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Development of a Spermatogenesis Specific Gene Driver Using the UAS-GAL4 System

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ABSTRACT

The goal of the project was to develop a tool that allows for targeted expression of genes in spermatogenesis. To do this the GAL4/UAS system was employed. This system places the coding region of the GAL4 gene under a tissue specific promoter, ProtamineB in this case. The construct is transformed into *Drosophila melanogaster* and crossed with an existing UAS-reporter strain to make visualization of successful transformants easy. Expression was searched for in the testes by dissection and various detection methods. Unfortunately no expression was seen in the testes despite the construct being successfully integrated into the genome. This information tells us about the timing of expression within late spermatogenesis, and that a tool can successfully be developed to target genes of interest within spermatogenesis.

EXECUTIVE SUMMARY

This research aims to develop a gene driver tool capable of discovering the intimate genetic mechanisms involved in spermatogenesis. Spermatogenesis in *D. melanogaster* is one of the most dramatic instances of cell differentiation. A comprehensive understanding of spermatogenesis has useful applications in medicine, public health, and agriculture. The spermatogenesis pathways between fruit flies and humans are highly conserved and a better understanding of the genes involved and their expression patterns could lead to a useful treatment for male infertility.

In addition to human therapies, a strong understanding of the genes involved in spermatogenesis will allow researchers to select target genes to produce sterile males. Genetic knockdown experiments can be done with a UAS-RNAi reporter strain to reduce population in common insect pests. As the global population increases and food becomes an increasingly scarce resource, crop yields must be maximized and insects can be detrimental. RNAi knockdown can also be used to reduce population of insects that spread disease, such as mosquitos. Sterile males can be engineered and released into the wild, effectively reducing the population of the next generation. It is our hope that this research can provide useful information and novel tools for further advancing the knowledge of spermatogenesis within *D. melanogaster*.

TABLE OF CONTENTS

Abstract 2
Executive Summary 3
Acknowledgements 5

Introduction 6
Materials and Methods 9
Results 16
Discussion 26
Figures 31

References 43

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INTRODUCTION

Discovering genetic mechanisms allows researchers to gain a better understanding of the biology of an organism. Knowledge of genes and their functions have broad, and incredibly useful applications in agriculture, medicine, and industry. The functions of most genes are carried out by the proteins that they encode. Eliminating expression of the gene and observing how the phenotype is affected is a common way to determine gene function. The most common way to eliminate gene function is to carry out a mutagenesis experiment to create a loss-of-function mutation. Traditionally, this is done by treating the model organism with a mutagen and then screening F1 or F2 progeny for abnormal phenotypes [i.e., a forward genetic screen (St. Johnston, 2002)]. Because the mutagenesis procedure causes random damage to the genome, one must typically screen large numbers of offspring before finding the rare mutant in a gene of interest. More recently methods for targeted mutation of specific genes have been developed [e.g., CRISPR/Cas9 (Jinek, *et al*., 2012)] but these methods require advance knowledge of what specific gene is to be mutated.

Overexpression, or ectopic expression (i.e., expressing a gene at an inappropriate time or in the wrong place), of a gene of interest can also be useful in determining that gene's function (Phelps and Brand, 1998). In this case, the approach is usually to clone the gene of interest into some type of expression vector and then create transgenic individuals that will express the gene either constitutively, or under the control of some type of inducible or tissue specific promoter (Prelich, 2012).

One area of interest in our lab is to understand the genetic control of the complex developmental process of spermatogenesis in the *Drosophila melanogaster* model system. Spermatogenesis is one of the starkest examples of cell differentiation, during which a round, diploid, unspecialized germline stem cell becomes transformed into an incredibly long, streamlined, haploid, motile spermatozoa, capable of being transferred to the female reproductive tract where it can survive for many days until it is used for fertilization of an egg (Fuller, 1998; Demarco, et al., 2014). Understanding how this dramatic developmental process happens, and how genes responsible function and interact has important implications for subjects such as male fertility, biological pest control, and developmental biology (Sutton, et al., 2016; Demarco, et al., 2014). Spermatogenesis in Drosophila begins at the apical tip of the testis with the division of germline stem cells into a new germline cell and a gonial cell (Figure 1). The gonial cells undergo four rounds of mitotic division, but do not fully separate, yielding a sixteen-cell "cyst" of interconnected primary spermatocytes. Following a growth phase in which the spermatocytes increase in size, each of the sixteen cells undergoes meiosis, resulting in a cyst of 64 round, haploid spermatids, each containing a prominent round nucleus and a structure called the nebenkern, consisting of fused mitochondria. At this stage, the 64 spermatids elongate in synchrony, and the nuclei condense and experience a dramatic shape change, eventually assuming dense, needle-shaped configurations. The spermatids then undergo individualization, during which the interconnected spermatid bundle is resolved into 64 separate sperm cells, after which they coil up for storage in the seminal vesicle (Fuller, 1998). The orchestration of these complicated events is controlled by the coordinated activity of many genes, not all of which have been elucidated. One goal of

our lab is to test collections of various candidate genes of interest for those that play an important role in some aspect of this complicated developmental process.

A powerful tool for manipulating gene expression of candidate genes of interest (GOI) is the GAL4/UAS binary system (Duffy, 2002). This system employs the *GAL4* gene from common baker's yeast, *Saccharomyces cerevisiae*. The GAL4 protein is a transcriptional activator that binds tightly to an upstream activation sequence (UAS) to activate the transcription of the downstream gene. To study a GOI the system is set up in such a way that the *GAL4* coding region is placed under the control of a known promoter sequence. This way, whenever the native gene would normally be expressed the *GAL4* gene is expressed in its place. Separately, the UAS sequence is placed in front of a GOI. If the GAL4 and UAS-GOI transgenic lines are crossed, individuals with both components will be produced in the F1. In these individuals, GAL4 will bind to the UAS and drive target gene expression in the desired time or tissue-specific manner (Figure 2). The GAL4/UAS system can target a specific gene of interest for study of expression in a controlled manner *in vivo*.

RNA interference is a powerful tool for understanding the function of specific genes within a pathway. A gene can be "knocked down" using RNAi and the function of the gene can be deduced based on the phenotype of the organism. RNA interference is a mechanism in which a double-stranded RNA molecule triggers the destruction of the mRNA that contains an identical sequence, thereby leading to silencing, or "knockdown," of the corresponding gene's expression (Hannon, 2002). In this process, a protein called Dicer cuts double stranded RNA (dsRNA) into short fragments and unwinds them. The RNA induced silencing complex (RISC) uses the short interfering RNA (siRNA) from

Dicer as a guide to bind to a corresponding sequence in an mRNA and cleaves it, thereby reducing the level of that targeted mRNA. Since the mRNA is destroyed no protein is made and the gene is effectively silenced (Bernstein, *et al.*, 2001; Hannon, 2002).

Using the UAS/GAL4 system in tandem with RNAi, genes of interest can be targeted for knockdown. In this approach, a transgene is created in which an inverted repeat sequence for a GOI is placed downstream of the UAS. By crossing this line to a GAL4 expressing strain, the F1 individuals will express the inverted repeat which will fold up into a dsRNA hairpin which will trigger RNAi, thus knocking down expression of the GOI (Figure 3; Yamamoto-Hino and Goto, 2013).

The goal of this project is to create GAL4 "driver" constructs and transgenic fly lines that can be used to target overexpression or RNAi knockdown of any gene of interest during spermatogenesis. To accomplish this, we sought to use the promoter region of the *ProtamineB* gene to drive expression of GAL4 in a spermatogenesis-specific manner. This gene is transcriptionally activated during the late stages of spermatogenesis, and its product, ProtamineB, replaces the histone-based nucleosomes and is responsible for the tight packaging of the paternal DNA in the sperm head (Raja and Renkawitz-Pohl, 2005). The hope was that transgenic lines containing the ProtamineB-GAL4 driver could be used in conjunction with UAS-GOI transgenic lines to target overexpression or knockdown of the GOI specifically during spermatogenesis. By examining the phenotypic effects of this on sperm development or function, the normal role of the GOI might be inferred.

MATERIALS AND METHODS

Gel Electrophoresis

Gel electrophoresis was performed using 0.7% agarose dissolved in 1X Tris-Acetate EDTA (TAE) buffer. 2μg/mL of ethidium bromide was added to the TAE buffer. 4μL of bromophenol blue dye was added to each DNA sample before it was loaded into the gel. A HiLo DNA ladder (Bionexus Inc.) was used to approximate DNA size of the sample. Normal gel run time was 30 minutes at 90 volts. Gels were visualized with a UV lamp.

Polymerase Chain Reaction (PCR)

PCR was set up in a 0.5mL Eppendorf PCR tube with the following ingredients: 4μL 5X GoTaq buffer, 2μL dNTP mix (2mM of each nucleotide), 1μL 5' primer (5μM), 1μL 3' primer (5μM), 2μL sample DNA (1/100 dilution of genomic or 1/1000 of plasmid), 10μL sterile, distilled water. The usual reaction was performed as follows:

- Denature the DNA for 5 minutes at 95°C, then return temperature to 72°C.
- Add 5μL of diluted Taq polymerase to a final volume of 25μL. Dilution was made with 19μL of sterile, distilled water, 5μL 5X GoTaq buffer, 1μL Taq polymerase.
- PCR cycles were started with the following format:
    - 94°C for 1 minute to denature DNA
    - 58°C for 1 minute to anneal primers
    - 72°C for 1 minute to extend DNA
    - Repeat 34 times
    - 72°C for 10 minutes for final extension

- Hold at 10°C

Run 10µL on a gel to test success of PCR. Table 1 contains the list of primers used for this project.

Table 1: Primers used and their sequences

Primer Name	Sequence (5’ to 3’)
Ubprom5 BF	AGATCTGGCCGGCCAACGCAGCGACAGGGATTC
Ubprom3 B	AGATCTGGATTATTCTGCGGGAAGAAAATAGAGATGTGG
GFPsense	AAGGTGAGCAAGGGCGAGGAGCTG
GFPantisense	TTACTTGTACAGCTCGTCCATGCC
GAL1s	TGACATGTCGGATGGCTTGC
GAL2as	GGAGCCTGTTAACGTTAGAG
GAL3s	GGTCTGTCTACTCTTGGGAG
GAL4as	CTCGAAACCTCCTCAATCTC
GAL5s	CAAACTCAAAATCGAATGCTGAG
GAL6as	CATACCTCTTCCAGTACTTG
GAL7s	GTAGCAACGGTCCGAACCTC
GAL8as	CACAGTTGAAGTGAACTTGC
ProtBPs	AGATCTGGCCGGCCGGTACCACTTTCGACCATATAAAG
ProtBPas	AGATCTCAACTAATGTTTAAAAAAACCGACTGCCCG
pW8.TS1upstream	CAAAGCCACATACACTTTTCGCTGC
pW8.TS1downstream	TATGAGGTTTGGCTTTCTGGATCATAG

DNA Ligation Reactions

Ligation reactions were generally performed using 5µL of DNA PCR product, 1.5µL 10X T4 DNA Ligase buffer, 1µL T4 DNA Ligase, 1µL of plasmid vector, and 6.5µL of sterilized, distilled water for a total volume of 15µL. Ligations were incubated overnight at 14°C.

Restriction Enzyme Digests

Restriction enzyme digests were usually performed with the following volumes and reagents: 5µL DNA of interest, 2µL of appropriate 10X buffer, 1µL of appropriate restriction enzyme, and 12µL sterile, distilled water. Digestion time was usually 1 hour at 37°C, but was occasionally extended when needed. Digest results were confirmed using gel electrophoresis.

Transformation of E. coli using electroporation

LB + Ampicillin plates were placed into an incubator at 37°C to warm up. Electroporation cuvettes were pre-cooled on ice and DH5α electrocompetent *E. coli* cells were removed from the -80°C freezer and placed on ice to thaw. 1µL of sample DNA was added to each tube of electrocompetent cells and gently mixed by pipetting. 300µL of sterile LB was pipetted into a 14mL sterile tube. Cells with added DNA were transferred into an electroporation cuvette and the cuvette was placed into the electroporator with voltage set to 1800V. After the electric pulse, 300µL of LB was added to the cuvette and mixed well. The cells were transferred to the 14mL tube and incubated at 37°C for 45 minutes to an hour. The cells were then transferred to a sterile glass tube. 15µL of ampicillin stock (10mg/mL), X-gal (50µL of 2% solution) and IPTG

(10µL stock) was added. Roughly 3mL of melted top agarose was then added, mixed, and then poured onto the pre-warmed agarose plate. After the agarose had hardened, the plate was inverted and placed in the 37°C incubator to grow overnight.

Plasmid Miniprep

1.5mL of terrific broth (TB) with Ampicillin (50µg/mL) was inoculated with bacteria containing the plasmid of interest in a 14mL sterile tube. Culture was grown overnight in shaker at 37°C. Culture was transferred into 1.5mL Eppendorf tube and spun in a microfuge for 3 minutes at 13,000 RPM. Supernatant was poured off and 200µL of resuspension buffer was added. The pipette tip was used to break up the pellet and resuspend cells. 200µL of lysis buffer was added, mixed by inverting, and let sit for about 5 minutes. 200µL of neutralizing buffer was added and mixed by inversion, then put on ice for 10 minutes. Next, the solution was spun in a microfuge for 7 minutes at 13,000 RPM. The supernatant was transferred to a new 1.5mL Eppendorf tube. 450µL of isopropanol was added and let sit on ice for 10 minutes. The tube was centrifuged for 10 minutes at 13,000 RPM. A small, white pellet was visible. Isopropanol was poured off and 500µL of 70% ethanol was added. Sample was spun briefly and the ethanol was poured off. The excess ethanol was blotted off with a Kimwipe. The pellet was vacuum-dried until all ethanol was evaporated, about 10 minutes. The DNA pellet was resuspended in 100µL TE and incubated at 37°C for 15 minutes. DNA was checked by restriction enzyme digest.

Plasmid Midiprep

Midipreps were done using Qiagen CompactPrep Plasmid Kits (cat # 12843 and 12863). The protocol for high-copy plasmid midiprep, included with the kit, was followed for experiments.

Microinjections

DNA Preparation

15µg of purified plasmid was mixed with 5µg of helper plasmid, Δ2-3 wc. The mixture was ethanol precipitated using 1/10 volume 3M NaOAC and two times 100% ethanol. The sample was centrifuged for 30 minutes at 13,000 RPM, and then washed with 70% ethanol. The pellet was resuspended in 50µL of injection buffer (5mM KCl, 0.1mM $NaPO_4$ buffer pH 7.5).

Embryo Collection

Embryos from w^{1118} stock flies were collected on apple agar plates (100mL apple juice, 100mL sterile, distilled water, 6g agar, 2mL glacial acetic acid, 2mL methyl-P-hydroxybenzoate in ethanol) smeared with yeast extract mixed with water. Flies were allowed to lie for 30 minutes before changing apple agar plates. Embryos were transferred to a microscope slide with a sliver of double-sided scotch tape (tape was moistened before transferring embryos). Roughly 12 embryos fit per slide. The embryos were oriented so the posterior end was hanging just off the tape using a moist, fine tip brush; if the chorion ruptured, or the embryo ruptured, it was discarded.

Injections

5µL of DNA mixture was placed into 0.5mL Eppendorf tube with 4µL red dye. A pulled glass needle was loaded by backfilling the needle. The loaded needle was connected to

plastic tubing connected to a 10mL syringe; to seal the needle the junction was wrapped in parafilm. The slide with embryos was placed on the microscope stage. The needle tip was aligned with the center of an embryo, and then pierced the embryo. The needle was pulled back as far as possible without leaving the embryo, and then the DNA was injected. A good injection did not rupture the embryo, and the DNA was visible inside the embryo. This process was repeated for each embryo. The tape slice was removed and placed in a food vial – flies that developed from these embryos were considered G_0 flies. Adult G_0 flies were crossed with wild-type flies. G_1 flies were screened for green-eyed transformants.

Dissections

Flies were dissected in PBS with fine tip forceps. Testes were fixed in 4% formaldehyde in PBS in depressive slides. Fixed testes were rinsed 3 times for 10 minutes in PBST (0.1% Tween). Testes were placed on microscope slide with a coverslip on top. Edges of coverslip were sealed with rubber cement. Testes were viewed under fluorescent microscope.

X-Gal Staining

Flies were dissected in PBS, and then fixed in 3% formaldehyde in PBS for 20 minutes. The tissues were rinsed three times with staining buffer (10mM NaH_2PO_4/Na_2HPO_4 pH 7.2, 1mM $MgCl_2$, 150mM NaCl, 5mM $K_4[Fe^{II}(CN)_6]$, and 5mM $K_3[Fe^{III}(CN)_6]$), and then incubated for 10 minutes at room temperature in staining buffer. The staining buffer was removed and replaced with 250µL of staining buffer containing 20µL of 1% X-gal in

dimethylformaldehyde. The depression slide is covered with a coverslip to prevent evaporation and it is placed in a moist chamber. Tissues were incubated at 37°C in the solution overnight, and then washed in PBS plus 1mM EDTA to stop the reaction.

RESULTS

The goal of this project was to create novel GAL4 "driver" constructs that could be used to target expression of various genes of interest (GOI) in cells undergoing spermatogenesis. This would allow researchers to assess what effects ectopic expression (or overexpression) of a specific GOI has on sperm development or function. These GAL4 drivers could also be used to drive targeted expression of hairpin RNA's to trigger RNA-interference and thereby "knockdown" expression of the GOI's to assess their role during spermatogenesis. These constructs will provide a useful genetic tool for dissecting the genetic control of spermatogenesis and further our understanding of this important developmental pathway. As a control, we also sought to construct a constitutive GAL4 driver that would direct expression of any GOI not only in the male germline but also in somatic cells throughout the fly.

The overall strategy for these experiments was straightforward: use PCR to amplify and clone a cassette containing upstream regulatory sequences, *i.e.*, promoter region from known genes that are expressed in a testis-specific manner (or constitutively in the case of the control), and clone that fragment upstream of the coding region of *GAL4*. This construct could then be subcloned into a transformation vector that would allow us to introduce this GAL4 driver into the *D. melanogaster* genome using germline

transformation methods. Once transgenic flies are created, the chromosome linkage of the insertions could be genetically mapped using segregation analysis, and homozygous stocks established. Finally, the GAL4 driver lines could be tested by crossing them to existing UAS-reporter lines such as *UAS-GFP* or *UAS-lacZ* to examine whether or not they effectively drive expression of the reporter in the expected testis-specific manner.

Creation of the pBS/GAL4 plasmid

For all of the constructs, the first step was to create a plasmid containing the *GAL4* coding region flanked by convenient restriction enzyme cutting sites that would allow us to first insert the promoter cassette upstream of *GAL4* and then cut out the Promoter-GAL4 fusion gene fragment for subcloning into a unique cloning site of a transformation vector.

The starting point for this step was an existing plasmid called pBS/Pros25-Gal4#11, which had a fragment of the *Pros25* proteasome subunit gene (also called *Prosa2*) upstream of the *GAL4* coding region (J. Belote, unpublished). This 0.6 kb *Pros25* fragment could be removed by digestion with *Bgl*II, followed by dilution and ligation to yield a plasmid that retained the *GAL4* sequences but was now missing *Pros25* DNA.

The pBS/Pros25-GAL4#11 plasmid was digested with *BglII* to release the 0.6 kb *Pros25* fragment and the digestion confirmed by gel electrophoresis. The digested DNA sample was then diluted 1/100 with TE and ligated to obtain the pBS/GAL4 plasmid with a single *BglII* restriction site, and missing the *Pros25* fragment. The ligation mixture was transformed into electrocompetent cells and plated on LB/Amp plates. A negative control

using no ligase in a separate reaction was also plated to confirm that the original digestion was effective. Ten colonies from the ligation plate were picked and grown overnight in TB/Amp and plasmid DNA isolated by miniprep. DNA was digested using *BglII* and electrophoresed to see of any of the ten clones were correct. Of the ten, eight showed the right digestion pattern of a single band at 6.1 kb. One of these, pBS/GAL4#10, was chosen for future experiments (Figure 4).

Construction of the ProtamineB-GAL4 Driver

Because the *ProtamineB* gene is highly expressed during the late stages of sperm development, we sought to use that gene's promoter to construct a GAL4 driver that would target spermatogenic expression. We first used PCR to amplify a 1.3 kb fragment that contained the upstream regulatory region and the transcription start site (Manier, et al., 2010), and then ligated the PCR product into the cloning vector pJET1.2 (Figure 5). The PCR primers, ProtBPs and ProtBPas, were designed to include *Bgl*II sites to allow subcloning into the *Bgl*II site upstream of the GAL4 coding region of pBS/GAL4#10, and a 5' *Fse*I site so that the ProtBp-GAL4 fragment could be subsequently cloned into the transformation vector pBac3xP3-EGFPaf. High fidelity polymerase was used in this PCR reaction, and the result was confirmed using gel electrophoresis. Colonies from the ligation plate were picked and grown overnight in TB/Amp and plasmid DNA was purified by miniprep. DNA was digested using *Bgl*II to confirm plasmid with two bands, 2.9 kb and 1.3 kb. This plasmid was named pJET2.1/ProtBp1.3.

The next step was the subcloning of the 1.3 kb *Bgl*II fragment of pJET2.1/ProtBp1.3 into the *Bgl*II site of pBS/GAL#10. To accomplish this, the pJET2.1/ProtBp1.3 plasmid was double digested with *Bgl*II, to release the 1.3 kb

fragment containing the ProtamineB promoter, and *Sca*I, to cut the pJET2.1 vector into two pieces to prevent it from simply religating into the vector without an insert. The pBS/GAL#10 plasmid was also cut with *Bgl*II, to open it up so that the ProtBp fragment could be inserted, and treated with Shrimp Alkaline Phosphatase (SAP) to prevent it from simply religating back together without any insert. In this experiment, the 1.3 kb. *Bgl*II fragment could insert in either orientation and so colonies were screened by PCR for those that had the insert in the proper orientation. For this, we used primers ProtBPsense and GAL2as. Because our initial screening of dozens of colonies failed to identify a clone with the insert we changed our approach to allow us to efficiently screen hundreds of colonies. In this approach we picked several hundred colonies and created Master Plates with a numbered grid of colonies for testing. These colonies were picked and pooled in groups of ten or more for growing up in TB/Amp. Plasmid DNA was then extracted from each pooled culture and used as template for PCR. Pooled samples that gave the correct size PCR product were identified and the corresponding colonies from the Master Plate were then individually grown up and tested by PCR to identify the correct clone. The DNA was also checked by restriction enzyme analysis to confirm its correct structure. In this way, clone pBS/ProtBp-GAL was identified (Figure 6).

The final step of this cloning procedure was to cut out the 4.5 kb ProtBp-GAL fusion gene fragment from pBS/ProtBp-GAL using *Fse*I and ligate that into the unique *Fse*I cloning site of the transformation vector pBac3xP3-EGFPaf (Horn and Wimmer, 2000). Again, we used the Master Plate/pooled colonies approach to screen hundreds of colonies, using the PCR primers GFPsense and ProtBpas, to identify positive colonies. The positive candidate clone was then checked by additional PCR reactions using primer

pairs GFPs and GFPas, ProtBpsense and ProtBpantisense, and GAL1s and GAL2as, as well as restriction digest analysis. The clone pBac3xP3EGFP/ProtBp-GAL was confirmed to be correct and was used for subsequent germline transformation experiments (Figure 7).

Creation of Transgenic Flies Carrying the ProtB-GAL4 Driver

Next step was to carry out the germline transformation to introduce the ProtBp-GAL construct into the genome of *D. melanogaster*. The transformation vector, pBacEGFPaf, carries sequences from the piggyback transposon that causes random insertion into the genome catalyzed by the piggyback transposase enzyme. The pBac3xP3EGFP/ProtBp-GAL plasmid DNA was co-injected with another helper plasmid, hspΔSst, which carries the piggyback transposase gene. Pre-blastoderm embryos from w^{1118} white-eyed stock were injected with the DNA mixture. Surviving larvae were grown into G_0 adults. The G_0 males and females were crossed with w^{1118} mates and the G_1 offspring were screened for the presence of the pBac3x3P-EGFP/ProtBp-GAL transgene detectable by fluorescent green-eyes (resulting from the eye-specific 3x3P-EGFP marker). These flies were individually crossed to w^{1118} mates, and subsequently made homozygous to establish stable stocks.

Approximately 2,000 embryos were injected resulting in about 150 surviving G_0 adults. Among the G_1 progeny five transformants were identified: 1A, 6A, 6B, 10A, and 10B. The chromosomal linkage of these transgenes was determined by segregation analysis. Transgenes 6A and 6B were determined to be X-linked, since green-eyed males when crossed with white-eyed females produced all green daughters and all white-eyed

sons (Figure 8). The other transgenes were found to be autosomally linked since these crosses yielded both green-eyed and white-eyed sons and daughters.

The chromosomal linkage of these autosomal transgenes (1A, 10A, and 10B) were determined by first crossing them to a double mutant strain, w^{1118}*; CyO/+; TM3, Sb/TM6B, Tb.* This strain displays two phenotypes, curly wings and stubble hairs, when crossed with the transgenic fly the trait restored indicates the chromosome that the transgene is located on. The w^{1118}*/Y; CyO/*; TM3, Sb/** males were collected and crossed to w^{1118} females. In all three cases the transgene was seen to be linked to chromosome 2 since all green-eyed offspring were straight winged (did not carry the *CyO* balancer) while all white-eyed offspring were curly winged (*CyO*) (Figure 9).

Testing the ProtBp-GAL Driver for Testis-specific Expression

The next step of this project was to test these ProtBp-GAL4 drivers to see if they could drive expression of a reporter such as Green Fluorescent Protein (GFP) in the expected testis-specific manner. The first approach was to make use of an existing UAS-GFP reporter line that was available from the Bloomington Stock Center. This UAS reporter, called *P{*w^+*, UAS-GFP.nls}* (stock #4776) has the coding region of GFP fused to a nuclear localization signal so that the expressed GFP protein is concentrated into the nucleus for easier visualization. Each of the five ProtBp-GAL4 driver lines was crossed to *P{*w^+*, UAS-GFP.nls}* and the resulting F1 hybrids were examined for testis-specific GFP expression. Males were aged for three days, and then testes were dissected out in PBS, fixed in 4% formaldehyde, washed and mounted in 10% glycerol. They were then examined under the fluorescent microscope for GFP signal. In all cases, no green fluorescence was detected. As a positive control, we also crossed the *P{*w^+*, UAS-*

GFP.nls} reporter line to a constitutively expressed GAL4 (e.g., tubulin-GAL4, Bloomington Stock #5138). In this case, the F1 flies showed broad GFP expression in most if not all tissues examined.

Because of these negative results, we sought to test other reporters that might be more sensitive to detection of low levels of GAL4 expression. Two additional GFP reporters were identified from the literature and stocks obtained from the Bloomington Stock Center (Pfeiffer, et al., 2010). One of these is designated *P{10xUAS, mCD8-GFP}att2* (stock #32184). It has two features that make it potentially more sensitive than the *P{w*$^{+}$*, UAS-GFP.nls}* reporter. First, it has 10 copies of the UAS sequence that can bind GAL4 and activate transcription of the GFP reporter gene. Second, the GFP coding region is tagged with the mouse mCD8 marker that will localize the GFP to the cell surface where it might be more easily detected. The second reporter stock is *P{10xUAS-IVS-myr-GFP}att2* (stock #32197). It also has 10 copies of the UAS sequence, and it also has sequences corresponding to an intron (IVS) that is thought to assist transport of the mRNA out of the nucleus leading to more efficient translation and higher levels of the GFP protein (Pfeiffer, et al., 2010). In this reporter, the GFP is also tagged with a sequence from the nuclear myristoyl protein that targets it to the nucleus. We crossed five ProtBp-GAL4 driver lines to both of these improved reporters and examined testes of the F1 hybrids for GFP signal but, again, there was no detectable GFP fluorescence (not shown).

Given the negative results with these GFP reporters, we decided to use a different reporter that is based on a histochemical staining method instead of fluorescence. That is, we obtained the *P{UAS-lacZ, Exel}* reporter from Bloomington (stock #8529) which

expresses the *E. coli lacZ* gene, encoding beta-galactosidase, under the control of the UAS regulatory sequence. Tissues expressing this enzyme can be stained with the chromogenic substrate X-Gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside) that turns blue in the presence of beta-galactosidase. We crossed our five driver lines with *P{UAS-lacZ, Exel}* mates and collected the F1 males. Testes and other tissues were dissected from adult males, fixed and stained with X-Gal overnight. These were then examined for blue stain using light microscopy. While there was some blue staining in the gut, there was no testis staining. Moreover, the gut staining was also observed in the negative controls (i.e., males that did not carry the ProtBp-GAL4 driver constructs).

Table 2: Injected Fly Data

Flies Embryos Injected	Surviving G_0 Adults	G_1 Transformants	Chromosomal Linkage
~2,000	150	1A	Chromosome 2
----------	----------	6A	X Chromosome
----------	----------	6B	X Chromosome
----------	----------	10A	Chromosome 2
----------	----------	10B	Chromosome 2

Construction of the Ubiquitin-GAL4 Driver

As a positive control an Ubiquitin-GAL4 driver was developed using a similar strategy as the ProtamineB-GAL4 driver. Ubiquitin is a protein expressed in virtually

every tissue of eukaryotic organisms. It is involved in controlled destruction of proteins. Since Ubiquitin is expressed in all tissues it is ideal for use as a positive control.

The first step was to isolate the Ubiquitin upstream region. For this, we chose to use the promoter from the polyubiquitin gene, *Ubi-63E* (Lee, et al, 1988), which is constitutively expressed throughout development. An existing plasmid, pBacUbn1s-GFP (ref), was used as a starting point. The Ubiquitin upstream region was amplified via PCR with the primers Ubprom3B and Ubprom5BF (Fig 10). Next the Ubiquitin upstream region was ligated into the blunted pJET2.1-cloning vector. The PCR primers, Ubprom3B and, Ubprom5BF were designed to include *Bgl*II sites to allow subcloning into the *Bgl*II site upstream of the GAL4 coding region of pBS/GAL4#10, and a 5' *Fse*I site so that the Ubprom-GAL4 fragment could be subsequently cloned into the transformation vector pBac3xP3-EGFPaf. High fidelity polymerase was used in this PCR reaction, and the result was confirmed using gel electrophoresis. Colonies from ligation plate were picked and grown overnight in TB/Amp and plasmid DNA was purified by miniprep. DNA was digested using *Bgl*II to confirm plasmid size with two bands 3.0 Kb and 2.0 Kb. This plasmid was referred to as pJET2.1/Ubprom2.0.

Next, the 2.0 Kb *BglII* fragment of pJET2.1/Ubprom2.0 was cloned into the *BglII* site of the pBS/GAL#10. To accomplish this, the pJET2.1/Ubprom2.0 plasmid was double digested with *Bgl*II, to release the 2.0 kb fragment containing the Ubiquitin promoter, and *Pst*I, to cut the pJET2.1 vector into two pieces to prevent it from simply religating into the vector without an insert. The pBS/GAL#10 plasmid was also cut with *Bgl*II, to open it up so that the Ubprom fragment could be inserted, and treated with Shrimp Alkaline Phosphatase (SAP) to prevent it from simply religating back together

without any insert. In this experiment, the 2.0 kb *Bgl*II fragment could insert in either orientation and so colonies were screened by PCR for those that had the insert in the proper orientation. For this, we used primers Ubprom5BF and GAL2as. This time we skipped right to screening hundreds of colonies with the master plate and PCR method described previously. Colonies that were positively identified by PCR were also checked with several restriction enzyme digests. Using this method clone pBS/Ubprom-GAL4#3 was identified (Figure 11).

Lastly, the 5.2 kb Ubprom-GAL fusion gene fragment from pBS/Ubprom-GAL4#3 fragment was cut out using *Fse*I and ligating that into the unique *Fse*I cloning site of the transformation vector pBac3xP3-EGFPaf (Horn and Wimmer, 2000). Again, we used the Master Plate/pooled colonies approach to screen hundreds of colonies, using the PCR primers GFPsense and Ubprom3B, to identify positive colonies. The positive candidate clone was then checked by additional PCR reactions using primer pairs GFPs and GFPas, Ubprom3B and Ubprom5BF, and GAL1s and GAL2as, as well as restriction digest analysis. The clone pBac3xP3EGFP/Ubprom-GAL4 was confirmed to be correct and was used for subsequent germline transformation experiments (Figure 12).

Germline Transformation of the Ubprom-GAL4 Construct

We attempted to create transgenic lines containing the constitutive Ubiquitin-GAL4 driver but have not been successful, to date. This far, we have injected approximately 1,500 w^{1118} embryos with pBac3xP3-EGFP/Ubprom-GAL4 plus the pBac-hsp [illegible] helper plasmid resulting in about 150 G_0 surviving adults. Male and female G_0 flies were crossed in groups of about 10 pairs and several thousand G_1 offspring were

scored for green fluorescent eyes, indicating successful transformation. Unfortunately, no green-eyed flies were found.

DISCUSSION

The purpose of this study was to develop a new genetic tool that could be used to better understand the genetic mechanisms controlling spermatogenesis in Drosophila. Specifically, we sought to create novel GAL4 lines that would drive expression of UAS-controlled target constructs in the late stages of spermatogenesis. These GAL4 "drivers" could be used to either (1) overexpress specific genes of interest (by crossing them to UAS-GOI transgenic lines), or (2) knockdown expression of the GOI (by crossing them to UAS-GOI RNAi hairpin transgenic lines), in a sperm specific manner. By examining the phenotypic effects of these manipulations on the functional role of the GOI in sperm development or function could be inferred. For example, if knocking down expression of a candidate GOI results in abnormal sperm, it can be assumed that the GOI plays an important role in spermatogenesis, and the nature of the abnormality might provide clues as to what its specific role is.

The construct to test this system was created by inserting the *ProtamineB* promoter region upstream of the GAL4 coding region. The *ProtamineB* gene encodes a sperm-specific chromosomal protein that is highly expressed during the later stages of spermatogenesis. As spermiogenesis proceeds, histone-containing nucleosomes are removed and replaced by Protamine proteins which results in a more tightly condensed chromatin structure and a shutdown of most, if not all, transcriptional activity. Because

of this highly specific spermatogenic expression pattern we chose to use the *ProtamineB* gene's promoter region to create a sperm-specific GAL4 driver.

To create the construct the GAL4 coding region was subcloned into the appropriate vector from an existing plasmid, and 1.3 kb of the *ProtamineB* upstream region was amplified and inserted in front of GAL4. This *ProtBpromoter-GAL4* fusion gene was then subcloned into a transformation vector that allowed for its introduction into the *D. melanogaster* genome. Five *ProtBpromoter-GAL4* transgenic lines were identified and the chromosomal linkage of the transgene integration in each line was determined by genetic mapping.

The five *ProtBpromoter-GAL4* driver lines were crossed to UAS reporter strains to check for testes specific expression. Four different reporter strains were used. Initial tests involved crosses with a standard UAS-GFP reporter in which the GFP protein contained a nuclear localization signal and whose expression was controlled by one copy of the UAS upstream activation sequence. Unfortunately, there was no detectable expression of the GFP in testes of flies carrying this reporter and any of the five ProtB-GAL4 drivers. To potentially increase the sensitivity of the system, we also tested two other GFP reporters. Both of these contained 10 copies of the UAS sequence that theoretically should increase the transcriptional response to the ProtB-GAL4 activator. One of these had the GFP tagged with a nuclear localization signal and the other had GFP tagged with a signal that localizes the protein to the cell surface. In spite of these improvements to the UAS reporter, there was still no detectable expression of the GFP reporter when crossed to the five ProtB-GAL4 driver lines. Finally, we tested a different UAS reporter that carried the *E. coli lacZ* coding region. Expression of this reporter could

be monitored by staining testes with the chromogenic substrate X-gal. It was thought that this might allow for more sensitive detection of GAL4 driven expression since the staining reaction could be extended over several hours and thus might detect lower levels of expression that can be detected by GFP fluorescence. But, unfortunately, even with this reporter there was no indication that the ProtB-GAL4 drivers were effective at driving sperm-specific expression of the UAS target.

One explanation for these negative results is that the *ProtBpromoter-GAL4* transgenes were not being expressed for some reason. Although we never directly tested GAL4 expression in our lines, for example by immunostaining testes with anti-GAL4 antibodies, we assume that GAL4 is being expressed because the same 1.3 kb *ProtamineB* upstream promoter region has been shown to drive robust expression of a GFP-tagged ProtamineB sequence in several transgenic lines (e.g., Manier, et al., 2010). It seems unlikely that this same sequence would fail to drive transcription of the GAL4 coding sequence in our *ProtBpromoter-GAL4* constructs. Another possible reason why our driver construct is not effective would be if there was some type of chromosomal position effect that is silencing the transgene, which had been inserted into a region of the genome that is not compatible with high levels of testes-specific transcription. While this type of chromosomal position effect gene silencing is known to occur in Drosophila (Castronuevo and Martin, 2002), it is relatively uncommon. Moreover, because we have tested five independently derive transformed lines it is highly unlikely that all of them would exhibit this effect on expression. Another explanation for our negative results would be that the *ProtBpromoter-GAL4* driver is working fine, but that there is something wrong with the UAS reporter. However, we used four different UAS reporters,

containing different numbers of UAS sequences, different subcellular localization signals, and two different reporter proteins (i.e., GFP and β-galactosidase). This rules out the possibility of a defective reporter line or detection method. One likely explanation for the failure of the ProtB-promoter-GAL4 driver to activate detectable expression of the UAS-reporters is suggested by previous results with another testis-specific driver, *beta-Tubulin85D-GAL4* (Cooper-White, 2012). That is, they report the failure of this testis-specific driver as possibly being related to the late spermatogenic expression of the driver relative to the shutdown of transcription that occurs as the histones are replaced by protamines and chromosomes condense. This author suggests that there is probably insufficient time for the GAL4 protein to accumulate to levels that can adequately activate the UAS target gene transcription before all transcriptional activity ceases. If this were the explanation, then one way around this would be to construct a driver that is expressed earlier in spermatogenesis, or ubiquitously, so that there is enough time for the GAL4 protein to accumulate and activate the UAS target.

It was for this reason that we created the Ubiquitin-GAL4 construct. The Ubiquitin upstream region was cloned in front of the GAL4 coding region. This fragment was then subcloned into the transformation vector and injected into eggs in the same manner as the Protamine B-GAL4 construct was. Unfortunately, despite injecting over one thousand flies, and screening thousands more, no green-eyed transformants have yet been found.

A future direction of this research is to use a gene driver that is expressed in an earlier stage of spermatogenesis than Protamine B. The genes controlling proteasome function are promising targets due to their expression in the spermatocyte stage, and

importance in cellular differentiation (Belote and Zhong, 2009). Studies of testis-specific proteasome genes have suggested that *Prosα3T* would be a good candidate for creating a testis-specific driver since its expression occurs at the primary spermatocyte stage, earlier than the time when ProtamineB is expressed (Ma, et al., 2002). The creation of a plasmid construct with GAL4 coding region downstream of the *Prosα3T* promoter region has been initiated, however this work is currently incomplete. Another approach is to use a gene driver that is constitutively expressed, such as ubiquitin. However, this may produce undesirable off target effects within the fly making this system ineffective.

FIGURES

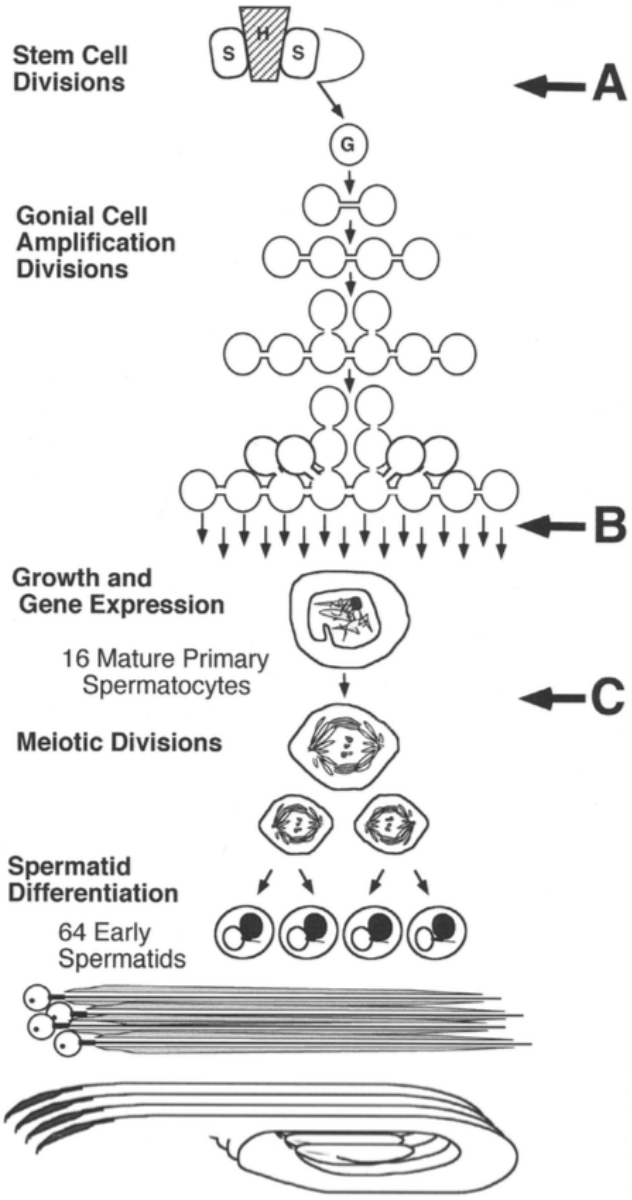

Figure 1: Spermatogenesis (Taken from Fuller, 1998). Shows division and differentiation from stem cells to mature sperm cell

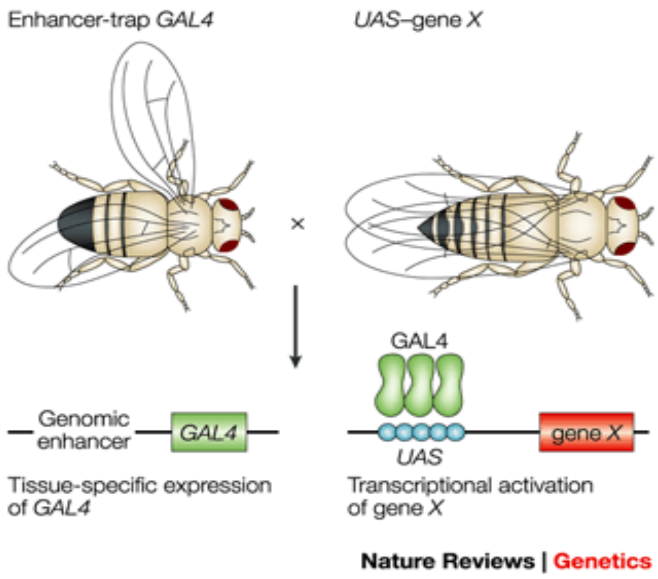

Figure 2: UAS/GAL4 System Overview (Taken from Johnson, 2002). A representation of the set up of our experiment. GAL4 is cloned under the tissue specific genomic enhancer (Protamine B). Upon crossing with a UAS-Reporter fly (UAS-GFP) expression will be driven by the GAL4 protein in the offspring.

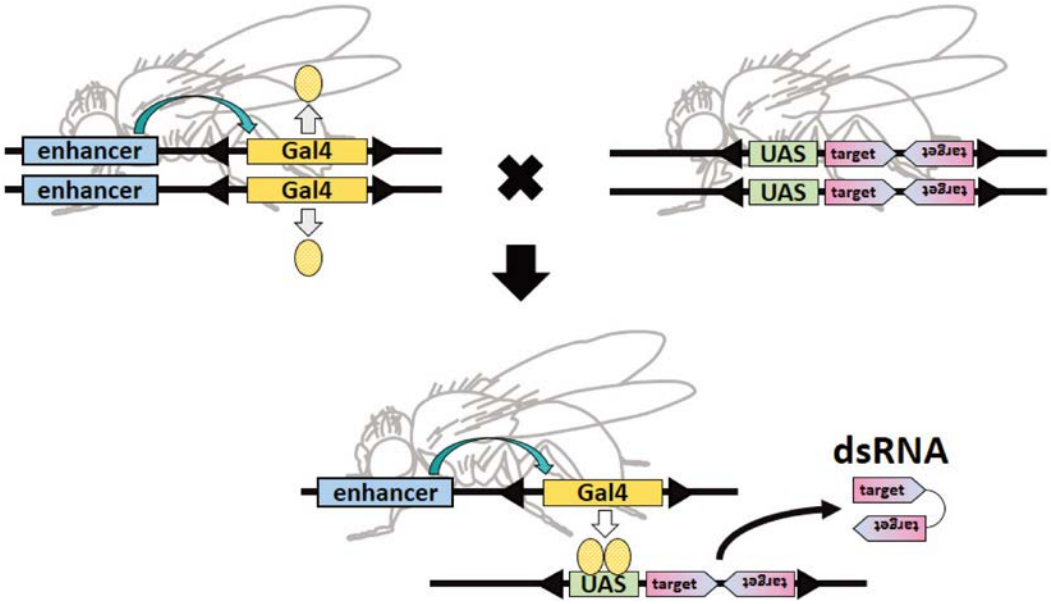

Figure 3: UAS-RNAi System Overview (Taken from Yamamoto-Hino and Goto, 2013). A representation of how a UAS-RNAi expression system would be set up. A hairpin RNA can be created by putting a gene and its inverse sequence back to back. Upon transcription the complementary base pairs will bind and form double stranded RNA (dsRNA) which is then recognized by the RNAi pathway and degraded

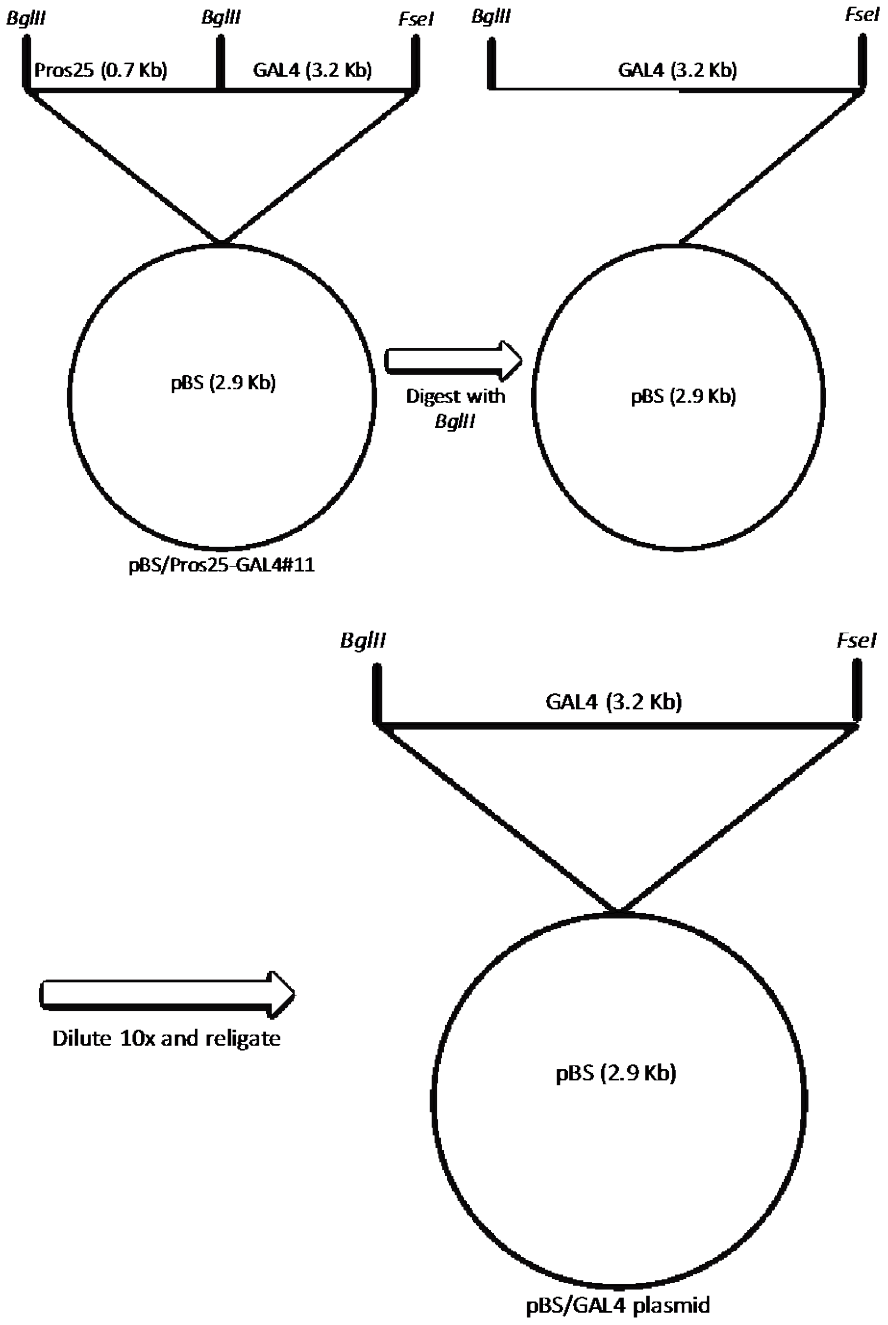

Figure 4: Creation of pBS/GAL4 plasmid. Digestion of the existing pBS/Pros25-GAL4 plasmid with the restriction enzyme *BglII* yields the GAL4 coding region in the pBS plasmid upon dilution and relegation. Restriction sites are indicated by vertical lines.

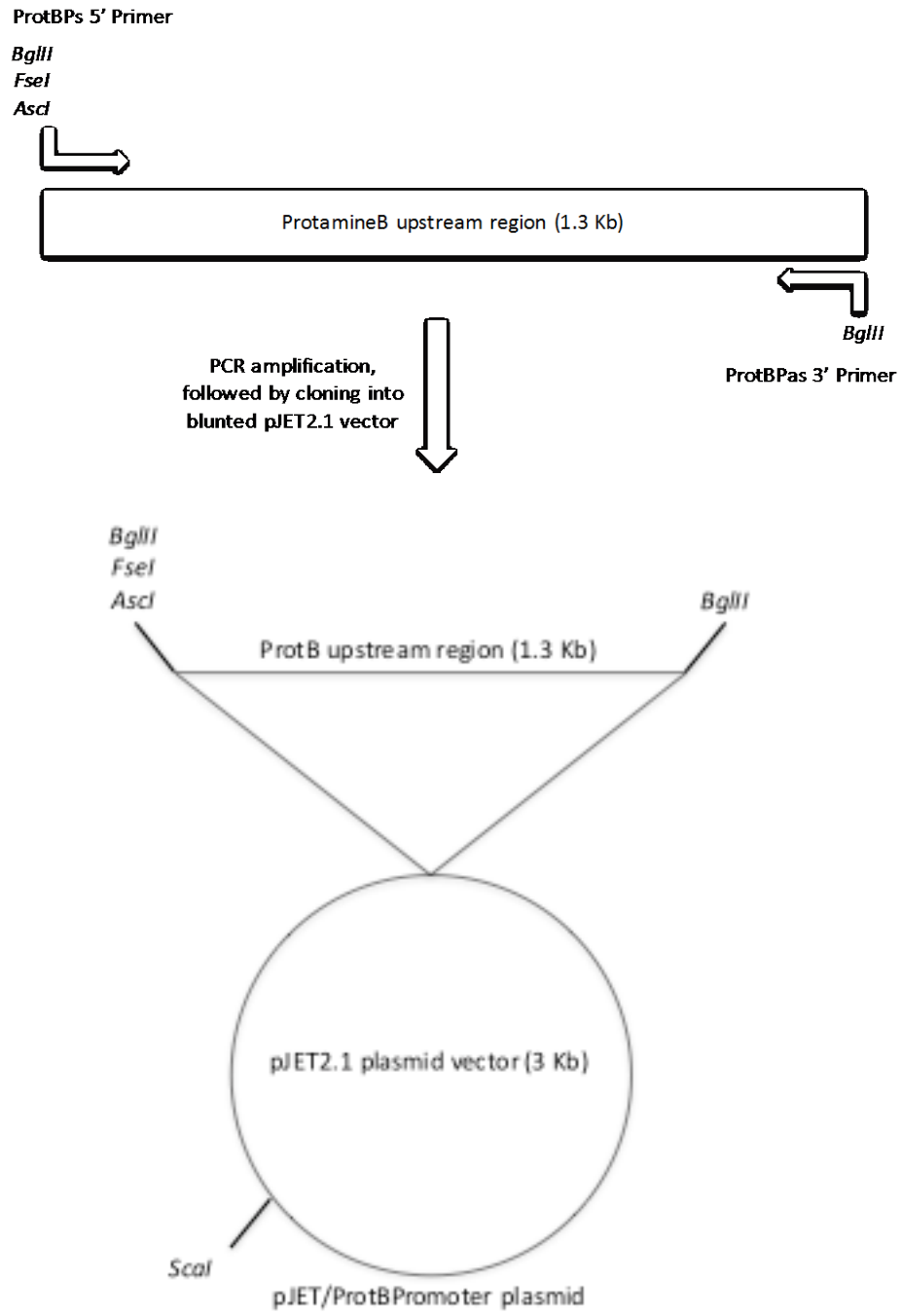

Figure 5: Amplification of ProtamineB upstream region via PCR and cloning into pJET2.1 plasmid vector. The ProtamineB upstream region was amplified by primers ProtBPs and ProtBPas (represented by block arrows). The amplified region was then cloned into the pJET2.1 plasmid.

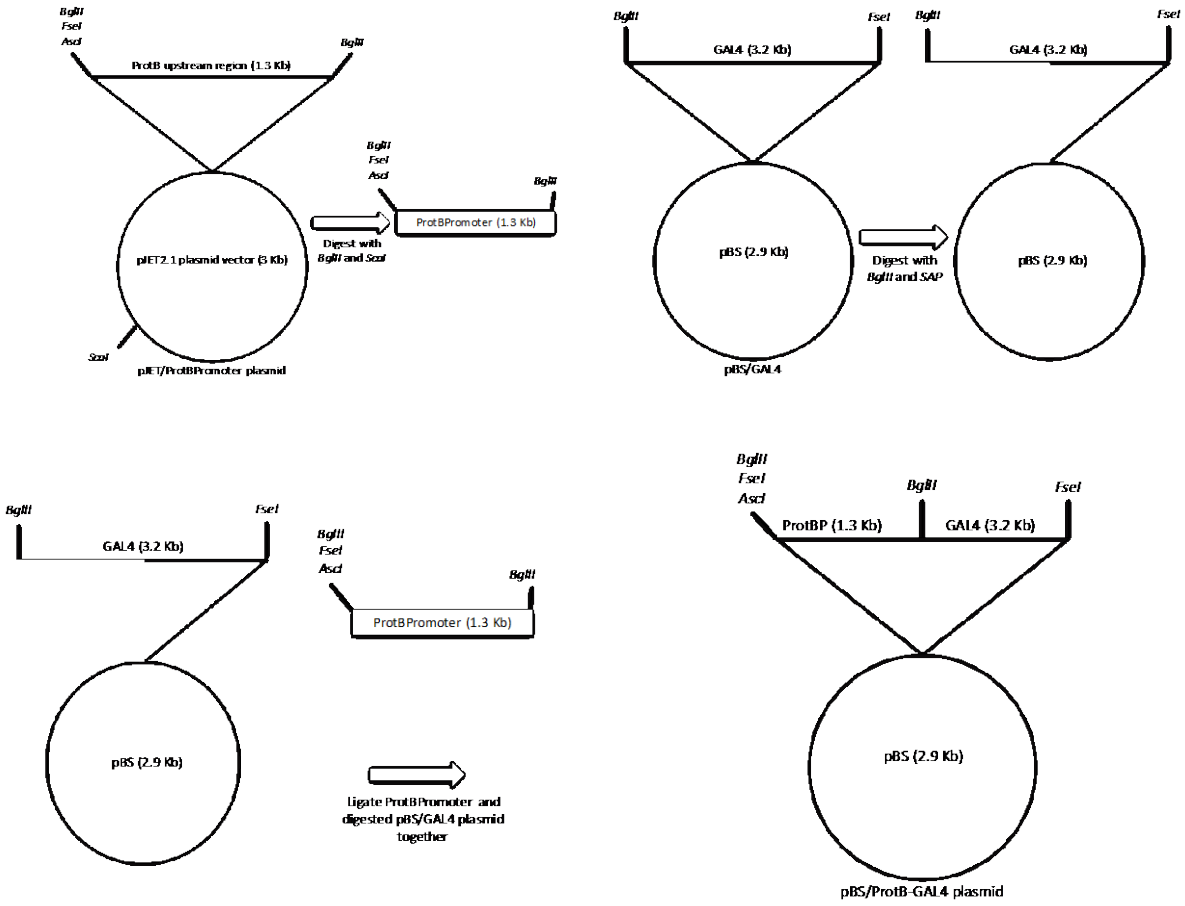

Figure 6: Creation of pBS/ProtB-GAL4 plasmid. The pieces from Figures 4 and 5 were combined into the pBS vector via ligation.

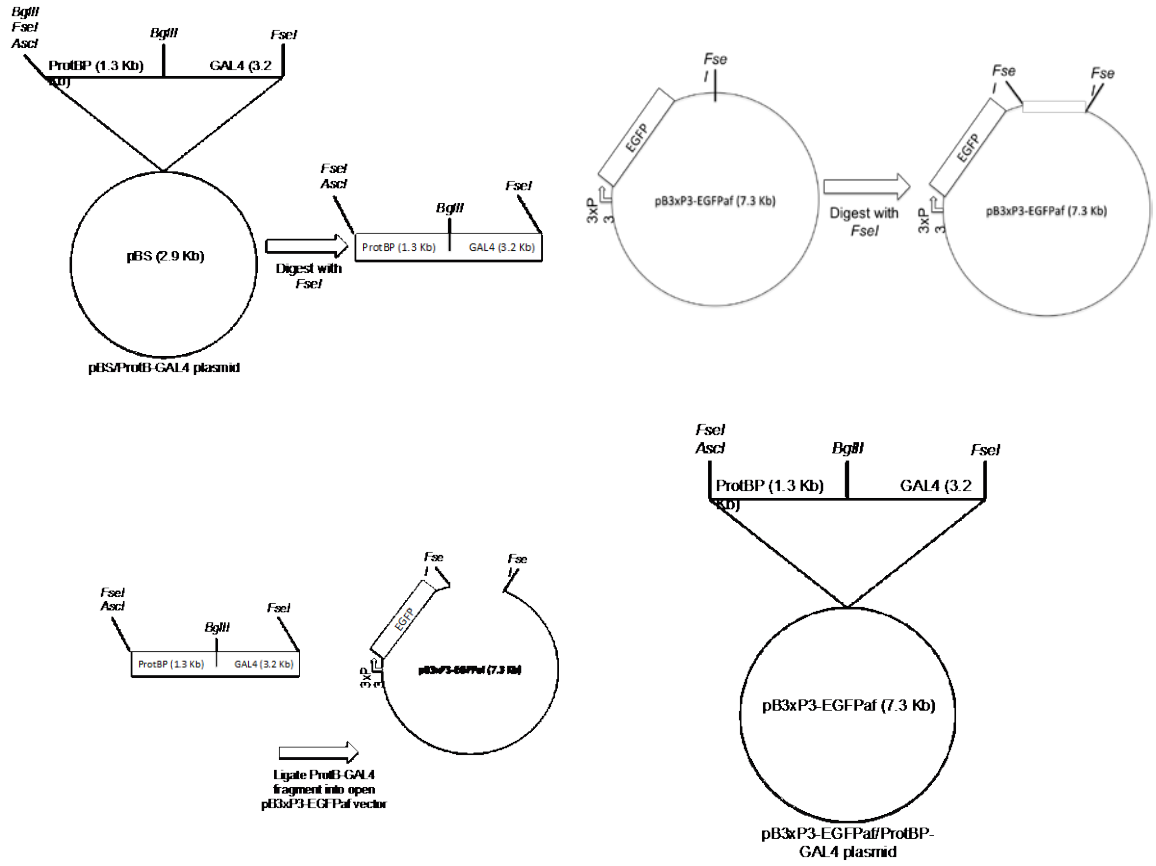

Figure 7: Cloning ProtB-GAL4 fragment into 3xP3/EGFPaf transformation vector.

The ProtB/GAL4 fragment was removed from the pBS vector via restriction enzyme digest with *FseI* and cloned into the fly transformation vector pB3xP3-EGFPaf.

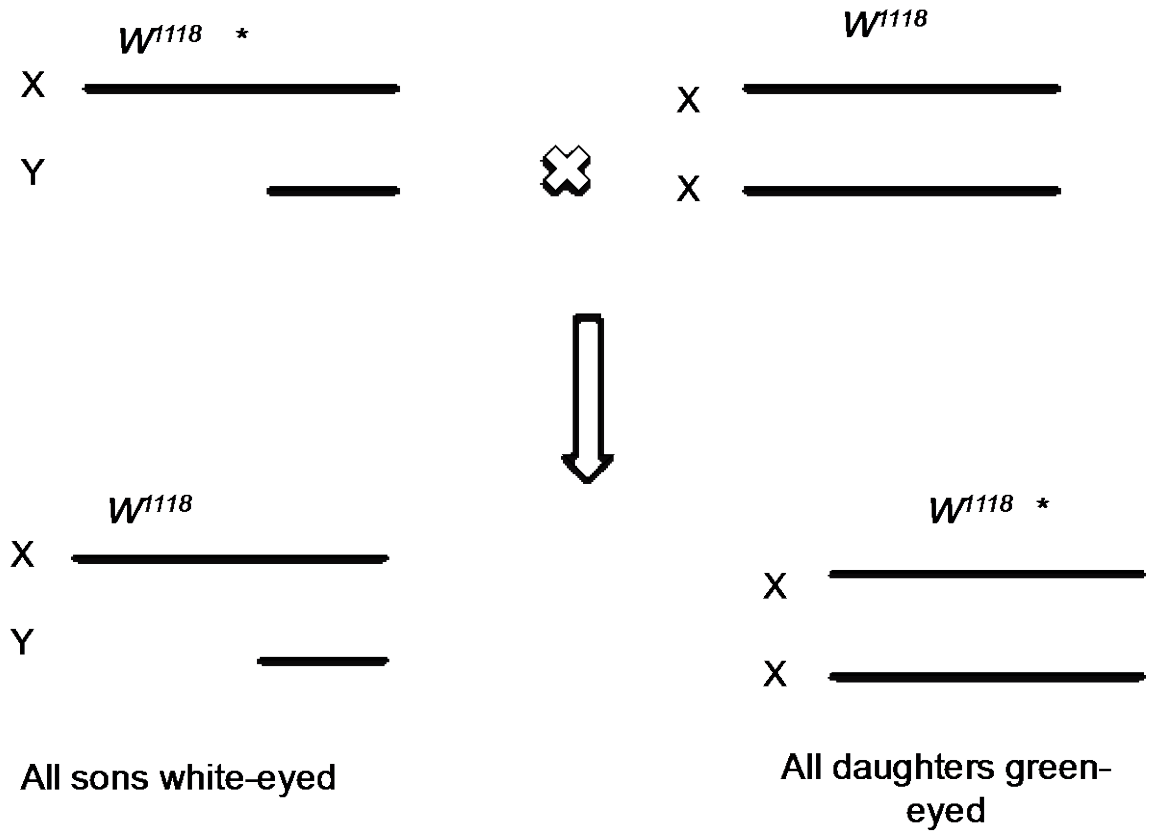

Figure 8: Sex linkage of transgenes 6A and 6B. Experimental determination of sex chromosome linkage of transgenes. * = inserted pBac3x3P-EGFP/Protbp-GAL4 transgene.

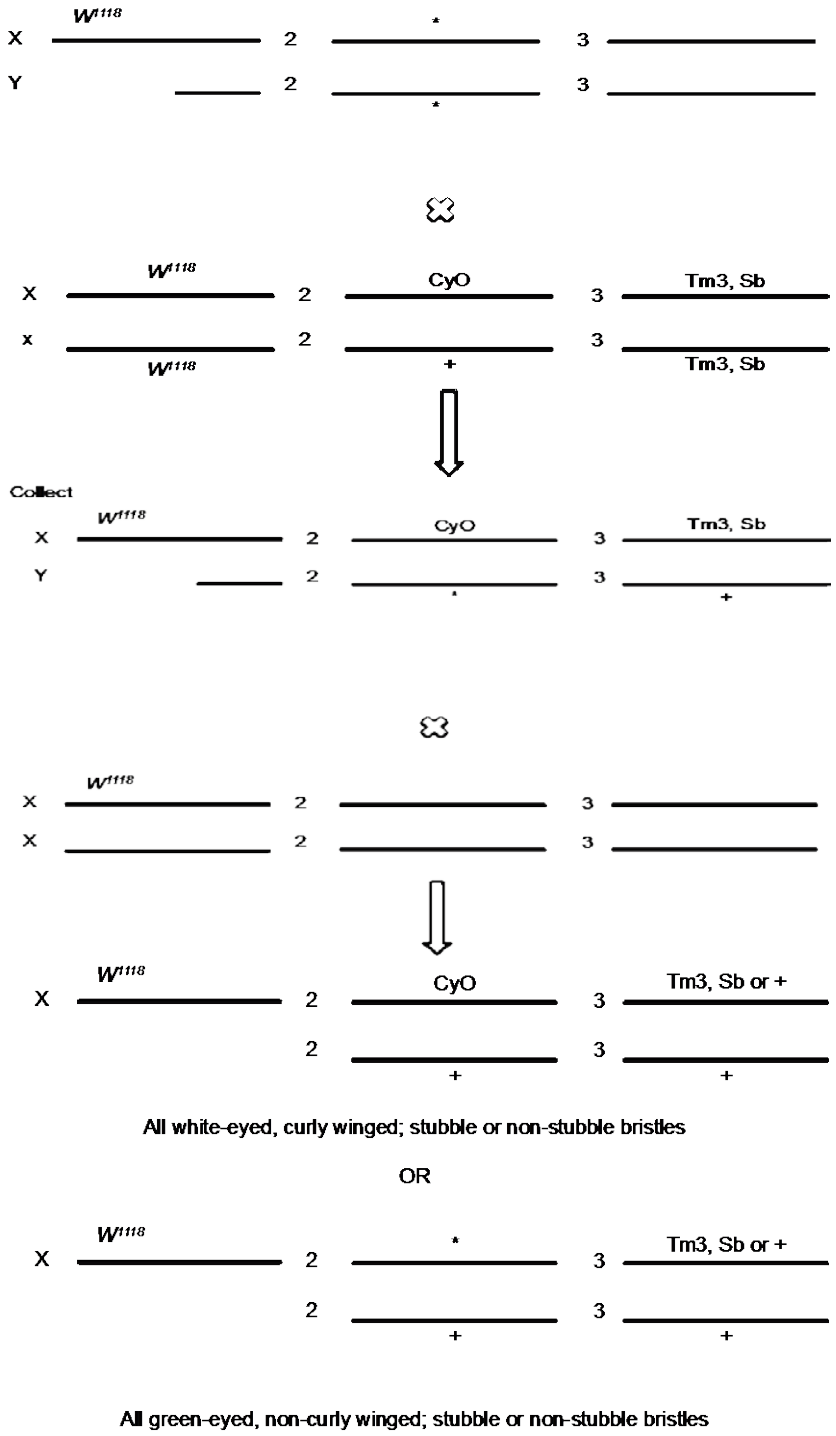

Figure 9: The 1A, 10A, and 10B transgene map to chromosome 2. Experimental determination of somatic chromosome linkage of transgenes. * = transgene insert in 1A, 10A, and 10B.

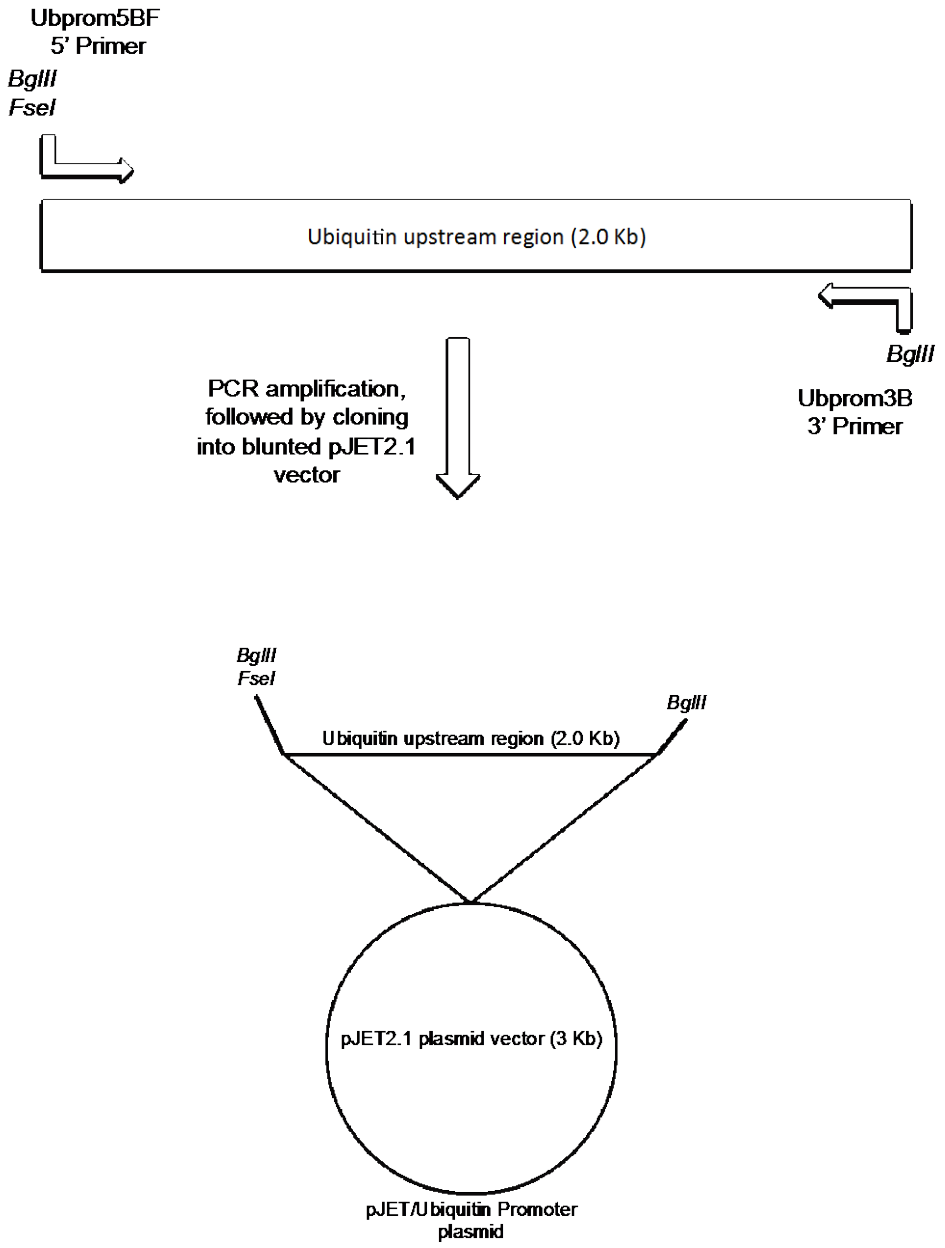

Figure 10: Amplification of Ubiquitin Upstream Region. In similar fashion to the ProtamineB steps, the upstream region of the Ubiquitin gene was amplified via PCR with primers UbProm5BF and UbProm3B.

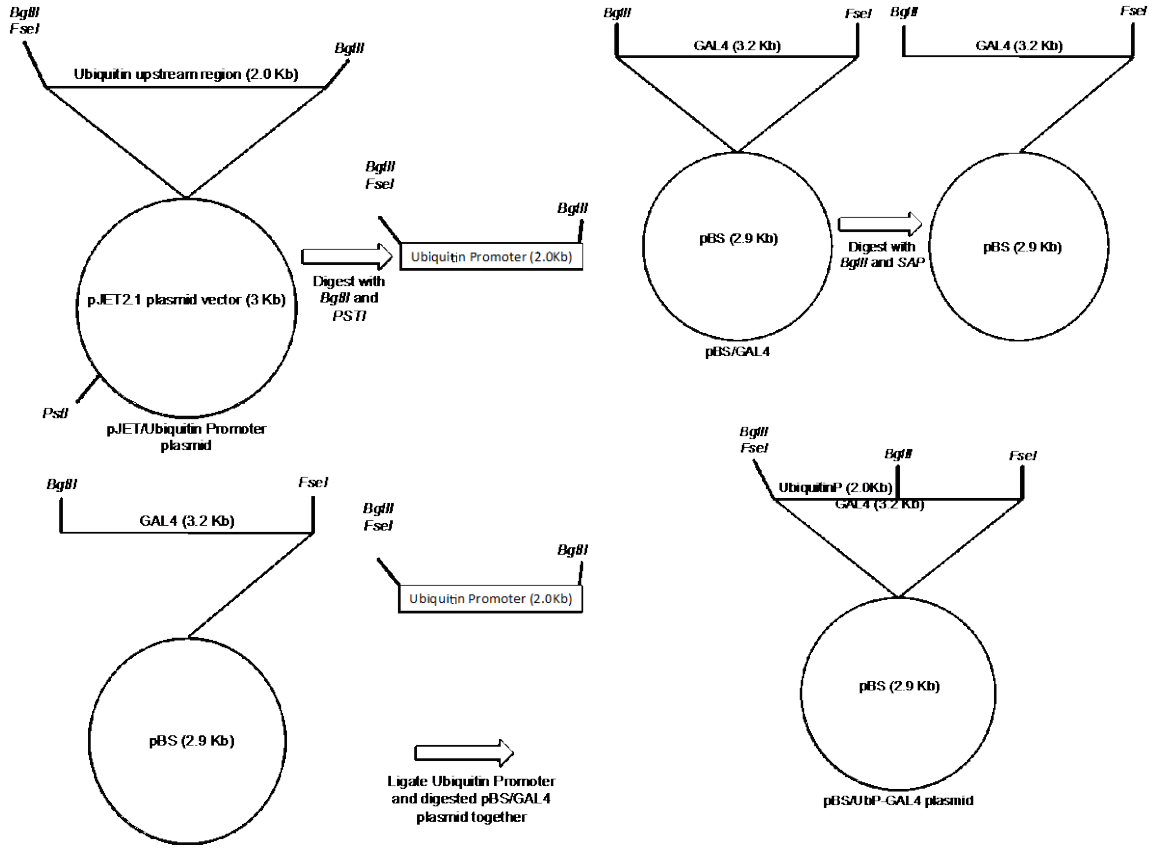

Figure 11: Creation of pBS/Ubiquitin-GAL4 plasmid. The GAL4 plasmid from Figure 4 was ligated with the amplified Ubiquitin region from Figure 8 to yield the pBS/UbP-GAL4 plasmid.

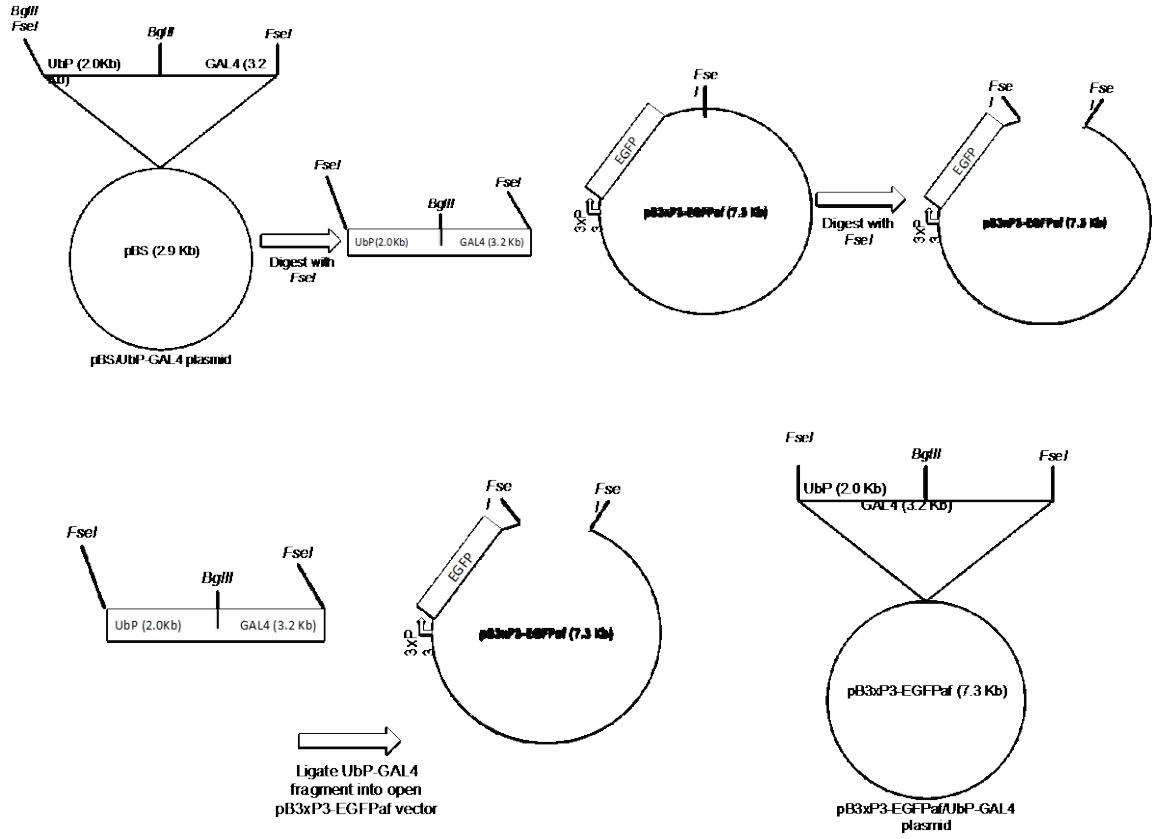

Figure 12: Cloning UbP-GAL4 fragment into 3xP3/EGFPaf transformation vector.

The UbP/GAL4 fragment was removed via digestion with FseI and inserted into the fly transformation vector pB3xP3-EGFPaf.

REFERENCES

Belote, J.M. and Zhong, L. (2009). Duplicated proteasome genes in Drosophila and their roles in spermatogenesis. *Heredity* 103: 23-31.

Bernstein, E. Caudy, A.A. Hammond, S.M. and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature, 409:* 363-366.

Castronuevo, P. and Martin, P.F. (2002). Localization of a position effect element that affects alcohol dehydrogenase transgene expression in *Drosophila melanogaster*. *DNA and Cell Biology* 21: 535-540.

Cooper-White, H. (2012). Tissue, cell-type and stage-specific ectopic gene expression and RNAi induction in the Drosophila testis. *Spermatogenesis* 2: 11-22.

Demarco, R.S. Ãsmund, H. Eikenes, K.H. and Jones, D.L. (2014). Investigating spermatogenesis in *Drosophila melanogaster*. *Methods* 68.1: 218-27.

Duffy, J.B. (2002). GAL4 system in *Drosophila*: A geneticist's swiss army knife. *Genesis* 34: 1-15.

Fuller, M.T. (1998). Genetic control of cell proliferation and differentiation in *Drosophila* spermatogenesis. *Cell & Developmental Biology* 9: 433-444.

Hannon, G.J. (2002). RNA interference. *Nature* 418.6894: 244-51.

Horn, C. and Wimmer, E.A. (2000). A versatile vector set for animal transgenesis. *Development Genes and Evolution* 210.12: 630-37.

Jinek, M. Chylinski, K. Fonfara, I. Hauer, M. Doudna, J.A. and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337.6096: 816-21.

Ma, J. Katz, E. and Belote, J.M. (2002). Expression of proteasome subunit isoforms during spermatogenesis in *Drosophila melanogaster*. *Insect Molecular Biol*ogy 11: 624-639.

Manier, M.K. Belote, J.M. Berben, K.S. Novikov, D. Stuart W.T. and Pitnick, S.S. (2010). Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. *Science* 328: 354-357.

Phelps, C and Brand A. (1998). Ectopic gene expression in *Drosophila* using GAL4 system. *A Companion to Methods in Enzymology* 14: 367-379.

Prelich, G. (2012). Gene overexpression: Uses, mechanisms, and interpretation. *Genetics* 190.3: 841-54.

Raja, S.J. and Renkawitz-Pohl, R. (2005). Replacement by *Drosophila melanogaster* protamines and Mst77F of histones during chromatin condensation in late spermatids and sole of sesame in the removal of these proteins from the male pronucleus. *Molecular and Cellular Biology* 25.14: 6165-177.

St. Johnston, D. (2002). The art and design of genetic screens: *Drosophila melanogaster*. *Nature Reviews Genetics* 3.3: 176-88.

Sutton, E.R. Yu, Y. Shimeld, S.M. White-Cooper, H. and Alphey, L. (2016). Identification of genes for engineering the male germline of *Aedes aegypti* and *Ceratitis capitata*. *BMC Genomics* 17.1: n. pag.

Yamamoto-Hino, M and Goto, S. (2013). *In Vivo* RNAi-based screens: Studies in model organisms. *Genes* 4: 646-665.

Zhong, L. and Belote, J.M. (2007) The testis-specific proteasome subunit Pros □6T of *Drosophila melanogaster* is required for individualization and nuclear maturation during spermatogenesis. *Development* 134: 3517-3525.