples were loaded on a 1% agarose gel, which was run in 1X TAE running buffer containing 5 µl ethidium bromide (EtdBr) at 120V for 1 hour.

Transformed Bacterial Cultures

A single colony of transformed B834(DE3) cells was cultured in a 250 ml Erlenmeyer flask containing 10 ml LB-kanamycin and was incubated overnight in a shaker at 37 °C. The 10 ml culture was used to inoculate 50 ml LB-kanamycin in a 500 ml Erlenmeyer flask. It was incubated in a shaker at 37 °C for about 30 minutes until the OD_{600} reading reached between 0.5 and 0.7. A 1 ml sample was

taken from the culture for a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis.

Isopropyl- β-D-thiogalactopyranoside (IPTG) Induced Protein Expression

The 60 ml culture was divided into two equal portions. One portion was induced for protein expression by adding 0.8 mM IPTG from a 100 mM stock solution, while nothing was added to the other. Both cultures were incubated in a shaker at 37 °C for 5 hours. A 1 ml sample was taken from both cultures every hour for SDS-PAGE analysis. The induced culture was then harvested by centrifugation at 4000 x g for 20 minutes. The supernatant was discarded while the pellet was stored at -20 °C and was thawed on ice for 15 minutes before further use.

SDS-PAGE Analysis of IPTG Induced Protein Expression

The hourly samples of IPTG-induced and uninduced cultures were microfuged at 10,000 x g for 1 minute. The supernatant was discarded and the pellet was resuspended in 100 µl 1X SDS-PAGE buffer (see Appendix). The samples were boiled for 5 minutes at 100 °C. They were vortexed for 10 seconds and centrifuged in a table-top microcentrifuge for 10 seconds. The samples, 20 µl per well, were loaded on a 10% polyacrylamide gel (see Appendix). Protein Plus Protein Standard (Bio-Rad, see Appendix), 5 µl, with 15 µl of 1X SDS-PAGE buffer was used for protein size reference. Excess wells were loaded with 20 µl of 1X SDS-PAGE buffer. The gel was run in 1X tris-glycine electrophoresis buffer

(see Appendix) at 80V for 30 minutes and at 120V for another hour. It was then washed with ultrapure water three times for 5 minutes each time on a shaker. The gel was stained in a Tupperware with Coomassie (Bio-Rad) for 30 minutes on a shaker. It was then destained with ultrapure water three times, each for 10 minutes and was stored in 4 °C.

Purification of βFTZ-F1 Protein

Sonication

The pellet was resuspended in buffer B (see Appendix) at the rate of 15 ml per gram wet weight. A 1 ml sample was taken for SDS-PAGE analysis. The solution was placed on ice and sonicated using the Sonifier Cell Disruptor with the microtip limit set at 2 for 45 minutes. Care was taken to prevent foaming. A 1 ml sample of the cell lysate was taken for SDS-PAGE analysis.

Affinity Tag Purification

The protein from the cell lysate was purified under denaturing conditions. The cell lysate was centrifuged at 10,000 x g for 20 minutes to remove cellular debris. The supernatant was discarded while the pellet was resuspended in 50% Ni-NTA agarose slurry (Qiagen) at a ratio of 1 ml of the slurry for every 2 ml of lysate. It was mixed on a shaker for 30-60 minutes at room temperature. The lysate-resin mixture was loaded into an empty 5 ml purification column (Qiagen) with the bottom cap still attached. After loading, the bottom cap was removed and the flow-through was collected in a 10 ml Falcon tube. A 1 ml sample was taken

from the flow-through for SDS-PAGE analysis. The lysate-resin mixture in the column was washed twice, each time with 4 ml wash-buffer C (see Appendix). The fractions were used for SDS-PAGE analysis. The recombinant protein was collected in 4 fractions of 0.5 ml elute-buffer D (see Appendix) followed by 4 fractions of 0.5 ml elute-buffer E (see Appendix). A 1 ml sample for the flow-through with each buffer was used for SDS-PAGE analysis.

SDS-PAGE Analysis of Purification Samples

Samples from various steps during the culture, induction and purification processes were run on a 6% polyacrylamide gel (see Appendix). The 1 ml samples collected before the addition of IPTG, and after the optimized induction time of 5 hours with IPTG were centrifuged at 10,000X g for 1 minute. The pellet and the supernatant were separated and analyzed as different samples. Each pellet was resuspended in 100 µl of 1X SDS-PAGE buffer, while 100 µl of the other samples were mixed with 100 µl of 1X SDS-PAGE buffer. All the samples were prepared for loading and loaded on the gel as explained before. The gel was run, washed, stained and destained as previously described. The samples run on the gel were as follows (Figure 12 in the Results Section):

Sample A: Pellet of the transformed B834(DE3) culture at OD_{600} 0.5-0.7, and before the addition of IPTG.

Sample B: Corresponding supernatant of sample A.

Sample C: Pellet of the culture after the 5-hour incubation with 0.8 mM IPTG.

Sample D: Corresponding supernatant of sample C.

Sample E: IPTG induced pellet resuspended in buffer B.

Sample F: Cell lysate after sonication.

Sample G: Wash buffer C flow-through.

Sample H: Elute buffer D flow-through.

Sample I: Elute buffer E flow-through.

βFTZ-F1, the Consensus Sequence and Gel-Shift Assay

Consensus Oligonucleotide Sequence

Two oligonucleotides with the following sequences (Set 1) were ordered. The sequence shown in bold is based on the consensus sequence, PyCAAGGPyCPu, of the βFTZ-F1 binding site (Horner *et al.,* 1995; Ueda *et al.,* 1992).

5'- GGT TGT C**TC AAG GTC A**CC GAG TC -3'
3'- CA G**AG TTC CAG T**GG CTC AGT TGG – 5'-------- Set 1

Annealing Reaction

Each oligonucleotide (27.5 nmol = 0.19 mg) was dissolved in 27.5 µl RNase free water to form a 1 mM solution. A 10 µM (i.e. 6.9 µg/100 µl) stock solution was prepared, from which 1.45 µl (i.e. 100 ng) of each oligonucleotide was used for the annealing reaction. The two oligonucleotides were mixed with 2.5 µl of 10X NTB (nick translation buffer, see Appendix), and dH_2O was used to make a total volume of 19 µl. The mixture was boiled at 100 °C for 5 minutes and placed at 37 °C for 20 minutes to allow the oligonucleotides to anneal.

End-Labeling Reaction

The end-labeling reaction was carried out after the annealing of the oligonucleotides. Each of the cold deoxynucleotides, dGTP, dTTP, dCTP and dATP, with the original concentration of 100 mM was diluted in RNase free water to form a 10 mM solution. For the labeling reaction, 2.5 µl of 10 mM dGTP, dCTP and dTTP and 2.5 µl of [α-^{32}P]-dATP (Perkin-Elmer) were mixed with 1 µl Klenow enzyme (DNA Polymerase I Klenow Fragment from USBiological) and incubated at 37 °C for 30 minutes. After the incubation, 175 µl of TE buffer (see Appendix) was added to the microfuge tube and the mixture was filtered through a NucTrap Probe Purification Columns (Stratagene) column to clean-up the probe. A microliter of the probe was used to check for radioactivity using a scintillation counter.

Binding Reaction

Six binding reactions, including a negative control containing no protein, were prepared as shown in Table 1. The reactions were placed on ice for 20 minutes. One microliter of the radioactive probe was added to each of the six reactions and placed at room temperature for 30 minutes.

Gel-Shift Assay

A 5% polyacrylamide 0.5X TBE gel (see Appendix) was pre-run at 4 °C and 100V for 30-60 minutes. 1X gel loading buffer, 2 µl, was added to each reaction

Table 1: Binding reactions of variable amounts of βFTZ-F1 protein with consensus oligonucleotide sequence.

	No Protein	**rxn 1**	**rxn 2**	**rxn 3**	**rxn 4**	**rxn 5**
10X Yao binding buffer (see Appendix) (µl)	2	2	2	2	2	2
50% glycerol (µl)	3	3	3	3	3	3
1µg/µl dI-dC non-specific competitor (µl)	2	2	2	2	2	2
βFTZ-F1 elute (µl) CONC	0	2	4	6	8	10
RNase free dH_2O (µl)	12	10	8	6	4	2
Radiolabeled Set 1 (µl)	1	1	1	1	1	1

and 20 µl of the samples were loaded per well. The gel was run in 0.5X TBE running buffer (see Appendix) at 4 °C and 100V for 1 hour and stopped when the loading dye reached half-way through the gel. The gel was then placed on Whatmann 3MM filter paper and was covered with a plastic wrap. It was dried using a gel dryer at 80 °C for 90 minutes under a vacuum. The dried gel on the filter paper was transferred to an autoradiography cassette. An X-ray film was exposed to the radioactive gel for 60 minutes at -80 °C. The autoradiogram was then developed.

RESULTS

Transformation of Competent B834(DE3) Cells with pET42a-*βFTZ-F1*

pET42a-*βFTZ-F1* was successfully introduced into B834(DE3) cells. The hence kanamycin resistant cells formed large, round, white colonies on LB-kanamycin plates. Smaller, white satellite colonies were also observed. The larger colonies grew in overnight liquid cultures, which were used to make plasmid minipreps for the restriction digest assay.

Restriction digests of the transformed B834(DE3) minipreps were carried out to determine the presence of the recombinant plasmid, pET42a-*βFTZ-F1*. The double digest with *Eco*R I and *Hin*d III showed two bands of 5.9 kbp and 2.5 kbp on the agarose gel (Figure 10). The two restriction enzymes cut the circular plasmid twice, giving rise to two fragments; the linear plasmid and the insert. The larger fragment corresponds to the pET42a vector while the smaller fragment corresponds to the *βFTZ-F1* ORF. Similarly, the single digest with *Xho* I linearized the recombinant plasmid with a single cut, giving rise to the expected size of 8.4 kbp (8384 bp) sequence that included the vector as well as the insert (Figure 10).

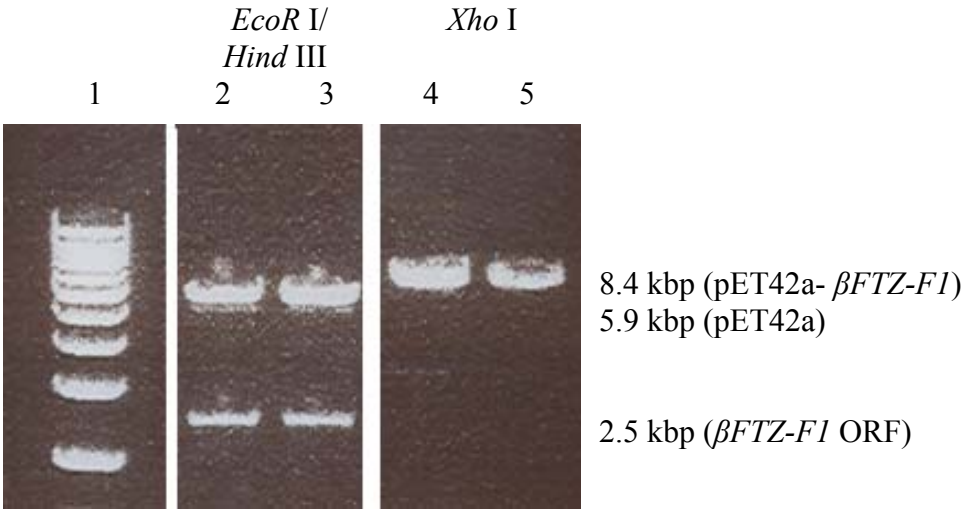

Figure 10: Restriction digests of transformed B834(DE3) cells. The 1% agarose gel containing EtdBr was exposed to UV radiation to observe the DNA bands. The double digest with *Eco*R I and *Hin*d III (lanes 2 and 3) show two bands. The bands at 5.9 kbp and 2.5 kbp correspond to the pET42a vector and the *βFTZ-F1* ORF respectively. The *Xho* I single digest (lanes 4 and 5) linearizes the recombinant plasmid giving rise to a single band of 8.4 kbp corresponding to the length of the sum of the vector and the insert. Lane 1 shows the size markers in 1 kbp increments starting from 2 kbp at the bottom of the gel.

IPTG Induced *βFTZ-F1* Expression

Transformed bacterial cells were successfully induced by 0.8 mM IPTG to overexpress *βFTZ-F1*. Larger colonies of transformed B834(DE3) cells were used to make 10 ml overnight cultures, which were used to inoculate 50 ml LB-kanamycin broth. The culture reached the OD_{600} of 0.5-0.7 within 30 minutes. The presence of IPTG in the culture gave rise to a distinct band of 130 kDa in the induced culture sample when compared to the uninduced (Figure 11). The predicted size of the βFTZ-F1 ORF from its sequence is 88 kDa. However Lavorgna *et al.* (1993) observed that the protein ran at 95 kDa. The amino-terminus of the protein consists of the GST-tag, the HIS-tag, the S-tag and other interspersed sequences that add up to 35 kDa. Hence, the IPTG induced sample shows the overexpressed protein that includes the 95 kDa βFTZ-F1 protein and the additional sequence at the amino-terminus, giving rise to a band at around 130 kDa.

The optimum time for induction with IPTG was determined to be 5 hours. Hourly samples from the induced and the uninduced (negative-control) culture were run on a 10% polyacrylamide gel. Apart from the distinct 130 kDa band corresponding to the βFTZ-F1 protein, the gel also shows a distinct pattern of protein expression with respect to the duration of IPTG induction. It was qualitatively observed that the βFTZ-F1 band in the hourly samples taken from the induced cultures increased in intensity from 1 hour to 5 hours (Figure 11).

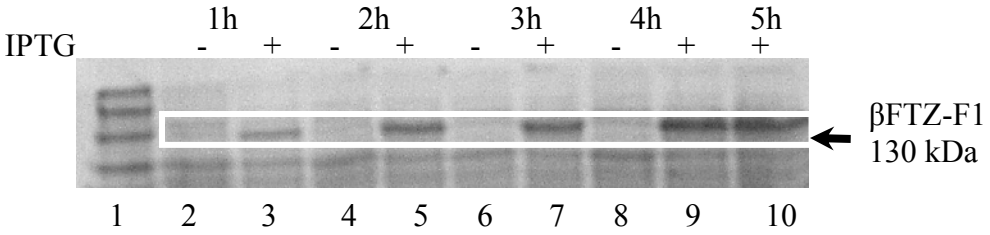

Figure 11: *βFTZ-F1* overexpression by IPTG induction. The 10% polyacrylamide gel used for an SDS-PAGE analysis shows the expression of *βFTZ-F1* in IPTG induced culture. The IPTG induced and uninduced samples are indicated. The duration of induction is also marked. The 130 kDa band that corresponds to βFTZ-F1 is seen in lanes (3, 5, 7, 9 and 10) that contain the IPTG induced samples. The negative control samples (lanes 2, 4, 6 and 8) were not induced by IPTG and hence do not show a prominent band. The intensity of the overexpressed protein band increases with an increase in the duration of induction with IPTG.

It was also observed that the intensity of the band did not show any qualitative difference between the samples at hours 5, 6 and 7 (Data not shown). Hence, 5 hours was determined to be the optimum duration for induction with IPTG.

Affinity Tag Purification of βFTZ-F1 Protein

Low level of βFTZ-F1 protein was successfully purified using the affinity tag purification process. After the 5-hour induction with IPTG, the pellet of transformed bacterial culture was resuspended in buffer B. However, the sample taken at this point (Figure 12, sample E) shows no band of βFTZ-F1, indicating the absence of soluble βFTZ-F1 protein. The suspension was then sonicated and the sample of the cell lysate (sample F) showed the reappearance of the βFTZ-F1 band of 130 kDa, suggesting an increase in the solubility of the protein. The cell lysate was then used for purification. The wash buffer C was used to remove impurities and untagged-proteins from the lysate. The flow-through (sample G) however contained the majority of the βFTZ-F1 along with the impurities. The elute buffers D and E contained low levels of the βFTZ-F1 protein (samples H and I). Buffer D, which has a higher pH than buffer E, is expected to contain monomeric βFTZ-F1 while buffer E is expected to contain mostly multimers and aggregates of βFTZ-F1.

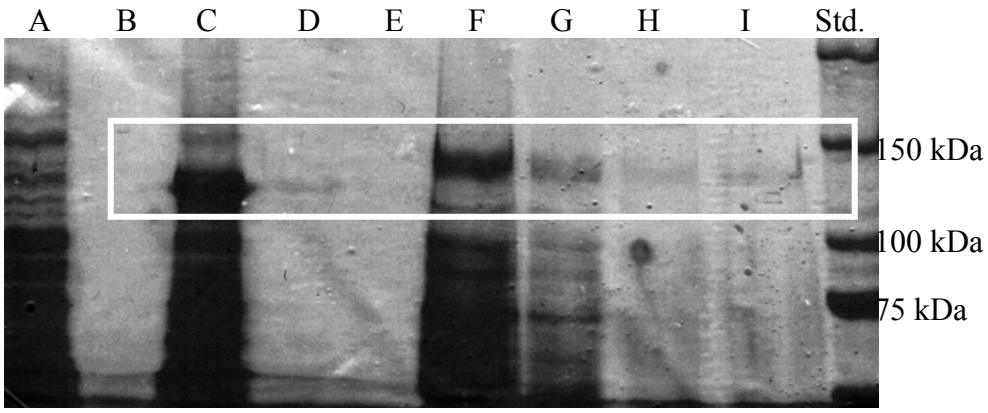

Figure 12: *βFTZ-F1* overexpression and protein purification. The 6% polyacrylamide gel used for the SDS-PAGE analysis shows the location of βFTZ-F1 from expression to purification. The lanes here correspond to the samples that are described in the materials and methods section (Page 38-39). Sample A contains the pellet of the transformed B834(DE3) cells after the OD_{600} reached 0.5-0.7 and before the addition of IPTG to the culture. It shows the presence of various proteins in the uninduced bacterial culture. B contains the corresponding supernatant of A. It is devoid of any bacterial protein. Sample C contains the pellet of the transformed cells after the 5-hour incubation with IPTG while D contains the corresponding supernatant. Sample C shows a distinct band at 130 kDa, which corresponds to the expected molecular weight of the overexpressed βFTZ-F1. The corresponding supernatant, sample D, shows a faint band of βFTZ-F1. Sample E contains the IPTG induced pellet resuspended in buffer B, and indicates the absence of soluble βFTZ-F1. Sample F contains the cell lysate after sonication. It shows the presence of the βFTZ-F1 protein. Sample G contains the flow-through of the cell lysate with the wash buffer C. The sample not only contains bacterial proteins washed-off as impurities, but also the majority of βFTZ-F1. Sample H and I are elutes showing low amounts of purified βFTZ-F1. The protein size standard is shown on the lane marked Std and three size standards are indicated.

βFTZ-F1, the Consensus Sequence and Gel-Shift Assay

The consensus oligonucleotide sequence was successfully labeled with α-^{32}P-dATP. The scintillation counter gave a reading of 116975.5 cpm for 1 μl of the labeled sample. The success of the radioactive labeling was calculated to $2.16x10^{7}$cpm for the entire mix of the probe.

The gel-shift assay showed a shift in band to a higher molecular weight. The intensity of the shifted band increased with an increase in βFTZ-F1 protein concentration. The negative control (Figure 13, lane 1) contained only the radiolabeled oligonucleotide. This sample showed one band of low molecular weight at the bottom of the gel. Lanes 2 to 6 contained βFTZ-F1 in increasing concentration as indicated in Figure 13. Lanes 4, 5 and 6 clearly show a shift in the band, the intensity of which was qualitatively observed to increase with the increase in βFTZ-F1 concentration.

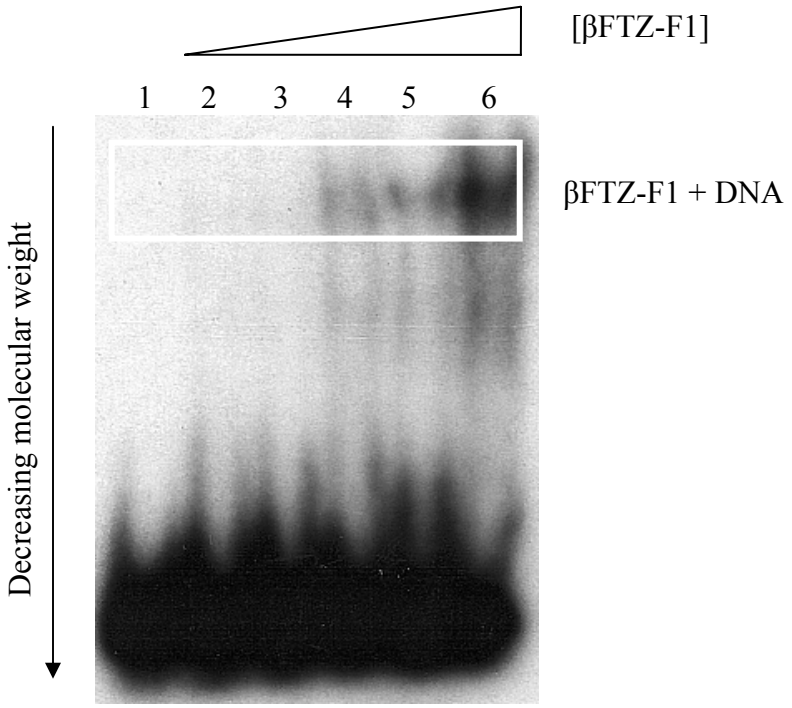

Figure 13: Gel-shift assay of βFTZ-F1 with radioactive consensus oligonucleotide sequence. Lane 1 is the negative control that contains only the radioactive oligonucleotide and no βFTZ-F1 protein. It shows only one band that corresponds to the radioactive oligonucleotide at the bottom of the gel. Lanes 2 to 6 contain the βFTZ-F1 protein in increasing concentration in addition to the radioactive oligonucleotide. Besides the radioactive oligonucleotide band, a shifted band of a higher molecular weight is observed clearly in lanes 4, 5 and 6. The shifted band, which is expected to be the result of βFTZ-F1 binding to radioactive oligonucleotide, increases in intensity with the increase in the βFTZ-F1 protein concentration.

DISCUSSION

The transformed B834(DE3) cells showed the presence of a 2.5 kbp insert (Figure 10). The size of the insert can be attributed to the *βFTZ-F1* ORF, which consists of 816 amino acids, and the 40 and 45 nucleotides located at the amino- and carboxy-termini respectively of the ORF. The insert was determined to be in frame.

The overexpression of *βFTZ-F1* in bacterial cells was successfully induced by IPTG (Figure 11). The 'pET' system, which stands for 'plasmid for expression by T7 RNA Polymerase,' is designed such that the phage T7 promoter, encoded by the vector, allows the expression of eukaryotic genes in *Escherichia coli* (Novagen). The T7 RNA polymerase is about five times more active than *E. coli* RNA polymerase, hence allowing genes controlled by the T7 promoter to be overexpressed. The T7 RNA polymerase is supplied to the system by the B834(DE3) cells. The B834(DE3) cells are lysogenic for a fragment of the DE3 phage. Among the components of interest, this fragment contains the *lacUV5* promoter and the T7 RNA polymerase. The *lacI* repressor encoded by the pET vector binds to the *lacUV5* promoter, hence inhibiting T7 RNA polymerase expression. The LacI repressor also binds to the *lac* operator, which is downstream of the T7 promoter in the pET vector. This binding inhibits the T7 RNA polymerase from moving beyond the T7 promoter. The addition of IPTG is important, as it binds to the *lac* repressor, prohibiting it from binding to two sites: the *lac* operator in the pET system and the *lacUV5* promoter on the DE3

fragment. Hence it enables the *E. coli* RNA polymerase to activate the expression of T7 RNA polymerase. The T7 RNA polymerase then uses the T7 promoter on the pET vector for the overexpression of the insert, in this case, βFTZ-F1.

The molecular weight of the overexpressed βFTZ-F1 predicted from its sequence is 88 kDa. However, Lavorgna *et al.* (1993) observed that the protein runs at 95 kDa due to anamolous mobility on SDS-polyacrylamide gels and/or posttranslational modifications. The expressed sequence has 289 additional amino acids at its amino-terminus, which adds about 35 kDa to the molecular weight of the βFTZ-F1 protein. Hence, the expected size of βFTZ-F1 was 130 kDa.

Although the high-level expression of βFTZ-F1 was successful, protein purification required denaturing conditions. βFTZ-F1 was believed to be present as inclusion bodies, which are cytosolic, insoluble aggregates of misfolded proteins that need to be solubilized and refolded for proper function of the protein. A quick analysis of 808 amino acids of βFTZ-F1, reported by Ohno and Petkovich (1993), showed that 54.8% of the amino acids were hydrophobic (Glycine included, see Appendix). High levels of hydrophobic residues may have contributed to the formation of inclusion bodies, consequently decreasing the solubility of the protein. Low solubility necessitated dissolution of the protein by sonication and its purification under denaturing conditions. Sample E in Figure 12 shows the absence of the 130 kDa βFTZ-F1 band before sonication. Sonication resulted in increased solubility of the βFTZ-F1 protein. The purification buffers (buffers B-E) contained 8 M urea, which created denaturing conditions for the

protein. Therefore, βFTZ-F1 was successfully purified under denaturing conditions.

The HIS-tag at the amino-terminus of βFTZ-F1 was used as the affinity tag for its purification. The affinity tag consists of six consecutive histidine residues that are recognized by Nickel ions (Ni^{2+}). Four of the six coordination sites of Ni are used by nitriloacetic acid (NTA) to chelate the ion. The remaining two coordination sites interact with the HIS-tag. The Ni-NTA agarose is composed of Ni-NTA coupled to Sepharose CL-6B (Qiagen), which reduces nonspecific binding. The purification buffers, buffers B to E, decrease in pH in that order (see Appendix). This gradual decrease in pH allows impurities and unbound proteins to be washed away from the lysate-resin mixture in the purification column, giving rise to a purer solution of βFTZ-F1. The decrease in pH due to an increase in proton concentration of the solution, results in the interaction of the protons with the histidine residues. Hence, positively charged histidine residues are formed. These residues repel the Ni^{2+}, resulting in a decrease in affinity of the resin to the HIS-tag. Hence, the flow-through collected from the purification column in buffers with the lowest pH, buffer D and E (Figure 12, samples H and I) contain the protein that binds to the Ni-NTA with the highest affinity. Hence, as expected, a purer form of βFTZ-F1 was obtained in these samples.

Unfortunately, the flow-through in the wash buffer contained the majority of the βFTZ-F1 protein along with the impurities. The low yield of pure βFTZ-F1

may be attributed to the HIS-tag used in the affinity purification process. The HIS-tag is made up of six amino acids. It is small, such that it does not interfere with the overall structure of the protein. However, this very characteristic increases the likelihood of the tag being sequestered by the final protein structure. The decreased accessibility to the affinity tag can result in the loss of large amounts of βFTZ-F1 protein during the washes (Figure 12, sample G). In order to have a higher yield of pure βFTZ-F1, the 220-amino acids long GST-tag can be used. Additionally, in order to increase the effectiveness of the protein in further experiments, the extra sequence upstream of βFTZ-F1 protein could be removed. The protein is also required to undergo proper refolding by the removal of urea used in the purification buffers. Furthermore, quantification of the protein would also enable us to make more conclusive results.

The purified βFTZ-F1 was used in a gel-shift assay with a consensus sequence. Set 1 (see Materials and Methods) is based on the consensus sequence, PyCAAGGPyCPu, to which βFTZ-F1 is known to bind. A bio-analyzing tool called Cis-analyst was used to conduct a quick search of the Set 1 oligonucleotide consensus sequence in and around the *E93* gene. Figure 14 shows the presence of the sequence (GGT TGT CTC AAG GTC ACC GAG TC) in several sites. Few sites were found more than 8 kbp upstream of the *E93* gene. Most of the sites, however, were found in intron regions of the gene, with the highest concentration of sites at the 3'-end of the first intron. This finding supports the study conducted

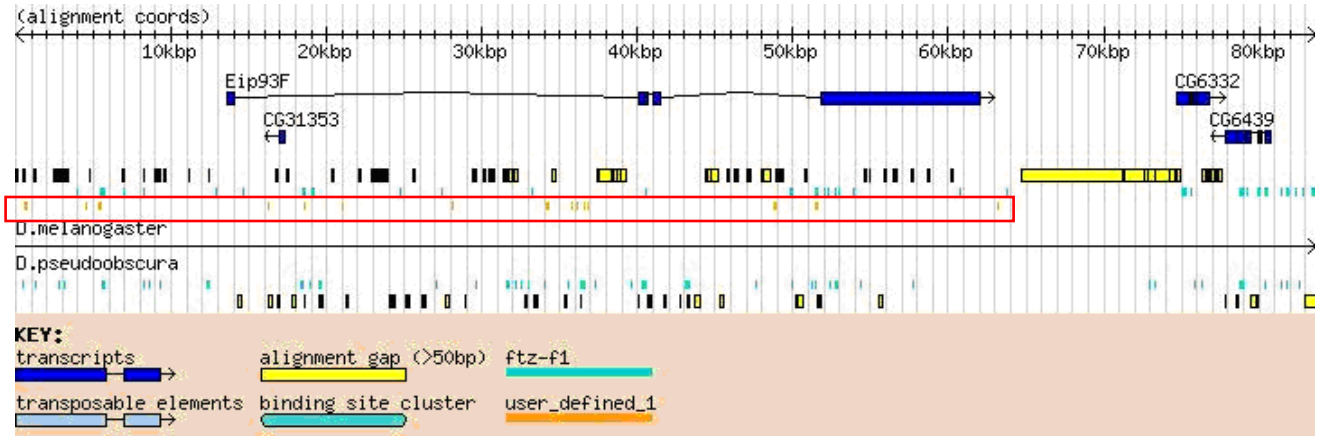

Figure 14: The sites of βFTZ-F1 binding consensus sequence (GGT TGT CTC AAG GTC ACC GAG TC) in and upstream of the *E93* gene. The *E93* gene is marked as Eip93F. The exons are shown in blue boxes while the solid lines indicate the intron regions. The sites where the consensus sequence was located are shown in orange. Sites that pertain to *Drosophila melanogaster* are within the red box. Although few sites are found more than 8 kbp upstream of the gene, most of the sites lie in the intron regions of *E93*.

by E. H. Baehrecke (unpublished), where βFTZ-F1 was shown to bind to the 3'-end of the first intron of *E93*.

The consensus sequence was used in gel-shift assays with βFTZ-F1 to assess the binding property of the purified protein. The two DNA strands that make up Set 1 are each 23-bases long and base-pair with each other in the annealing reaction. Four nucleotides remain as 5' overhangs in each strand. [α-^{32}P] dATP is used for end-labeling of Set 1 such that each double-stranded DNA would ideally contain four radioactive dATPs. However, the concentration of the DNA in the binding reaction was 0.48 μM, while that of the [α-^{32}P] dATP was only 0.28 μM. Hence, only half of the double-stranded oligonucleotide sequence was labeled with one radioactive dATP. In order to amend this, at least 5 μl of [α-^{32}P] dATP can be used in the end-labeling reaction to ensure that each double-stranded DNA incorporates at least one radioactive dATP. The use of higher amounts of the radioactive dATP is not required since even low amounts of [α-^{32}P] dATP gave rise to highly radioactive DNA (Figure 13).

In a polyacrylamide gel, a sample that contains radiolabeled DNA, but no protein, shows only one radioactive band at the bottom of the gel. The idea behind gel-shift assays is that, in the absence of SDS, the protein cannot migrate on a polyacrylamide gel unless it is bound to DNA. The presence of a protein that binds to DNA retards the migration of the DNA due to higher molecular weight of the DNA-protein complex (Figure 13). Hence, a radioactive band that migrates relatively less on the gel can be observed. The presence of a protein that binds to

the radioactive DNA causes a shift of band. In such a case, an increase in protein concentration increases the intensity of the shifted band. Migration of larger amounts of DNA is retarded, as larger amounts of protein bind to it.

Figure 13 shows a gel-shift assay carried out with the radioactive consensus sequence and βFTZ-F1. The experiment conducted, however, did not include proper controls for any conclusive result. Although the intensity of the observed shift of band increased with an increase in βFTZ-F1 concentration, the shift may have occurred due to nonspecific binding of βFTZ-F1 to the DNA or due to DNA aggregation at higher protein concentrations. Therefore, in order to eliminate such uncertainties, further experiments need to be carried out.

Competition reactions will provide a better understanding of the interaction between the purified βFTZ-F1 and its consensus sequence. Unlabeled consensus sequence, in excess, can be used to compete away the protein from the radiolabeled consensus sequence. If βFTZ-F1 binds to the consensus sequence, as suggested by Figure 13, we would expect the excess unlabeled consensus sequence to compete βFTZ-F1 away from the radiolabeled sequence, hence giving rise to a relatively faint shift of band, if any. This result would show that the purified βFTZ-F1 has retained its binding property. Additionally, to check for non-specific binding, another competition reaction can be carried out. Another set of oligonucleotides can be designed. This set of oligonucleotides would be of the same length as Set 1 and would be designed by replacing the conserved nucleotides of the consensus sequence. βFTZ-F1 protein is expected not to bind to

this sequence. Hence, when used in excess in a competition reaction with the radioactively labeled consensus sequence, the radioactive consensus sequence is expected to compete βFTZ-F1 away. This would give rise to a shifted band of relatively high intensity. Such results would show specificity of βFTZ-F1 to the consensus sequence and support the gel-shift observations shown in Figure 13.

The next step of the project would be to test our hypothesis. Similar gel-shift assays would be carried out with the *E93* sequence. Fragments of *E93* would be used to determine parts of the gene that would give rise to a shift in bands. The fragment(s) would then be used with βFTZ-F1 in DNaseI protection assays. These assays would enable us to determine the exact nucleotide sequence of βFTZ-F1 binding site(s) on *E93*.

Such studies would provide a better understanding of the interactions between βFTZ-F1 and *E93* and a mechanism of achieving competence. As mentioned earlier, competence can be achieved in various ways. βFTZ-F1 could provide *E93* with the competence to respond to ecdysone by competing with EcR for binding sites on *E93*. The sites at the intron region could have a higher affinity to βFTZ-F1 compared to EcR. Hence, in the presence of βFTZ-F1, EcR would then bind to the promoter region of *E93* to initiate transcription (Mathur, 2003). A second possible mechanism involves histone modification. Thyroid hormone receptors have been shown to carry out histone deacetylation, hence repressing transcription. In the presence of the hormone, histone acetyltransferases are activated to initiate transcription (Wolffe, 1997). βFTZ-F1 may follow a similar

pathway to enable *E93* to respond to ecdysone. A third mechanism by which βFTZ-F1 can provide *E93* with competence is through a change in conformation of the DNA. βFTZ-F1 can bind to sites on *E93* recognized by the early genes that repress its transcription. The absence of βFTZ-F1 before the late larval ecdysone pulse allows the binding of the early genes to repress *E93* expression. However, in the late prepupal pulse, βFTZ-F1 may bind to *E93* to change the conformation of the gene, inhibiting the binding of the repressor.

In conclusion, βFTZ-F1 provides competence to *E93* to respond to ecdysone. βFTZ-F1 has been shown to be a competence factor in mosquitoes. A mammalian homolog of βFTZ-F1 has also been shown to act as a competence factor. The goal of our study is to find the binding site(s) of βFTZ-F1 on *E93*. It will enable us to gain more insight into the molecular mechanisms that allow βFTZ-F1 to function in order to provide *E93* with the competence to respond to ecdysone. Studies conducted with βFTZ-F1 and *E93* can prove to be a good model to gain a better understanding of the mechanisms by which a gene can achieve competence to respond to hormonal signals. It would also allow us to understand competence factors for hormonal signals across species. Furthermore, it will enable us to recognize the complex mechanisms of stage- and tissue-specific responses brought about by a single hormone signal.

APPENDIX

Buffers and Solutions

Buffers for QIAprep Spin Miniprep Kit Protocol (Qiagen)
Complete information about the contents of the buffers has not been provided by the manufacturer.

Buffer P1:
RNase A is added before use

Buffer P2:
Contains NaOH

Buffer N3:
Contains GuHCl and CH_3COOH

Buffer PE: No information provided

Buffer EB:
10 mM Tris.HCl, pH 8.5

Buffers (B, C, D and E) for purification under denaturing conditions:
100 mM NaH_2PO_4
10 mM Tris-Cl
8 M Urea
Buffer pH B = 8.0, C = 6.3, D = 5.9 E = 4.5

1X DNA loading buffer:
80% Glycerol
1 mM EDTA
0.4% Bromophenol blue
0.4% Xylene cyanol

10X Nick translation buffer:
0.5 M Tris.Cl pH 7.4
50 mM $MgCl_2$
0.1 M 2-mercaptoethanol

Precision Plus Protein® Standards (Bio-Rad):
30% (w/v) glycerol
2% SDS
62.5 mM Tris pH 6.8
50mM DTT
5 mM EDTA
0.02% NaN_3

1X SDS gel-loading buffer:
50 mM Tris.HCl (pH 6.8)
100 mM dithiothreitol (DTT)
2% SDS
0.1% bromophenol blue
10% glycerol
Note: DTT should be added to the buffer just before use.

0.5X TBE running buffer:
0.045 M Tris-borate
0.001 M EDTA

TE buffer:
10 mM Tris.HCl pH 8.0
1 mM EDTA pH 8.0

Tris-glycine electrophoresis buffer:
25 mM Tris
250 mM glycine (pH 8.3)
0.1% SDS

10X Yao buffer:
1 M KCl
0.2 M Hepes pH 7.5
0.02 M DTT
1% NP-40

Procedures and Other Information

Table 2: Preparations for restriction digest with *Xho* I or *Eco*R I and *Hin*d III:

	***Eco*R I/*Hin*d III double digest**	***Xho* I digest**
pET42a- βFTZ-F1 (µl)	5	5
10x BSA (µl)	2	2
10x NE2 (µl)	2	2
Eco RI (µl)	0.5	-
Hind III (µl)	0.5	-
Xho I (µl)	-	1
dH2O (µl)	10	10

Table 3: Tris-glycine SDS-Polyacrylamide Gel Electrophoresis:

	10% Resolving	**6% Resolving**	**5% Stacking**
H_2O (ml)	4.0	5.3	2.7
30% (37.5 :1) acrylamide mix (ml)	3.3	2.0	0.67
1.5 M Tris (pH 8.8) (ml)	2.5	2.5	
1.0 M Tris (pH 6.8) (ml)	-	-	0.5
10% SDS (ml)	0.1	0.1	0.04
10% Ammonium persulfate (ml)	0.1	0.1	0.04
TEMED (ml)	0.004	0.008	0.004

Table 4: Polyacrylamide TBE gel:

	5% Resolving	**5% Stacking**
0.5X TBE (ml)	5.6	2.74
30% (37.5 :1) acrylamide mix (ml)	1.7	0.67
1.5 M Tris (pH 8.8) (ml)	2.5	-
1.0 M Tris (pH 6.8) (ml)	-	0.5
10% Ammonium persulfate (ml)	0.1	0.04
TEMED (ml)	0.008	0.004

Table 5: The number, percentage and characteristics of each amino acid in the 808 amino acid long βFTZ-F1 (Ohno and Petkovich, 1993):

Aminoacid	No.	Percentage	Characteristics
Arginine (R)	38	4.7	Polar (+)/Hydrophilic
Asparagine (N)	38	4.7	Polar/Hydrophilic/Neutral
Aspartate (D)	27	3.3	Polar (-)/Hydrophilic
Cysteine (C)	25	3.1	Polar/Hydrophobic/Neutral
Glutamine (Q)	43	5.3	Polar/Hydrophilic/Neutral
Glutamate (E)	47	5.8	Polar (-)/Hydrophilic
Glycine (G)	53	6.6	Aliphatic/Neutral
Histidine (H)	26	3.2	Aromatic/Polar(+)/Hydrophilic
Isoleucine (I)	36	4.5	Aliphatic/Hydrophobic/Neutral
Leucine (L)	78	9.7	Aliphatic/Hydrophobic/Neutral
Lysine (K)	36	4.5	Polar(+)/Hydrophilic
Methionine (M)	19	2.4	Hydrophobic/Neutral
Phenylalanine (F)	14	1.7	Aromatic/Hydrophobic/Neutral
Proline (P)	58	7.2	Hydrophobic/Neutral
Serine (S)	103	12.8	Polar/hydrophilic/Neutral
Threonine (T)	47	5.8	Polar/Hydrophilic/Neutral
Tryptophan (W)	4	0.5	Aromatic/Hydrophobic/Neutral
Tyrosine (Y)	19	2.4	Aromatic/Polar/Hydrophobic
Valine (V)	36	4.5	Aliphatic/Hydrophobic/Neutral

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