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THE ROLE OF βFTZ-F1 IN THE DISSOCIATION OF THE FAT BODY OF *DROSOPHILA MELANOGASTER* DURING THE PREPUPAL/PUPAL TRANSITION

by

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A Paper Presented to the

Faculty of Mount Holyoke College in

Partial Fulfillment of the Requirements for

the Degree of Bachelors of Arts with

Honor

Department of Biological Sciences

South Hadley, MA 01075

May 2008

This paper was prepared

under the direction of

Professor Craig Woodard

for eight credits.

ACKNOWLEDGEMENTS

I would like to thank Craig Woodard for his guidance and support throughout this process. It has been a wonderful and challenging experience for which I am deeply grateful. I'd also like to thank Amy Springer for her encouragement and support to write the thesis. If not for her, this thesis would probably not have been written. Thanks to Megan Nunez and Amy Springer for being a part of my thesis committee.

Special thanks to the Woodard fly lab, especially the other seniors. It was wonderful working with everyone and having people to commiserate with about my thesis.

Of course, a thank you goes out to my parents who promised to love me even if I did not write a thesis. Thanks also to my friends for their support.

I'd like to thank the Mount Holyoke Biology department for funding my research.

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ABSTRACT

Larval fat body dissociation is a major event during the metamorphosis of *Drosophila melanogaster*. The dissociation of the fat body results in a separation of fat tissue into individual cells distributed throughout the body and occurs at the same time as many other changes in the morphology of the fly. Despite detailed studies of the morphological changes that occur in the fat body, there is still much unknown concerning the genetic regulation of this event. Thus, the focus of my work is on possible genes controlling the transition of the fat body to individual fat cells. Many of the events of larval fat body dissociation occur simultaneously to events controlled by *βftz-f1*, such as abdominal muscle contractions and head eversion. By ectopically expressing *βftz-f1* using the UAS/Gal 4 system, I observed the levels of RNA expression of *βftz-f1* and the early genes *E93*, *E74A*, and *BR-C*. The results indicated that, while *βftz-f1* was ectopically expressed, the early genes are most likely not being expressed in the fat body.

INTRODUCTION

The study of fat: from fly to human

Obesity has become a major problem for humans, particularly for people living in the United States. This problem has led to increased investigations into fat and how the metabolic process works. As of 2002, a quarter of the people in the U.S. were considered obese and 55% of U.S. citizens were considered overweight (Wood, 2006). An excess of fat in the body poses many health risks, including type 2 diabetes and heart problems. Other organisms, including *Drosophila melanogaster*, have been used in the study of the fat.

The larval fat body in *Drosophila* serves as nutrient storage for the animal in preparation for metamorphosis (Schlegel and Stainier, 2007). As the fly uses the fat cells for energy, the cells die. The remaining cells left in the adult fly serve to feed the animal until it can reach another food source. They are also implicated in hormone production; the presence of larval fat cells is necessary for the adult males to participate in reproduction (Shlegel and Stainier, 2007).

Drosophila uses an insulin signaling network to regulate its fat body (Shlegel and Stainier, 2007). Insulin is used in humans to regulate the intake of glucose into muscle and fat cells where it is stored as glycogen (Wood, 2006). *Drosophila* has cells that function similarly to cells in the endocrine pancreas and

hepatocytes (cells that compose the majority of the mass in the liver). These cell types monitor and control the regulation of the fat body (Schlegel and Stainier, 2007).

There are two types of cells that control the regulation of lipid stores based on what state the animal is in: the fed state or the fasted state (Schlegel and Stainier, 2007). The fed state is controlled by insulin producing cells (IPCs). These cells release insulin which promotes the conservation of energy in the fat body. The IPCs are countered by adipokinetic hormone (AKH). Triacylglycerol (TAG) stores are activated when the animal is in need of energy (Schlegel and Stainier, 2007). AKH is a neuropeptide that functions in similar ways to glucagon and β-adrenergic agonists, both of which are found in vertebrates. AKH is present in the fasted state and is released by corpora cardia (CC) cells. The enzyme, TAG lipase, is activated by AKH (Schlegel and Stainier, 2007). TAG lipase partially hydrolyzes TAG into diacylglycerol molecules. Diacylglycerol is further broken down into free fatty acids (FAs). Partial oxidation converts the FAs into ketone bodies which can be used as an energy source by nearly any type of cell (Schlegel and Stainier, 2007). When TAG lipase is activated, it serves to increase the phosphorylation of lipid storage proteins (Schlegel and Stainier, 2007). TAG lipase phosphorylates the proteins with the help of cyclic AMP and protein kinase A. The mechanism used to phosphorylate proteins is similar to the mechanism used by mammals in the activation of lipolysis through β-adrenergic (Schlegel and Stainier, 2007). Another similarity between the metabolic process in humans

and flies is the regulation of triacylglycerol accumulation by the gene, *Adp*. The human ortholog to this gene is *WDTC1*.

Through the study of the metabolic process of *Drosophila melanogaster*, we can gain a greater understanding of the genetic mechanisms that control the metabolic process of humans.

Drosophila melanogaster **as a model organism**

Drosophila melanogaster has been used in research over the past hundred years. The fruit fly is used for many different reasons. For one, it is a small organism and thus does not take up much space in a laboratory. It is also easily and cheaply cared for by a researcher; many flies can exist on relatively little food. The ease and magnitude of reproduction is also appealing; it is possible for one female fly to lay up to several hundred eggs (Brookes, 2001). The short life cycle of the fruit fly allows for many different generations of flies to be observed without waiting years between one generation and the next (Fig. 1). Lastly, the fruit fly genome has been almost completely mapped. The mapping of the fly genome has allowed researchers to more easily manipulate the genes in *D. melanogaster*. The genome of *Drosophila* is approximately 180 Mb and contains four sets of chromosomes: an X/Y chromosome and three autosomes, 2, 3, and 4 (Adams *et al*., 200).

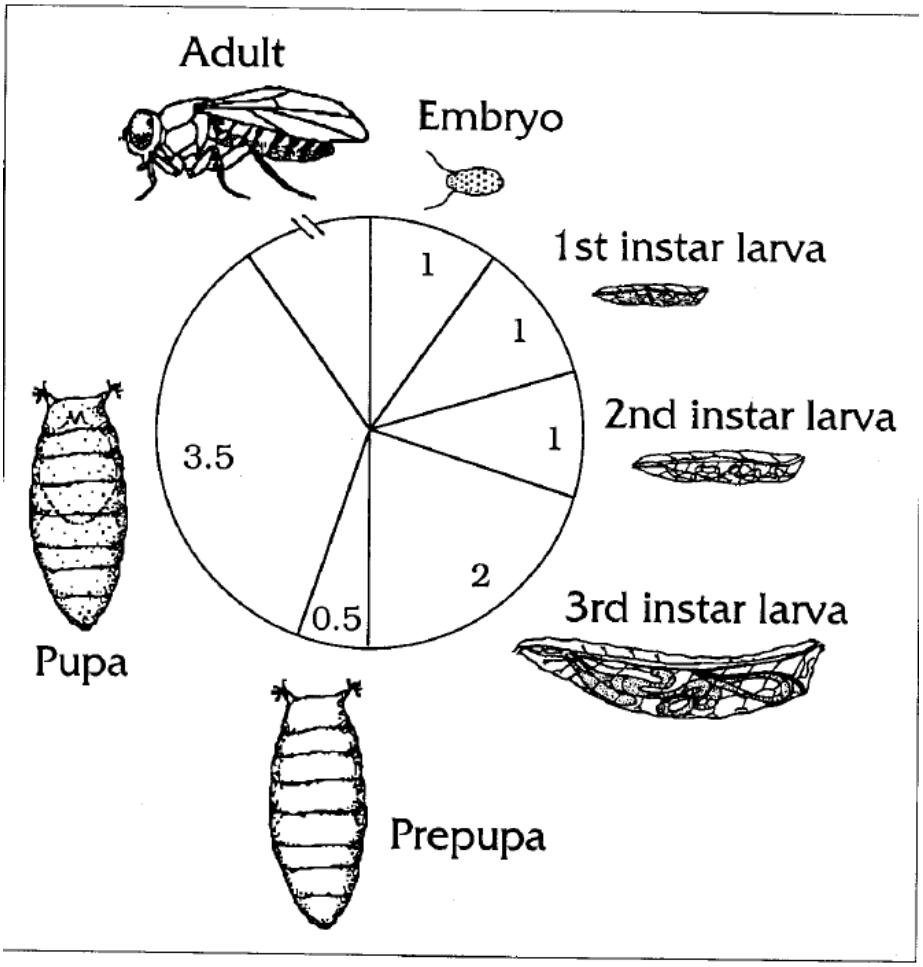

Figure 1: Life cycle of *Drosophila melanogaster*.

The life cycle of *Drosophila* is a relatively short affair. The whole process takes approximately 9-10 days to complete, during which *Drosophila* undergoes metamorphosis (Andres and Thummel, 1992).

Hormonal control of metamorphosis

During the life cycle of *Drosophila melanogaster*, the fly undergoes a complete rearrangement of its body structure. The larval tissues of *Drosophila* go through programmed cell death and are replaced by adult structures originating from imaginal progenitor cells (Andres and Thummel, 1992). These cells are epithelial sacs that arise during embryogenesis and will eventually form the adult body of the fly (Fortier *et al*., 2003). Two hormones control the events of metamorphosis: ecdysone and Juvenile Hormone (JH) (Riddiford, 1993). The presence of ecdysone is required in the restructuring of nearly all of the larval tissues (Andres and Thummel, 1992).

There are three known isoforms of ecdysone (Riddiford, 1993). The ecdysteroids are produced in the prothoracic glands and are then deposited in the haemolymph, mostly in the form α-ecdysone (King-Jones and Thummel, 2005). This isoform is then modified by surrounding tissues into the active form of 20-hydroxyecdysone (20E) (King-Jones and Thummel, 2005).

The steroid 20-hydroxyecdysone, which will henceforth be referred to as ecdysone, is instrumental in controlling metamorphosis of *D. melanogaster*. This steroid controls a vast array of processes in the fruit fly including apoptosis, reproduction, behavior, morphology, and physiology (King-Jones and Thummel, 2005). Ecdysone both activates cell growth and development in certain tissues, while at the same time causing programmed cell death in others (Buszczak and

Segraves, 2000). Ecdysone controls the transcription of seven nuclear receptors: *Ecdysone Receptor* (*EcR*), *Drosophila hormone receptor 3* (*DHR3*), *DHR4*, *DHR39*, *E75*, *E78*, and *ftz-f1* (King-Jones and Thummel, 2005).

The orphan nuclear receptor protein, EcR, binds with the Ultraspiracle protein to form a heterodimer (Buszczak and Segraves, 2000). (An orphan nuclear receptor is a nuclear receptor for which there is no known ligand.) This heterodimer is then bound by the ligand, ecdysone. The complex initiates the activation of genes known as the early genes, while at the same time repressing transcription of the late genes (Riddiford, 1993). The genes activated during the larval-pupal transition are *Broad-Complex (BR-C)*, *E74*, and *E75* (Beckstead *et al*., 2005). During the prepupal-pupal transition, the genes *BR-C*, *E74*, *E75*, and *E93* are activated when ecdysone binds the heterodimer of EcR and Ultraspiracle (Buszczak and Segraves, 2000). The early genes activate transcription factors that then initiate expression of the late genes at the same time as they suppress the early genes.

The levels of Ultraspiracle expression remain steady throughout development indicating that it is the fluctuations of EcR controlling events of development (Riddiford, 1993). Ultraspiracle also forms heterodimers with other NRs (King-Jones and Thummel, 2005). The protein, EcR, influences the expression of close to one third of the genes expressed during metamorphosis (Beckstead *et al*., 2005).

The titer of ecdysone fluctuates in *Drosophila melanogaster* throughout development (Fig. 2). There are believed to be a total of six pulses. These pulses occur in the embryonic stage, the three larval stages, before puparium formation, and during the prepupal stage (Andres and Thummel, 1992). Of the six pulses, there are two major pulses of ecdysone that take place during metamorphosis; one occurs during puparium formation and the other during the prepupal-pupal transition.

The larval-prepupal transition is induced by a pulse of ecdysone during the late third larval instar (Buszczak and Segraves, 2000). During this period the larval midgut undergoes programmed cell death and morphogenesis of the legs and the wings of the adult organism begins.

A second pulse of ecdysone, occurring 10-12 hours after puparium formation (APF), causes the prepupal-pupal transition (Buszczak and Segraves, 2000). At this time, movement of the gas bubble, formed during the prepupal stage, takes place and the bubble travels to the posterior end of the fly (Robertson, 1936; Fortier *et al*., 2003). The cuticle at the posterior end begins to pull away from the puparium (Fortier *et al*., 2003). Contractions of the abdomen induce multiple events. These events include forcing the gas bubble from the posterior to the anterior end of the fly, eversion of the head, and elongation of the legs and wings (Fortier *et al*., 2003). The movement of the gas bubble from posterior to anterior serves to separate the cuticle from the hypodermis (Robertson, 1936).

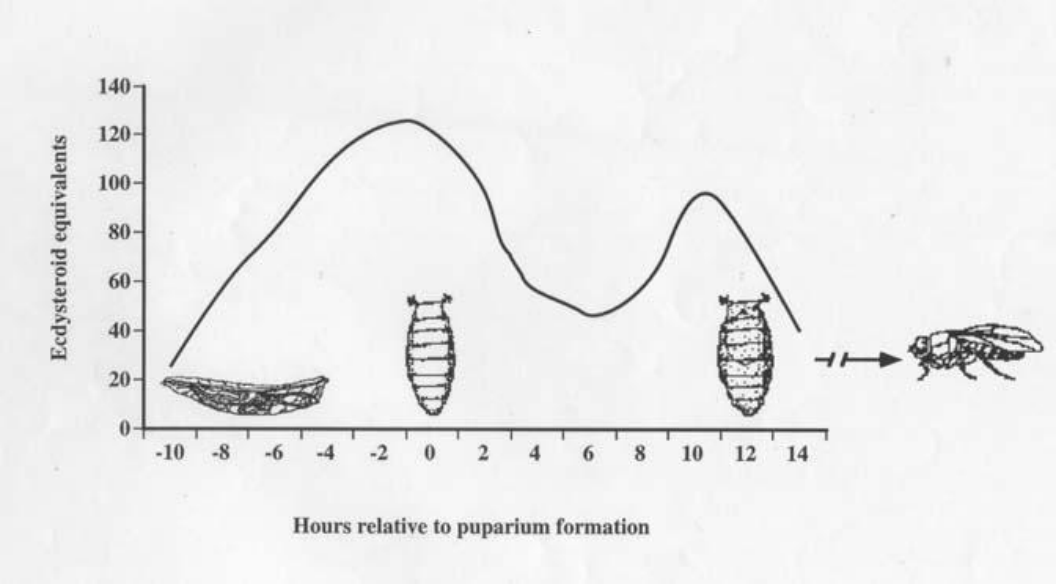

Figure 2: Ecdysone titer during *Drosophila* metamorphosis.

Two major pulses of ecdysone occur during metamorphosis. These peak at 0 hours APF and 10-12 hours APF. Both of these peaks correspond to major developmental events in metamorphosis, i.e. puparium formation and the prepupal to pupal transition.

Programmed cell death occurs in the larval salivary glands of the animal (Buszczak and Segraves, 2000).

Ecdysone controls the fate of cells through three different classes of cell death genes (Buszczak and Segraves, 2000). One of these classes, involved in cell death during the embryo stage, is the activators: *head involution defective* (*hid*), *grim*, and *reaper* (*rpr*). Apoptosis is prevented by the second class of cell death genes called inhibitors (Buszczak and Segraves, 2000). Genes included in this class are *Drosophila inhibitor of apoptosis-1* (*diap1*) and *Drosophila inhibitor of apoptosis-2* (*diap2*). The third class of genes is effectors (Buszczak and Segraves, 2000). Ecdysone promotes programmed cell death by up-regulating the activators and down-regulating the inhibitors. Other genes, such as *βftz-f1* and *E74*, are also believed to be involved in regulation of the effectors and activators (Buszczak and Segraves, 2000).

Nuclear receptors

Nuclear receptors contain a receptor function, DNA-binding sites, and transcriptional activation sites all within the same molecule (King-Jones and Thummel, 2005). They share a highly conserved DNA-binding domain (DBD) and a C-terminal ligand-binding and dimerization domain (LBD) that is less conserved (King-Jones and Thummel, 2005).

Ftz-F1, an orphan nuclear receptor, is one of 18 nuclear receptors (NR) found in *D. melanogaster* (Pick *et al*., 2006). The NRs, for the most part, control the activation of regulatory cascades. These cascades are regulated by the two hormones, JH and ecdysone (Pick *et al*., 2006).

Ftz-F1 regulates transcription of the gene *fushi tarazu* (*ftz*) (Woodard *et al*., 1994). The gene *ftz* is involved in embryonic segmentation and is expressed in the embryo in a pattern of seven stripes (Yamada *et al*., 2002; Pick *et al*., 2006).

There are two protein isoforms of Ftz-F1: αFtz-F1 which is 130-kDa and βFtz-F1which is 97-kDa. The two isoforms are consistent with the basic structure of nuclear receptors in that they have a DBD and an LBD (Pick *et al*., 2006). These domains are identical in both isoforms (Pick *et al*., 2006). αFtz-F1 has a longer N-terminal sequence than βFtz-F1, although the importance of this has not been discovered (Pick *et al*., 2006). Their presence in the organism also occurs at different times. αFtz-F1 is present in the early stages of the embryo at the same time as *ftz*, whereas βFtz-F1 is present in the late stages of the embryo and in successive stages in development at times when *ftz* is not expressed (Yamada *et al*., 2002).

Embryonic pattern formation is controlled by the transcription of specific genes under the control of αFtz-F1 (King-Jones and Thummel, 2005). βFtz-F1 is an orphan receptor (Buszczak and Segraves, 2000). This protein isoform of Ftz-

F1 is involved in the larval molts and also serves as a competence factor for ecdysone in the activation of the early genes (Lee *et al*., 2002).

The expression of *E93* is an example of a stage-specific response of a gene in which competence to ecdysone is obtained by βFtz-F1 (Broadus *et al*., 1999). The gene is activated by the pulse of ecdysone that occurs during the prepupal to pupal transition, after *βftz-f1* is expressed during the mid-prepupal stage (Broadus *et al*., 1999). Without βFtz-F1, *E93* is not competent to ecdysone and thus is not expressed in the animal during the previous pulse of ecdysone that occurred before puparium formation (Broadus *et al*., 1999).

The role of fat tissue in insects

Fat storage occurs primarily in adipose tissue (Loftus, 1999). Adipose tissue serves a dual role as both a source of energy and a source of hormones for adipocytes, fat cells (Loftus, 1999). The fat body in insects is a prominent organ that is arranged into thin layers of tissue to maximize the area of the fat body in contact with the hemocyte (Kilby, 1965). The area of contact between the fat tissue and the hemocyte allows for metabolites to pass easily from the fat tissue to the blood.

During the larval stage of development, a large amount of time is devoted by the animal to feeding. The organism needs to store as much energy reserves as

it can to prepare for metamorphosis. Nutrients are stored in the fat body in the form of fat, glycogen, and protein. These are present in the cytoplasm of the fat cells (Kilby, 1965).

The fat body of *Drosophila melanogaster*

The fat body of *Drosophila melanogaster* is derived from the mesoderm layer of the embryo in which the cells are set out in a metameric repeating pattern. It serves to sustain the continued growth and development of the fly during the period in which it has no access to food, i.e. from the prepupal stage to adult form (Aguila *et al*., 2007). The fat cells remain in *Drosophila melanogaster* throughout metamorphosis and are present in the adult fly (Hoshizaki, 2005). The larval fat cells are present in the animal for a total of 12 days (Butterworth *et al*. 1988). Fat body dissociation transforms the fat body from a connected tissue into a group of individual cells (Hoshizaki, 2005).

The embryonic fat body, as shown in Fig. 3, consists of three areas: the lateral fat body, the ventral commisure, and the dorsal fat-cell projection (Hoshizaki, 2005). The lateral fat body occupies the greatest amount of space and is composed of single-cell layers of fat cells (Hoshizaki, 2005). This bilateral layer extends between the body wall and the gut and is interrupted by the presence of holes in the tissue (Hoshizaki, 2005). The dorsal fat-cell projection runs parallel to the lateral fat body in the anterior direction and is connected with the

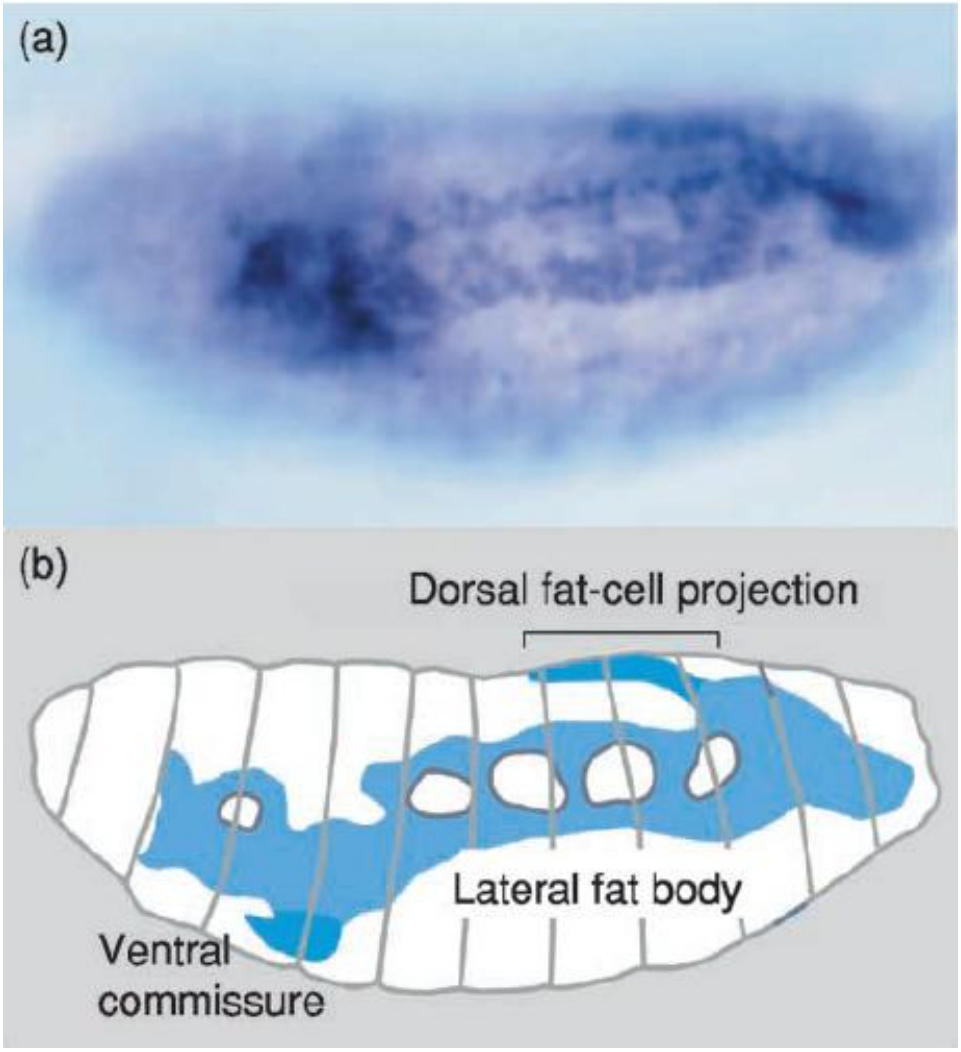

Figure 3: Structure of the fat body in the embryonic stage *of D. melanogaster.*

(A) Photograph taken of the larval fat body of an embryo in stage 16. The embryo was stained with Serpent protein (Hoshizaki, 2005). (B) Schematic diagram of the fat body in the embryo. The fat body is represented in blue. The lighter area shows the lateral fat body and the regions of dark area are representative of the dorsal fat-cell projection and the ventral commisure (Hoshizaki, 2005).

lateral fat body on the dorsal side of its posterior end (Hoshizaki, 2005). Extending from the lateral side of the anterior end of the fat body is the ventral commisure (Hoshizaki, 2005).

The larval fat body is involved in the maintenance and growth of *D. melanogaster*. The behavior of the organism is controlled based on the animal's nutritional needs (Hoshizaki, 2005). One class of growth factors made in the fat body is imaginal disc growth factors (IDGFs) (Hoshizaki, 2005). The exact functions of IDGFs are unknown. However, results from recent studies have supported the belief that IDGFs act with insulin to promote imaginal disc proliferation (Hoshizaki, 2005). Adenosine deaminase-related growth factors are another class of growth factors specifically produced by the fat body (Hoshizaki, 2005). These are also believed to promote imaginal disc proliferation (Hoshizaki, 2005).

The fat body of *Drosophila melanogaster* undergoes many morphological changes during development (Fig. 4). When the organism is a wandering third instar larva, the fat body consists of a tissue of interconnected fat cells that is located in the hemolymph between the body wall and the midgut (Hoshizaki, 2005). The shape of the cells, shown in Fig. 5, is polygonal and flat (Nelliot *et al*., 2006). Starting at 0 hours APF and concluding at 6 hours APF the fat body, still an intact tissue, retracts from the anterior portion of the fly (Hoshizaki, 2005). During this time the cells lose their flat, polygonal shape and become more

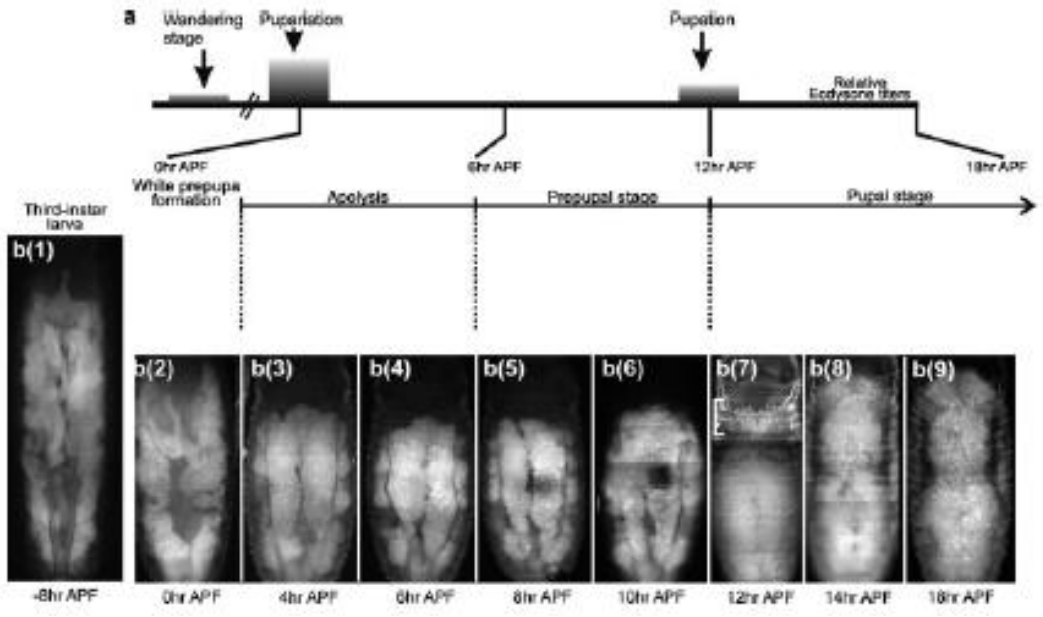

Figure 4: Morphological changes in the fat body of *D. melanogaster* during metamorphosis.

(A) Relative levels of ecdysone during metamorphosis. The two major pulses of ecdysone occur at pupariation and pupation. B(1-9) Pictures of the fat body taken at different intervals in development from the mid-third larval instar until 18 hours APF (Nelliot *et al*., 2006).

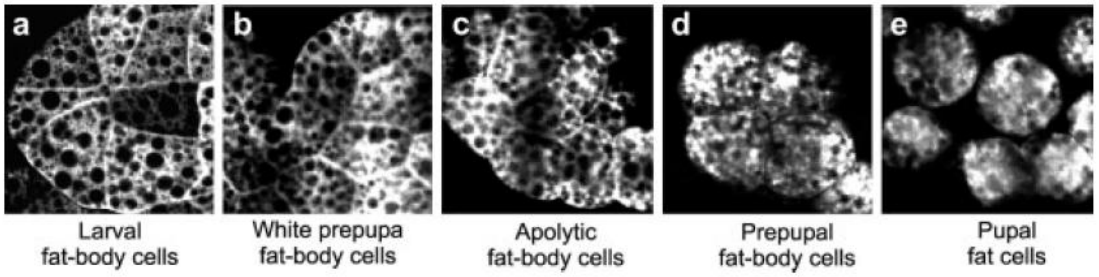

Figure 5: Change in the shape of the fat cells during metamorphosis.

Fat cells undergo a change in shape during dissociation. As the cells dissociate, they adopt a rounder appearance. The cells were taken from different stages in development: (A) third-instar larva (B) white prepupa (C) apolytic animal (D) prepupa (E) early-stage pupa (Nelliot *et al*., 2006).

spherical (Nelliot *et al*., 2006). Twelve hours APF the fat cells begin to appear looser and start to dissociate (Hoshizaki, 2005). Muscle contractions, responsible for eversion of the head, aid in the separation of the fat cells. Separation occurs from the anterior to the posterior end (Hoshizaki, 2005). Fat body dissociation concludes 18 hours APF (Hoshizaki, 2005). The fat cells in the newly eclosed adult exist as individual cells or in small free-floating groups (Nelliot *et al*., 2006). Failure of the fat body to dissociate leads to death in *Drosophila melanogaster* (Nelliot *et al*., 2006).

During metamorphosis, the change in the function of the fat body is mirrored by changes in its structure (Hoshizaki, 2005). Before the late third larval instar, the fat body is an intact tissue designed to supply the animal with storage proteins that are released into the hemolymph (Hoshizaki, 2005). Later, ecdysone induces the readsorbption of the proteins supplied by the fat body to the hemolymph back to the fat body and stores them in storage granules (Lepesant, 1978; Hoshizaki, 2005). The storage granules provide a ready supply of polypeptides and amino acids to the organism as it restructures its body (Hoshizaki, 2005).

The number of fat cells in a newly eclosed adult fly is less than the number of fat cells present in the larval stage by a difference of approximately 1,200 fat cells (Hoshizaki, 2005). The larval fat cells are depleted by 90% within the first two days of the life of the eclosed adult fly and vanish completely after

four days (Hoshizaki, 2005). These larval fat cells are then replaced by adult fat cells that arise from imaginal discs (Hoshizaki, 2005). The mechanism for fat cell death during metamorphosis is unknown, but is believed to be caused by programmed cell death (Hoshizaki, 2005).

The early Genes: *BR-C, E74A, E75A, E93*

Binding of ecdysone to the heterodimeric complex of the Ultraspiracle protein and EcR during the first major pulse of ecdysone causes the activation of the genes *Broad-Complex* (*BR-C), E74A,* and *E75A* (Lee *et al*., 2002). These genes are known as the early genes and their primary role is believed to be in activating transcription of the late genes (Lee *et al*., 2002).

Mutants that are missing *BR-C* are typically unable to begin metamorphosis and thus die as wandering third instar larvae (Crossgrove *et al*., 1996). BR-C protein accumulates in both imaginal and larval tissues (Fletcher and Thummel, 1995). Activation of the late genes is directly mediated by BR-C and late gene cis-acting regulatory elements. The two early genes *BR-C* and *E74* are believed to regulate some of the same events, such as puparium formation, pupation, and the activation of early genes (Fletcher and Thummel, 1995). The levels of expression of *BR-C* and *E74A* in the animal both peak at the same time, i.e. before puparium formation (Fletcher and Thummel, 1995). One specific event

regulated by *BR-C* and *E74A* is programmed cell death in the salivary glands. BR-C and E74A activate the genes *rpr* and *hid* (Lee *et al*., 2000).

E74A is believed to have an important role in the events controlled by ecdysone because it binds to numerous other early and late ecdysone-induced puffs (Karim and Thummel, 1999). Expression of E74A occurs in practically all of the larval tissues during the late third-larval instar. The importance of the gene is seen in mutants for *E74A* and *E74B.* These mutants typically die during either the pupal or the prepupal stage (Fletcher *et al*., 1995). Thus, *E74* is necessary for both puparium formation and for pupation.

The gene *E93* plays a critical role in the pathways leading to programmed cell death by the activation of specific genes (Lee *et al*., 2000). In mutants that have a loss-of-function for *E93*, programmed cell death does not occur in the salivary glands and defects in the destruction of the larval midgut are seen (Lee *et al*., 2000).

In a recent experiment, it was discovered that E75 is a thiolate hemoprotein (de Rosny *et al*., 2006). Hemoproteins are involved in the regulation of multiple functions in an organism; these include the transportation of storage of oxygen, nitric oxide transport, redox reactions, and electron transfer (de Rosny *et al*., 2006). E75 is able to bind both NO and CO (de Rosny *et al*., 2006).

The larval serum protein 2 (Lsp-2)

There are two major larval serum proteins, of the family arylphorins, present in *Drosophila melanogaster* during the third larval instar: Lsp-1 and Lsp-2 (Benes *et al*., 1990). *Lsp-2* is expressed solely in the fat body beginning in the third larval instar and continuing throughout the organism's life (Benes *et al*., 1990; Antoniewski *et al*., 1995). The expression of *Lsp-2* is regulated by the presence of ecdysone. Expression of the gene reaches its peak during the mid-third larval instar and declines before puparium formation. Finally, at the point at which the head everts, levels of Lsp-2 have reached a steady state at a low level of expression and remain at this level for the rest of the life of *Drosophila* (Antoniewski *et al*., 1995).

Previous findings of Deborah Hoshizaki

Unpublished work done by Deborah Hoshizaki reports on morphological changes in the larval fat body of *Drosophila melanogaster* when *βftz-f1* is ectopically expressed solely in the fat body of a mid-third instar larva. Premature fat body dissociation was shown to occur in the fat body of the modified animals (Fig. 6). Four hours APF, the fat cells begin to adopt a rounder shape and their adhesion to each other weakens. Two to four hours later, the actual dissociation of the cells is seen to occur, approximately four to six hours earlier than in a wild-type fat body.

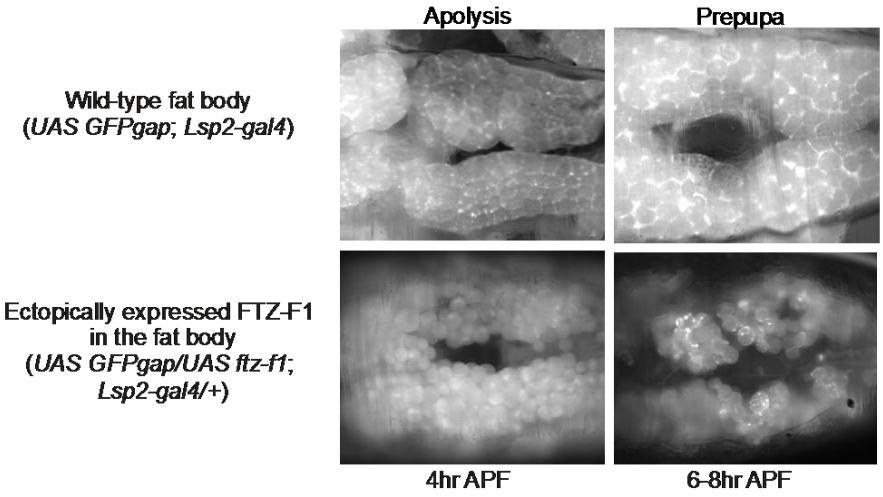

Figure 6: Premature fat body dissociation caused by the ectopic expression of *ftz-f1* in the larval fat body of *Drosophila*.

Unpublished work done by Deborah Hoshizaki examines the larval fat body in wild type flies and modified flies in which *ftz-f1* is ectopically expressed solely in the fat body. Ectopic expression of *ftz-f1* induces premature fat body dissociation.

Hoshizaki also found that the presence of ecdysone is required for fat body dissociation (Fig. 7). Tissues from the fat bodies of wild-type and modified *D. melanogaster* were cultured in Schneider's media at 25°C for 8 hours. For each condition, the larval fat body cultured without ecdysone did not dissociate. The larval fat body in which ectopic expression of *βftz-f1* occurred showed clear signs of dissociation. The wild-type larval fat body cultured with ecdysone did not dissociate.

Aim of my project

Despite knowing a great deal about the morphological changes in the larval fat body of *Drosophila* during metamorphosis, little is known about the genetic mechanisms controlling the event. In my research, I have attempted to discover more about possible changes in gene expression leading to the dissociation of the fat body.

The experiments I performed were based on the observation that many of the major events of larval fat body dissociation occur at the same time as events controlled by *βftz-f1* in the prepupal-pupal transition. The actual dissociation of the fat body begins during prepupal development, before the prepupal pulse of ecdysone. This corresponds to the presence of βFtz-F1 and the activation of the early genes *BR-C*, *E74A*, *E75*, and *E93*. Later in development, abdominal muscle contractions, believed to be controlled by βFtz-f1 or βFtz-F1 induced genes, serve

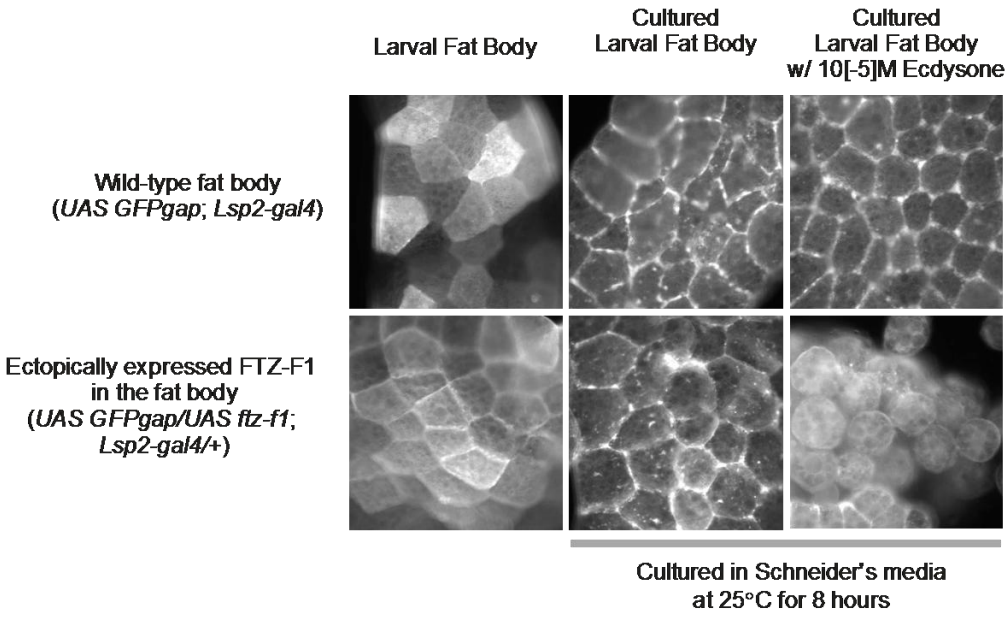

Figure 7: Presence of ecdysone needed to induce premature fat body dissociation.

Ectopic expression of *ftz-f1* in a cultured fat body is not sufficient for fat body dissociation. The presence of ecdysone is required. The images show the dissociation of fat bodies *in vitro*. Fat bodies did not dissociate in the absence of ecdysone. When cultured with ecdysone, the fat body in which *βftz-f1* was ectopically expressed was the only one to dissociate.

to force the dissociated larval fat cells into the head area (Fortier *et al*. 2003).

My hypothesis is that larval fat body dissociation is controlled by βFtz-F1 or βFtz-F1 induced genes. Deborah Hoshizaki has already shown that the fat body dissociates prematurely when *βftz-f1* is ectopically expressed in the mid-third larval instar. I studied RNA expression from whole animals of *Drosophila* through northern blot analysis. Knowing that premature fat body dissociation does occur the presence of elevated levels of RNA will indicate the possibility that the gene is being ectopically expressed and is involved in the process of fat body dissociation.

MATERIALS AND METHODS

Virgin collection from two stocks of *D. melanogaster*

I began my experiment by collecting female virgins from two stocks of flies provided by Deborah Hoshizaki. The first stock, *UAS ftz-f1 LA 276*, was homozygous for transgene with a UAS promoter attached to a *βftz-f1* gene. The second stock, *GFPgapLsp2-gal4*, was homozygous for transgene that had an Lsp2 promoter attached to a *Gal 4* gene. When crossed together, *βftz-f1* is ectopically expressed in the mid-third larval instar using the UAS/Gal 4 system.

To insure that the flies were virgins, I kept them in a 25°C incubator during the day and collected them at least every 8 hours. Overnight they were transferred to an 18°C incubator and collected at least every 18 hours. The virgins were separated by stock and sex and kept in vials until ready to be crossed.

Ectopically expressing *βftz-f1* in modified animals

In order to ectopically express *βftz-f1*, the two strains of flies were crossed. The virgin females from the stock *UAS ftz-f1 LA 276* were crossed with the males from the *GFPgapLsp2-gal4* stock and the males from the *UAS ftz-f1 LA 276* stock were crossed with the females from the *GFPgapLsp2-gal4* stock. The flies were

kept in vials containing fly food and yeast. The first generation was collected as the modified animals.

Isolation of RNA from whole animals

RNA was collected from five stocks of flies at three different developmental stages. The control stocks consisted of *UAS ftz-f1 LA 276*, *GFPgapLsp2-gal4*, Canton S wild-type (CS), and w^{1118} (wild-type flies with white eyes). The modified animals were the fifth stock. The flies were collected when they were wandering third instar larvae, 0 hour prepupae (PP), and aged 4 hour PP. Wandering third instar larvae were larvae that had left their food source and begun crawling on the side of the tubes. They were white in color and larger than the other larvae. Zero hour PP were distinguished by shortening in their length, broadening in their width, and eversion of the anterior spiracles (Demeree, 1915). At this stage, they still were white in color. Aged 4 hour PP were collected by putting 0 hour PP on a moistened petri dish. The petri dishes were put into Tupperware containers for four hours at 25°C.

The collected flies were homogenized by placing ~20-25 whole flies into RNase-free microfuge tubes, adding 1ml TRIzol reagent, and grinding them with a POLYTRON homogenizer. The microfuge tubes were kept in a -80°C freezer.

After removing the microfuged tubes from the freezer, they were allowed to thaw until they reached room temperature (RT). Phase Lock Gel-Heavy tubes were centrifuged for 30s at 1500 x g to ensure that the gel was settled onto the bottom of the tubes. The homogenized fly tissue was added to the Phase Lock Gel-Heavy tubes and the tubes were incubated at RT for 5 minutes. 0.2 ml of chloroform was added to the tubes which were then shaken for 15 seconds to mix. The tubes were again centrifuged for 10 minutes at 12000 x g in a 4°C refrigerator. At this point, the Phase Lock Gel separated the homogenized tissues into a clear, aqueous upper layer and a phenol-chloroform bottom layer. The phenol-chloroform layer was discarded and the aqueous layer was transferred to a separate microfuge tube and 0.5 ml of isopropyl alcohol was added to precipitate the RNA out of solution. The samples were mixed by inverting the microfuge tubes and were then left at RT for 10 minutes. Centrifugation of the samples for 10 minutes at 12,000 x g at 4°C caused an RNA pellet to become visible at the bottom of the tube. The supernatant was decanted from the microfuged tube. The pellet was air dried for approximately 5 minutes. The pellet of RNA was resuspended in 20 µl of 75% ethanol and then centrifuged at 7500 x g for 5 minutes at 4°C. The 75% ethanol was created using 3 parts ethyl alcohol at 200 proof with 1 part nuclease free water. The isolated RNA was again stored in a -80°C freezer.

Quantification of isolated RNA

To the microfuge tubes containing the isolated RNA, 30 µl more of nuclease free water was added. The tubes were then heated at 37°C for approx. 20 minutes. They were then centrifuged for 5 minutes at maximum speed. Using a spectrophotometer, the concentration of RNA was quantified by measuring the levels of absorbance at 260 nm. The amount of RNA to be added to each lane of the gel was determined by the amount of solution in the microfuge tube with the least amount of RNA. This was w^{1118} 3rd larval instar. There was 18 µl of isolated RNA solution for w^{1118} 3rd larval instar. Based on its concentration, as determined by the spectrophotometer, 18 µl of solution contains 18.3 µg of RNA. The amount of solution to be run on the formaldehyde gel for each of the microfuged tubes containing isolated RNA was calculated so that 18.3 µg of RNA would be in each lane of the gel. The concentrations of the isolated RNA solutions and the amount added to the formaldehyde gel for each condition is shown in Table 1.

Table 1. Amount of isolated RNA solution to run on formaldehyde gel

Stock	Developmental Stage	ng/µl	Amount run on gel (µl)
modified animals	3rd instar	2326.2	7.8
	0h PP	3740.2	4.9
	aged 4h PP	2544.1	7.2

CS	3rd instar	2849.3	6.4
	0h PP	1299.7	14.1
	aged 4h PP	1774.5	10.3
w^{1118}	3rd instar	1016.75	18.0
	0h PP	1547.9	11.8
	aged 4h PP	2205.8	8.3
UAS ftz-f1 LA 276	3rd instar	2005.3	9.1
	0h PP	1694.9	10.8
	aged 4h PP	1677.8	10.9
GFPgap Lsp2-gal4	3rd instar	3063.2	6.0
	0h PP	2291.8	8.0

Formaldehyde gel electrophoresis

The gel was prepared by mixing 150 ml distilled water and 2 g of agarose. This solution was boiled in a microwave until it was clear. Ten ml 20x MOPS buffer (see appendix) and 10 ml distilled water was added to the solution when it had reached RT. Next, 36 ml of formaldehyde was added to the solution. The solution was poured into the electrophoresis apparatus and allowed to set.

A cocktail was made of the sample buffer with 16 µl 20x MOPS, 56 µl formaldehyde, 160 µl formamide, and 12 µl 10mg/ml EtBr. 15.5 µl of the buffer and the amount of isolated RNA determined in Table 1 was mixed together and then put into a hot water bath at 70°C for 5-10 minutes. The loading buffer was added in 1 µl portions to each microfuge tube and the lanes were loaded. The gel was run at ~80 volts for 2.5 hours in 50 ml 20x MOPS and 950 ml distilled water.

It was then removed from the electrophoresis apparatus and imaged with UV light.

Northern blot

The gel was submerged in 20x SSC (see appendix) and put onto a gyratory shaker at a very low speed for 30 minutes. Meanwhile the northern blot was prepared by wetting a tent-like piece of blot paper laid across a raised surface in a rectangular Tupperware dish that had been filled with 100ml 2x SSC. The blot paper was wet with 20x SSC and the gel was inverted on top of the blot paper. The upper portion of the blot was removed with a clean razor and the gel was massaged to disperse any bubbles between it and the paper beneath. A piece of nylon hybridization membrane was placed in 2x SSC and then put on top of the gel. Again, the hybridization membrane was massaged to disperse any bubbles. Two more pieces of blotting paper were wet with 20x SSC and placed on top of the hybridization membrane. A tube was rolled across the top to remove any bubbles between the layers. Parafilm was placed around the edges of the gel to ensure that the 2x SSC would be drawn up through the gel and onto the hybridization membrane, instead of around the gel. A stack of paper towels was placed on top of the blotting paper to induce movement of the 2x SSC upwards through the materials by capillary action. A piece of glass was placed on top of the stack and it was left overnight.

The next day the gene screen was removed from the stack of materials used to create the blot and placed into 2x SSC. A piece of blot paper was cut and wet in 2x SSC. This piece of blot paper was then removed and run between two fingers to remove the excess liquid. The hybridization membrane was placed on top of the blot paper which was placed on top of a piece of glass. The hybridization membrane was then put into a UV stratalinker. The blot was wrapped in saran wrap, put into a freezer bag, and stored at -80°C.

Restriction digestion of recombinant plasmids and isolation of DNA through gel electrophoresis

The recombinant plasmids used for the restriction enzyme digests were provided by Craig Woodard. To prepare the restriction digest, the reagents needed for the digests were combined in a microfuge tube and incubated at 37°C for a minimum of two hours. In each probe preparation, the restriction enzymes were added last because of their sensitivity to heat. The restriction digest of *E74A* was left overnight at 37°C because the NE Buffer used only was only 50% effective for SalI. Table 3 shows the reagents used for each restriction digest.

Table 3. Reagents used in restriction digests

rp49	
Component	*Volume (µl)*
rp49 plasmid DNA	5.0
10X BSA	2.0
RNace-It Cocktail	0.5
NE Buffer 2	2.0
Xba I	0.5
Hind III	0.5
distilled water	9.5
E74A	
Component	*Volume (µl)*
E74A plasmid DNA	5.0
10X BSA	2.0
RNace-It Cocktail	0.5
NE Buffer 3	2.0
Bgl II	0.5
Sal I	0.5
distilled water	9.5

BR-C	
Component	*Volume (µl)*
BR-C plasmid DNA	5.0
10X BSA	2.0
RNace-It Cocktail	0.5
NE Buffer 2	2.0
STU I	0.5
BAMH I	0.5
distilled water	9.5
βftz-f1	
Component	*Volume (µl)*
βftz-f1 plasmid DNA	5.0
10X BSA	2.0
RNace-It Cocktail	0.5
NE Buffer 2	2.0
EcoR I	1.0
distilled water	9.5
E93	
Component	*Volume (µl)*

E93 plasmid DNA	5.0
10X BSA	2.0
RNace-It Cocktail	0.5
NE Buffer 4	2.0
Acc I	1.0
distilled water	9.5

The restriction enzyme digests were mixed with 1 µl of loading buffer and each was loaded onto a low-melting temperature agarose gel. A 1 kb DNA ladder (see appendix) was used as a reference for the molecular weights.

The gels were made by mixing 75 ml 1x TAE and 0.6g of low-melting temp agarose. The mixture of TAE and low-melting temp agarose was then heated in the microwave until the solution boiled and became clear. After heating, 5 µl of EtBr, at a concentration of 10mg/ml, was added and the agarose was then poured into the electrophoresis apparatus and allowed to set at 4°C for a minimum of 30 minutes.

The gels were submerged in 1x TAE and 10 µl 10mg/ml EtBr was added to the positive end of the apparatus. They ran at 80 volts for approximately 1.5-2 hours. Images of the gels are shown in Figures 8-12.The gels were imaged using the Fujifilm LAS-3000 imaging system.

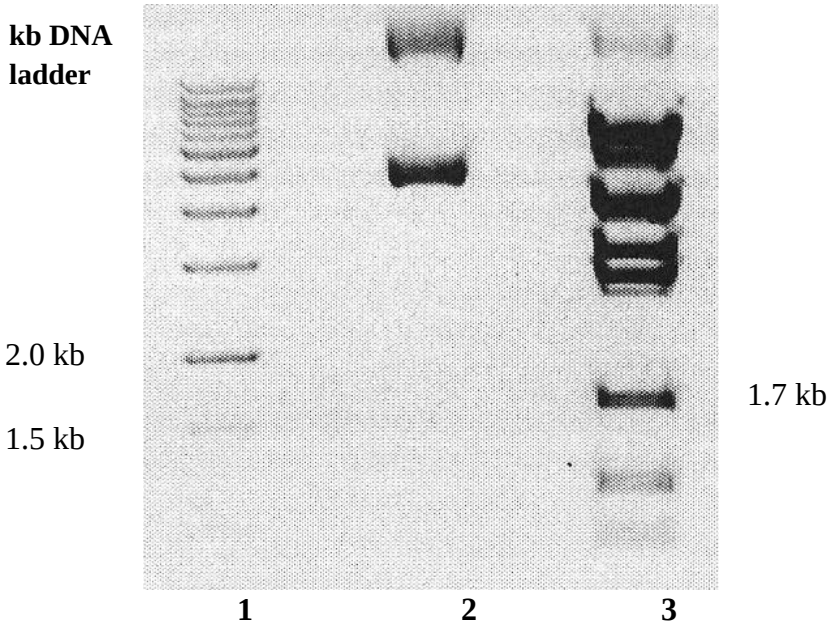

Figure 8. Separation of *βftz-f1* restriction digest on low-melting temp agarose with gel electrophoresis.

A plasmid containing *βftz-f1* was digested using the restriction enzyme EcoRI. The digest was then run on a low-melting temp agarose gel and the band at 1.7kb was cut out and refrigerated at 4°C. The digest of *rp49* did not work on this gel. Lane 1: 1 kb DNA ladder; Lane 2: failed digest of *rp49*; Lane 3: EcoRI digest of *βftz-f1*. A negative image was taken of this gel.

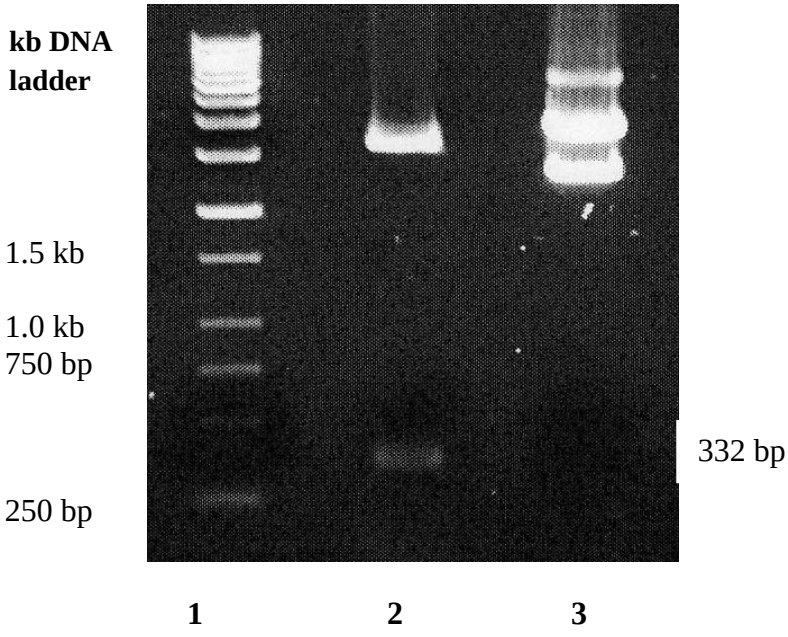

Figure 9. Separation of *rp49* restriction digest on low-melting temp agarose with gel electrophoresis.

Plasmids containing *rp49* were digested using XbaI and HindIII. The restriction enzyme digest was then run on a low-melting temp agarose gel and the band at 332 bp was cut out and stored in a 4°C refrigerator. Lane 1: 1 kb DNA ladder; Lane 2: XbaI/HindIII digest of *rp49*; Lane 3: EcoRI digest of *βftz-f1*.

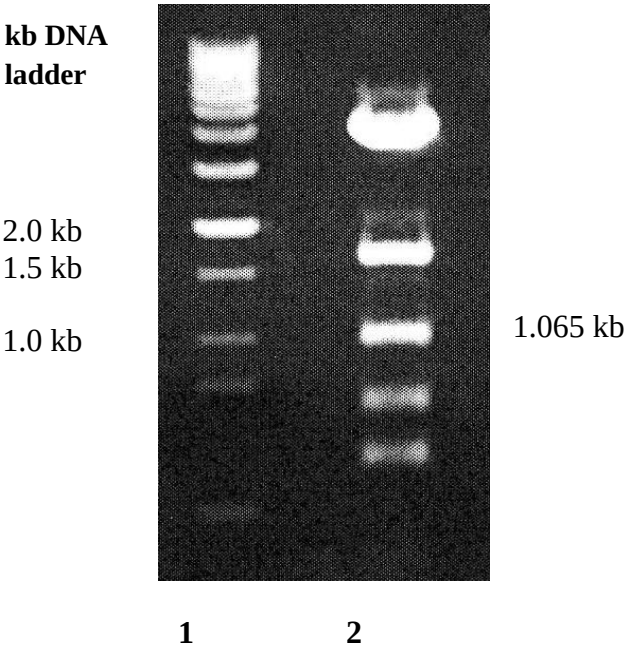

Figure 10. Separation of *BR-C* restriction digest on low-melting temp agarose with gel electrophoresis.

The restriction enzymes StuI and BamHI were used to digest plasmids containing *BR-C*. The digest was run on a low-melting temp agarose gel and the band at 1.065 kb was cut out and stored at 4°C. Lane 1: 1 kb DNA ladder; Lane 2: StuI/BamHI digest of *BR-C*.

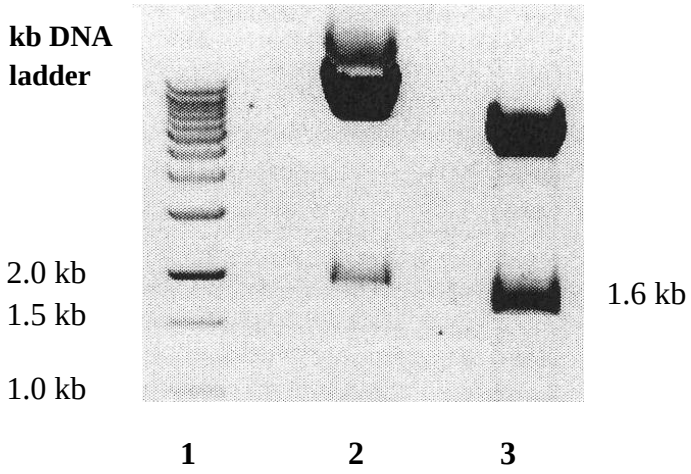

Figure 11. Separation of *E93* restriction digest on low-melting temp agarose with gel electrophoresis.

Plasmids containing *E93* were digested with the restriction enzymes AccI. The digest was run on a low-melting temp agarose gel and the band appearing at 1.6 kb was cut out of the gel and kept in refrigerator at 4°C. The digest of *E74A* did not work on this gel. Lane 1: 1 kb DNA ladder; Lane 2: failed digest of *E74A*; Lane 3: AccI digest of *E93*. A negative image was taken of this gel.

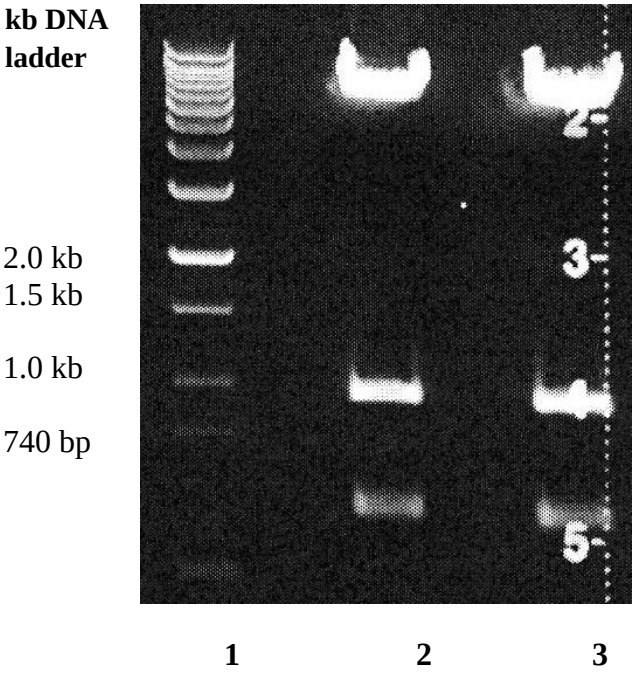

Figure 12. **Separation of *E74A* restriction digest on low-melting temp agarose with gel electrophoresis.**

Plasmids containing *E74A* were digested using the restriction enzymes BglII and SalI. The digest was run on a low-melting temp agarose gel and the band at 940 bp was cut out of the gel and stored at 4°C. Lane 1: 1 kb DNA ladder; Lane 2: BglII/SalI digest of *rp49*; Lane 3: BglII/SalI digest of *rp49*.

The band containing the restriction enzyme digests were cut from the rest of the formaldehyde gel and stored at 4°C. The band for *βftz-f1* was at 1.7 kb; the band for *rp49* was at 3*32* bp; the fragment of *BR-C* was cut out at 1.065 kb; the *E93* band was at 1.6 kb; the fragment of *E74A* was 940 bp.

Preparation of radioactively-labeled probes

The reagents used to radioactively-label the probes with ^{32}P were kept on ice during the experiment. Klenow DNA polymerase was kept at -20°C and cold tips were used to pipet the reagent into the solution.

The radioactively-labeled probes were prepared from the DNA obtained through the restriction enzyme digests. First, the gel slices were heated at 70°C to melt the low-melting temp agarose. In a microfuge tube, 10 µl of the probe DNA was mixed with 14 µl distilled water and 10 µl random nonamers. The solution was heated at 100°C to denature the probe DNA. It was then spun briefly and incubated at 32°C for a minimum of 10 minutes. During this time, the nonamers annealed to sequences of nucleotides on the probe DNA. Addition of 10 µl 5x dATP buffer, 5 µl dATP, and 1 µl Klenow DNA polymerase began polymerization of a new DNA template that included radioactively-labeled adenosine nucleotides. Finally, 2 µl stop solution was added to the reaction mixture.

The radioactively-labeled probe was then purified using a NucTrap Probe Purification column and a 10 ml syringe. To prime the column, 100 µl of TEN buffer (see appendix) was pushed through using the syringe to force the liquid down through the packed column. All of the radioactively-labeled probe solution, approximately 50 µl, was added to the column. Finally, another 50 µl TEN buffer was added.

To determine the level of radioactivity, 1 µl of the probe was placed onto a piece of filter paper in a plastic vial. After pipetting the sample, the tip was also released into the vial and a lid screwed on top. The plastic container was then placed in a Liquid Scintillation Counter (LSC). Beta emissions from the ^{32}P were measured by the LSC. This information was used to determine how much of the probe to add to the blot in order to have 1,000,000 counts/1 µl.

Prehybridization and hybridization of the blot

The hyb buffer was made fresh for each prehybridization. It consisted of 5ml formamide, 2ml 10x PIPES Buffer, 1ml 10% SDS (see appendix), and 2ml distilled water. After the blot was washed briefly with 2x SSC, it was placed in a cylinder with the hyb buffer and 100µl of herring sperm DNA that had been incubated at 100°C for a minimum of 10 minutes. The cylinder was then placed into the hybridization oven at 42°C and left for a minimum of two hours.

After the two hours, the radioactively-labeled probe was mixed with equal parts herring sperm DNA and incubated at 100°C for 10 minutes. This mixture was then immediately added to the cylinder and left overnight. The blot was imaged using a phosphor screen.

Washing and detection of the northern blot

After hybridization, the blot needed to be washed in order to remove any unhybridized probe. The blot was first washed with 2x SSC 0.1%SDS, a low stringency wash, and left at RT for 5 minutes. The blot was then placed in a 50/50 mixture of 2x SSC 0.1% SDS and 0.1x SSC 0.1% SDS. This wash was done at 55°C in a hot water bath for 30 minutes with gentle shaking. Additional washes varied between a repeat of the second wash with another 50/50 mixture at 55°C for 30 minutes or a 15 minute wash using only the 0.1x SSC 0.1% SDS solution at 55°C. The need for a third wash was determined by running a Geiger counter over the blot and looking for radioactivity around the edges of the blot where there should be no RNA.

Following the washes, the radioactively-labeled probe was detected on the blot using a storage phosphor screen. The phosphor screen was erased first and then placed over top of the blot, which had been covered in saran wrap, in a cassette. The cassette was left in the dark for approximately three to five days. The screen was then imaged using the Storm Gel and Imaging System.

Stripping the northern blot

To prepare the blot for a new radioactively-labeled probe, the blot first needed to be stripped of the old probe. This was done by immersing the blot in a solution of boiling TE (see appendix) for a period of 30 minutes. The blot was then ready to be labeled again with a different probe. One solution of TE was used for the stripping. Evaporated water was simply replaced each time by adding distilled water to bring the total volume of the buffer back to 1 L.

RESULTS

Formaldehyde gel

The isolated RNA from the crosses was run on a formaldehyde gel (Fig. 13). Each lane of the gel appears to have been evenly loaded with equal amounts of RNA.

Northern blot

The RNA run on the formaldehyde gel was transferred to a piece of nylon hybridization membrane. A UV stratalinker was used to crosslink the RNA with the blot. The radioactively-labeled probes were hybridized with the blot and the blot was imaged under a phosphor screen. Figure 14 shows the results obtained from the northern blots.

The rp49 bands all appear to be distinct and of the same intensity. The rp49 probe was used as a control to ensure the quality of the RNA and a relatively even concentration of RNA in each lane. If the quality of the RNA had not been good, the bands would have appeared to be smeared and the edges of the band would be less distinct.

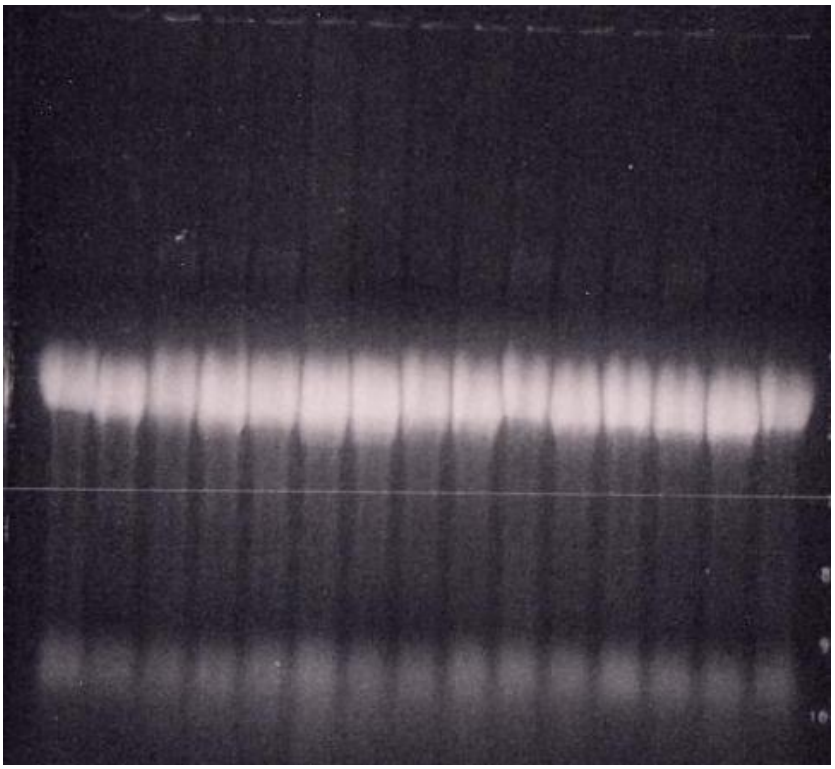

Figure 13. Formaldehyde RNA gel.

Image of the formaldehyde RNA gel. Isolated RNA was fractionated using gel electrophoresis on a formaldehyde RNA gel. The lanes appear to have run evenly and to have been evenly loaded. Lanes 1-5: 3rd instar larvae; Lanes 6-10: 0 hour PP; Lanes 11-15: aged 4 hour PP.

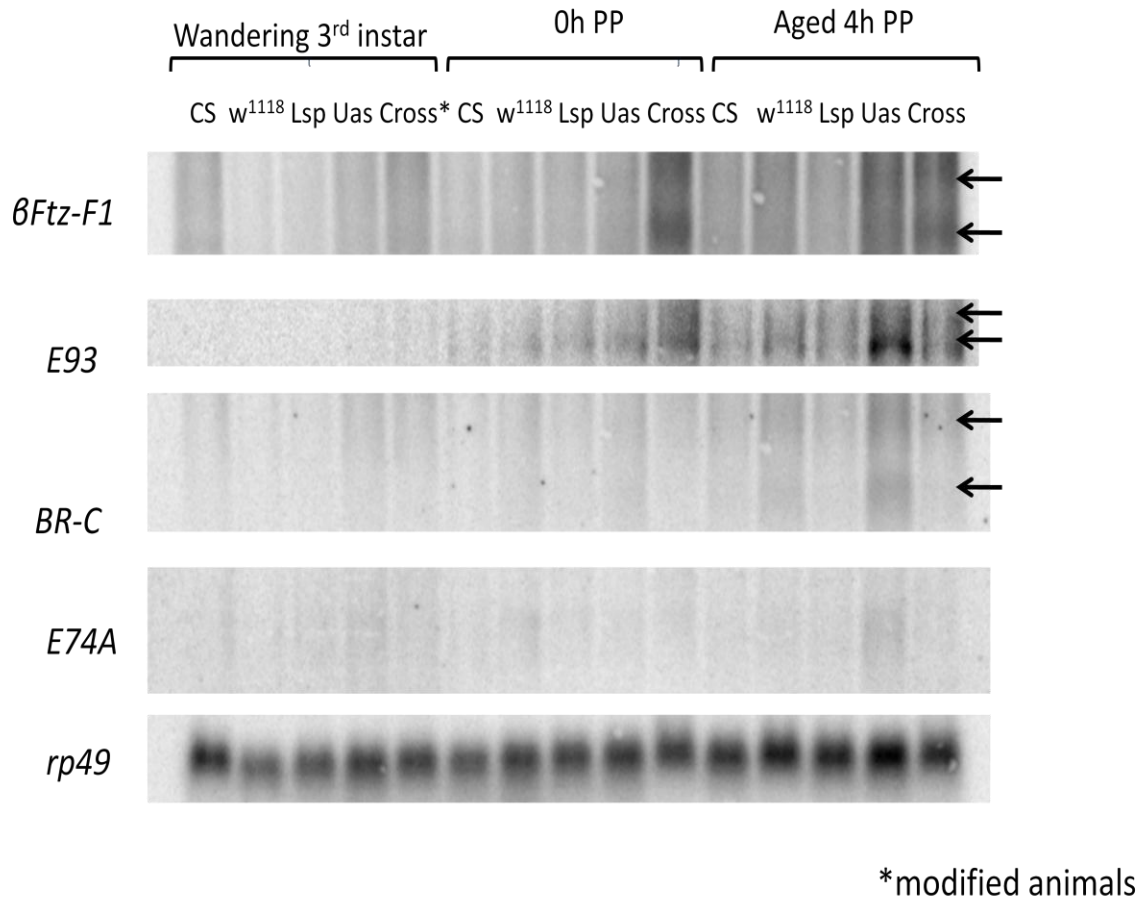

Figure 14. Northern blot data for *βftz-f1, E93, BR-C, E74A*, and *rp49*.

Transcription of the early genes, *βftz-f1*, and *rp49* during third larval instar, puparium formation, and prepupal development. Isolated RNA was transferred to a nylon hybridization membrane where it was then hybridized with different probes. The lanes are grouped according to stock and developmental stage. The arrows point to the bands, indicating the presence of the radioactively-labeled probe, present on the northern blot.

The levels of *βftz-f1* RNA expression are clearly elevated in the modified animals for 0 hour PP and aged 4 hour PP. The bands appear much darker and

more distinct in those lanes than in the others. In wild-type animals, *βftz-f1* is typically expressed at ~6 hours APF. For the third instar larvae of all the stocks, including the modified animals, expression of *βftz-f1* appears to be equal. The darkness of the bands appears to be uniform.

The levels of RNA of the early genes do not appear to be elevated in the modified animals at any developmental stage. There does appear to be darker bands seen for the aged 4 hour PP in the lane with RNA from the *UAS ftz-f1 LA 276* stock. This is believed to be due to an increased concentration of RNA in that particular lane and not due to an increase in the expression of any of the genes. The levels of expression of some of the early genes were much lower than expected when compared with the experiment done by Andres *et al*. on the temporal expression of genes controlled by the presence of ecdysone (1993).

DISCUSSION

Using northern blots to analyze expression of genes in *Drosophila melanogaster*

In this experiment, RNA levels were analyzed using northern blot analysis. Northern blots are easier to do than real-time PCR, but they require much more RNA. I wanted to see the levels of RNA of *βftz-f1* and the early genes and how they were affected throughout the body by the ectopic expression of *βftz-f1*. By using whole animals, there was easily enough RNA to do northern blots.

There are certain disadvantages to using northern blots. One of the disadvantages is that the RNA is very susceptible RNases and can be degraded easily. Furthermore, the blots have to stripped after each hybridization before hybridizing with a new probe. The stripping process is harsh on the blot and the blot is only able to be stripped a certain number of times. Lastly, the sensitivity of northern blots is less than that of real-time PCR because the RNA comes from the whole animal rather than a specific tissue.

The function of the early genes and their levels of expression on the northern blots

The early genes are induced by increases in levels of ecdysone during metamorphosis. There are two major pulses of ecdysone: the pulse at 0 hours APF and the pulse at 10-12 hours APF. At both of these pulses the early genes *BR-C*, *E74A*, and *E75A* are induced. The gene *E93* is only activated during the second pulse of ecdysone. βFtz-F1 acts as a competence factor for the early genes during the second major pulse of ecdysone. One of the major roles of the early genes is to regulate the transcription of the late genes. *E93* is different in that it plays a direct role in the initiation of programmed cell death.

The importance of the early genes in the development and metamorphosis of *Drosophila* is clearly established. Mutants for the genes *E74A* and *BR-C* typically die before they reach the pupal stage. Mutants for *E93* experience no programmed cell death in the larval salivary glands and defects in the programmed cell death of the larval midgut.

The results obtained from this experiment do not support the original hypothesis that one of the early genes is involved in the dissociation of the larval fat body despite their prominent role in the development of *Drosophila melanogaster*. None of the early genes are expressed at elevated levels in the modified animals. If one of the genes had elevated levels of RNA in the modified animals, the conclusion drawn would be that it was possible the early gene,

induced by βFtz-F1, is part of the mechanism of dissociation. Due to limited time, the blot was not hybridized with a radioactively-labeled *E75A* probe. Further work to be done would be to perform the hybridization and examine the relative levels of *E75A* RNA expression.

Ectopically-induced *βftz-f1* and the fat body

The results indicate that *βftz-f1* is being expressed ectopically in the modified animals using the UAS/Gal 4 system. The levels of *βftz-f1* RNA are approximately the same in all stocks during the third larval instar. At this point, one can assume that ectopic expression of the gene had not occurred. The Lsp-2 protein does not reach maximum levels of expression until the mid-third larval instar. The wandering third instar larvae, when collected, may not have had enough time to begin expression of ectopic βFtz-F1 at high enough levels to be visible on the northern blot.

The 0 hour PP had clearly elevated levels of *βftz-f1* RNA as did the aged 4 hour PP. At these stages, the UAS/Gal 4 system caused the ectopic expression of the gene resulting in elevated levels of *βftz-f1* RNA compared to the control stocks used in which the two transgenes were not incorporated into the genome of the flies.

The timing of the fat body dissociation and the fact that the fat body dissociates when *βftz-f1* is ectopically induced support the belief that *βftz-f1* acts as a competence factor for an unknown gene that controls the timing and events of larval fat body dissociation.

Hormonal control of larval fat body dissociation in *D. melanogaster*

No matter which genes are controlling fat body dissociation, it appears as if they are regulated by both ecdysone and *βftz-f1*. The images obtained by Deborah Hoshizaki show that fat body dissociation does not take place without the presence of ecdysone. The data obtained from her experiments supports the theory that the mechanism for fat body dissociation is hormone-regulated.

Integrins and their function in tissues

Genetic control of cell adhesion molecules is, most likely, involved in the dissociation of the larval fat body. Cell adhesion molecules are involved in the adhesion of cells to form tissues in the body of an organism.

Integrins are a family of cell surface receptors (Brown, 2000). These receptors serve to form connections between cell layers and the extracellular matrix (ECM) (Brown *et al*., 2000). The ECM serves to either hold tissues together or maintain separation between tissues (Brown, 2000). Signals emitted

from the ECM promote proliferation or differentiation of tissues (Brown *et al*., 2000).

Integrins consist of two single-pass transmembrane subunits: an α- and a β-subunit (Brown *et al*., 2000). There are 5 known α-subunits and two known β-subunits (Brown, 2000). The subunits are produced in the endoplasmic reticulum and form a stable, non-covalently linked heterodimer (Brown *et al*., 2000).

Studies on the functions of integrins in *Drosophila* show that integrins are involved in cell migration; in particular, the migration of primordial midgut cells (Brown, 2000). It is possible that the migration of the fat body from the anterior to the posterior part of the fly during the prepupal stage is controlled on some level by integrins.

***DHR39*: A possible gene involved in fat body dissociation**

Another nuclear receptor, DHR39, is homologous to Ftz-F1 (Pick *et al*., 2006). There is 63% similarity between the DBD of DHR39 and Ftz-F1 and 22% similar in the LBD. The NR contains a binding site for Ftz-F1 (Pick *et al*., 2006). There is still little known about this nuclear receptor. *DHR39* is characterized as an early-late puff. Unlike early puffs which are induced in a matter of minutes after the increase in ecdysone titer, early-late puffs are induced approximately 2 hours after a pulse of ecdysone (Huet *et al*., 1995).

The binding site for Ftz-F1 indicates a relationship between the nuclear receptor and the gene, which as stated before, is likely to be a requirement for the gene controlling fat body dissociation. The timing of the activation of the gene roughly corresponds with the timing to fat body dissociation. Activation of the early-late puffs occurs 2 hours after an increase in the ecdysone titer. The increase in ecdysone titer occurs at approximately 10-12 hours APF and the dissociation of the fat body occurs at 12 hours APF. The maximum range between these two events does not rule out the possibility that an early-late puff could be involved in controlling fat body dissociation.

Future Work

Due to time constraints, I was unable to prepare an *E75A* probe and hybridize it to my northern blot. Although *E75A* is not a likely gene involved in fat body dissociation, levels of expression would still need to be tested to rule out the involvement of any of the early genes.

This experiment should, most likely, be performed again. The levels of RNA expression obtained from the northern blots, specifically for *BR-C*, are much fainter than is expected. The levels of *E93* and *E74A* expression appear to correspond with the endogenous expression of the genes (Andres *et al*., 1993; Lee *et al*., 2000). The quality of the RNA on the blot is assured by *rp49*, so the most likely problem lies with the plasmid used for the *BR-C* probe.

The work done in this experiment supports my hypothesis that *βftz-f1* is being ectopically expressed in the modified animals. However, the results obtained did not show elevated levels of early gene expression. This indicates that while βFtz-F1 is, most likely, acting as a competence factor to ecdysone for a gene controlling fat body dissociation, that gene is not one of the early genes.

APPENDIX

Abbreviations

APF	After Puparium Formation
bp	base pair
CC	corpora cardia
DBD	DNA-binding domain
ECM	extracellular matrix
EcR	Ecdysone Receptor
EtBr	Ethidium bromide
IPC	insulin producing cells
JH	Juvenile Hormone
kb	kilo base pair
kDa	kilo Dalton
LBD	ligand-binding and dimerization domain
LSC	Liquid Scintillation Counter
Mb	Megabase
NR	Nuclear Receptor
PP	Prepupa
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
SSC	Sodium chloride sodium citrate
TAE	Tris-acetate-EDTA

DNA Marker

1 kb DNA ladder	50µl ladder 50µl loading buffer 400µl distilled water

Solutions for ^{32}P labeling

TEN buffer	0.5 ml 1M Tris Cl 0.1 ml 0.5M EDTA 1.0 ml 5M NaCl distilled water to 50 ml

Solutions for northern blot

20x SSC	1600 ml distilled water 176.4 g sodium citrate 350.6 g NaCl pH to 7.0
10% SDS	100 g SDS distilled water to 1 L heat to 68°C pH to 7.2
Tris EDTA (TE)	6.67 ml 1.5M Tris Cl 2 ml 0.5M EDTA 100 ml 10% SDS distilled water to 1 L

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