IDENTIFICATION OF MODIFIERS OF THE SF1 CHROMATIN BOUNDARY

by

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(Under the Direction of Haini N. Cai)

ABSTRACT

Insulators are elements that regulate genes through regulatory proteins and interactions with other insulators. A novel insulator discovered in *Drosophila melanogaster*, known as SF1, has been the primary focus of our lab. SF1 is located between two genes that are important for early embryogenesis, indicating that it may play a role in regulating these genes. In this study, we used a reverse genetic screen to identify any potential modifiers of SF1. We also investigated the interaction properties of SF1 with other elements in the Ftz-Antennapedia region. This study has led to the discovery of several candidate genes that have affected SF1 function. In addition, several novel elements exhibit cancellation with SF1. These latest findings have brought us closer to a better understanding behind the mechanisms responsible for the activity of SF1.

INDEX WORDS: Insulator, *in situ* hybridization, embryo fixation, Reverse genetic screen, RNAi

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CHAPTER 1

INTRODUCTION

1.1: General principal of gene regulation

Development requires genes to be activated or suppressed in specific tissue at specific time of an organism's life. Distinct DNA and protein components play important roles in regulating gene expression. Enhancers are DNA elements located outside transcribed region. They contain recognition sites for tissue-specific DNA binding proteins called transcription factors, including activator and repressors. Once bound by activators, an enhancer can interact with a gene promoter located at distance and activate mRNA synthesis from the promoter. Thus, enhancers direct tissue- and stage- specific gene expression.

The mechanisms of enhancer-promoter communicate is not known. However, many eukaryotic gene enhancers are known to function in a distance- and orientation-independent fashion. Some enhancers also display little promoter specificity, meaning that they can activate multiple genes simultaneously. This could cause mis-regulation among neighboring genes.

1.2: Insulators and their influence on gene regulation

In addition to activators and repressors, genes are regulated by other elements. Insulators are protein- DNA complexes that function in regulating gene expression through intra- and interchromosomal interactions (Corces et al., 2009). Insulators are defined by enhancer-blocking activity and barrier activity (Corces et al, 2009). The enhancer-blocking activity involves

obstructing distal enhancers from contacting promoters on the opposite side of an insulator (Felsenfeld and Wallace, 2007). Previous studies have demonstrated that when an insulator is present between an enhancer and promoter, then the expression of the enhancer's target gene is significantly reduced (Cai et al, 2001). The barrier activity protects genes from the influences of silent chromatin. An example of the chromatin barrier function is the observation that scs and scs′ elements can protect the *miniwhite* gene against chromosome positioning effect or CPE (Cai et al., 2009).

Interestingly, experiments showed that insulators identifled in yeast exhibits only function as barriers. Those from the telomeres and the HML and HMR loci possess different barrier elements that block the spread of silencing these regions. It was determined that the HMR domain is flanked by terminal repeats of *TY* elements. Another discovery was that a *t*RNA gene neighboring the HMR locus is a component of one of the boundaries. It was noted in a separate study that *t*RNA genes in *S. cerevisiae* yeast mediate barrier activity at different rates (Lunyak, 2008). Some tRNAs that are incapable of functioning as barriers alone have been shown to produce the barrier function when more than one copy of the tRNA is present (Kamakaka and Valenzuela, 2006). In a separate study, the deletion of tRNA genes that flanked a silent chromatin region resulted in the spread of heterochromatin (Scott et al., 2006). Acetylases and chromatin remodeling factors aid the insulator elements in protecting against the spread of heterochromatin by competing with heterochromatic deacetylases (Kamakaka and Valenzuela, 2006). One model states that insulator elements in yeast form large stable DNA-bound complexes by blocking the spread of silent chromatin.

One of the most characterized insulators in vertebrates is the CTCF insulator. CTCF's involvement in enhancer blocking was first discovered in studies of the chicken *ß-globin* gene

cluster (Felsenfeld et al., 2000). It was later learned that the insulator function was conserved in both mice and human clusters (Caiafa and Zlatanova, 2009). Previous studies also show that there are between 14,000 and 20,000 CTCF binding sites in higher eukaryotes (Caiafa and Zlatanova, 2009). Additional studies have identified CTCF-binding sites and other partners that are capable of interacting with CTCF (Kamakaka and Valenzuela, 2006). It is believed that CTCF plays a crucial role as a "master organizer" of the genome. According to several studies, CTCF is also capable of forming loops *in cis* and can bridge sequences *in trans* (Kamakaka and Valenzuela, 2006). The positions and the functions of the CTCF sites in vertebrates indicate that CTCF plays a critical role in chromatin organization (Caiafa and Zlatanova, 2009).

There are currently five identified insulators in *Drosophila*. The insulator proteins involved in each insulator's activity defines the insulator types. The scs and scs′ insulators are one of the first insulators to be discovered in Drosophila. The heat shock hsp70 locus is flanked by the scs and scs′ insulators. The DNA-binding protein responsible for scs insulator function is Zeste-White 5 (ZW5). ZW5 is a zinc finger protein that plays a separate role in cell viability. The zw5 gene is recessive lethal, but ealier studies have observed that hypomorphic alleles of zw5 results in pleiotropic effects on the development of several structures of the adult fly body. The scs′ insulator functions with help from the BEAF 32 protein. The BEAF32 gene encodes two isoforms, BEAF 32A and BEAF32B. In prior studies, BEAF 32A mutations do not show significant phenotypic defects, but BEAF 32B mutations cause lethality. One of the most characterized insulators in Drosophila is the gypsy insulator, which is located in the gypsy retrotransposon. Binding sites for the zinc finger protein, Suppressor of Hairy-wing [Su(Hw)] are contained within the gypsy insulator. The Su(Hw) protein interacts and forms a protein complex with two other proteins, Mod(mdg4)2.2 and CP190 (Corces et al., 2001). Some

proteins, such as Mod(mdg4)2.2, are incapable of binding DNA directly and instead must bind with DNA-binding insulator proteins to form a complex on the insulator, thus aiding in insulator function. CP190 can bind to DNA, but has a higher affinity for the Su(Hw) and Mod(mdg4)2.2 proteins. Previous studies have shown that when the Su(Hw) protein is absent due to gene knockout, then the result is female sterility. However, null mutations of the mod(mdg4) gene has resulted in lethality. Lethality is also observed in the mutant CP190 background (Corces et al., 2004). These results illustrate the importance of the gypsy insulator function in Drosophila development. Another well-studied insulator is the Frontabdominal-8 insulator (Fab-8). Fab-8 is known to interact with the Drosophila CTCF protein (dCTCF), which is a homolog of the CTCF insulator protein found in vertebrates. As with most of the known insulator proteins, dCTCF is also a zinc-finger protein with 12 zinc fingers. The dCTCF protein has also been found on other insulators in the bithorax complex, such as Mcp and Fab-6. When the dCTCF gene is mutated, the results are lethal, with abdominal homeotic phenotypes (Mohan et al., 2007). The Frontabdominal-7 or Fab-7 insulator is located within the Abdominal B locus (Abd-B).

Thus far, the enhancer blocking and chromatin barrier functions have been used to identify new insulators. Though insulators have been studied for over 30 years, the mechanisms that are responsible for their functions are not well understood. For this reason, insulators are studied to learn more about how they influence genes in an organism.

1.3: The SF1 insulator and its potential role in Hox gene regulation

A set of genes that are vital for development called Homeotic or Hox genes are expressed in specific tissues of the embryo during early development, which determines segment identity as the embryo matures (Lohmann et al., 2007). For instance, expression of a Hox gene

in the anterior region of an embryo would drive the development of anterior features. The Hox genes code for transcription factors that have highly conserved DNA-binding domains called homeodomains (Lohmann et al., 2007). The identified Hox genes in *Drosophila* include *labial (lab), proboscipedia (pb), Deformed (Dfd), Sex-comb reduced (Scr), Antennapedia (Antp), Ultrabithorax (Ubx), Abdominal-A (Abd-A), and Abdominal-B (Abd-B)*. When looking at the expression patterns of Hox genes in *Drosophila*, well-defined domains can be observed for each Hox gene along the anterior-posterior axis (Lohmann et al., 2007). The Hox gene homologs also determine tissue identity along the anterior-posterior axis in other organisms, such as mice and humans (Lohmann and Hueber, 2008). Previous experiments have shown that when the expression of a gene that controls body segmentation is altered, then the body segmentation is also changed in developing larva. One of the most well known Hox gene-related experiments involved the knockdown of *Ubx*, which resulted in the development of an extra pair of wings where the halteres would typically form (Lewis, 1978). In many cases, however, body segment transformation occurs when multiple paralogous Hox genes are mutated together (Lohmann and Hueber, 2008). There are mechanisms in place to regulate the expression of the homeotic genes.

Two groups aid in the regulation of Hox genes: the Polycomb group (PcG) and the Trithorax group (TrxG) (Duboule and Soshnikova, 2009). The PcG and TrxG form a protein complex consisting of histone methyltransferase activity and proteins that bind to methylated histone lysine residues according to earlier biochemical studies (Duboule and Soshnikova, 2009). Together, the PcG and TrxG gene products help regulate the Hox genes. The PcG and TrxG proteins bind to sites on the polycomb response elements (PREs) and trithorax response element (TREs), respectively. When TrxG proteins bind to sites on TREs, then the active subdomain that the PRE resides is maintained in the active state. In other body segments where the subdomains

are not active, PcG proteins bind to PREs, which maintain a repressed state (Kamakaka and Valenzuela, 2006). PcG proteins repress Hox gene expression during certain stages of development, while TrxG proteins are responsible for keeping Hox genes active at the right time (Duboule and Soshnikova, 2009). During the later stages of development, PcG and TrxG proteins form complexes with other proteins that bind to the same histone mark. Binding at these marks are necessary for attaining inheritable long-term repression of target genes (Duboule and Soshnikova, 2009)

A novel insulator that is of interest in this study is called SF1. The SF1 boundary was characterized using several different assays that are designed to test for enhancer-blocking activity. One method involved testing the effects of SF1 when positioned between an enhancer and a promoter. The RNA expression directed by the blocked enhancer was significantly lower than the RNA expression directed by the non-blocked enhancer in early fly embryos (Cai et al., 2003). Because the SF1 insulator is also active throughout the adult stage of the fruit fly, adult enhancer-blocking assays were designed to determine the enhancer-blocking activity through changes in the phenotype of flies. In an earlier study, several transgenic fly lines were tested for enhancer-blocking activity in adult flies. In the adult assay, the tested plasmids contained the SF1 boundary or the *gypsy* boundary. A plasmid containing lambda DNA served as the negative control, because lambda DNA doesn't have insulator activity. Each of the elements tested were placed in between two enhancers and a promoter. One of the enhancers was for the yellow gene, which is a gene that adds pigment to a part of the fly body when it is expressed. The type of yellow enhancer that contacts the yellow promoter influences the phenotype of a feature on the fly. When the yellow enhancer was blocked from contacting the yellow promoter by an insulator, the result was adult flies with lighter pigment (Cai et al., 2003). Without yellow

expression, the body, wings, and bristles would be a yellow color. The SF1 insulator is capable of strong enhancer-blocking activity when tested in enhancer-blocking assays, but unlike most insulators, SF1 does not exhibit cancellation when positioned beside another insulator element such as the *gypsy* insulator, Fab-8, or another SF1 insulator. It is possible that there is a specific insulator element that SF1 can exhibit the cancellation effect with through interaction. Another key piece of information about SF1 that remains unknown is the regulatory factors that are responsible for SF1's insulator activity during early fly embryogenesis. The reason for addressing this question is because the endogenous location of the SF1 insulator. It is hypothesized that the SF1 insulator requires one or more regulatory proteins to function as an insulator. It is imperative to learn more about what proteins are required for SF1 to function as an insulator.

The SF1 insulator is located between the *scr* and *ftz* genes in the Antennapedia complex (ANT-C) and is the first insulator to be discovered in this genomic region (Cai et al., 2003). Enhancers that communicate with either scr or ftz flank SF1. Though the enhancers flanking SF1 are within close proximity of both *scr* and *ftz*, they specifically communicate with their appropriate promoters (Ohtsuki et al., 1998). One element that aids in *Scr* and *ftz* gene regulation is the promoter-tethering element (PTE) near the *Scr* gene (Calhoun et al., 2002). The PTE directs the *Scr*-distal T1 enhancer to specifically interact with the *Scr* promoter. When the PTE function is removed, then the result is non-specific interactions by the T1 enhancer (Calhoun et al., 2002). The discovery of these elements provided more insight into the mechanics responsible for regulating the *Scr-ftz* region; however, there were still unanswered questions about the regulation of this gene region. One question was concerning the other *scr* and *ftz* enhancers and their regulation in the ANT-C. It is known that some of the enhancers of

the *scr* gene are located on the other side of the neighboring *ftz* gene. Likewise, there are *ftz* enhancers that are in closer proximity to the *scr* gene. The PTE element does not account for the activity of the other enhancers in the same region. There must be one or more elements that influence which promoters the *scr* and *ftz* enhancers communicate with.

It is believed that the SF1 insulator may be responsible for regulating gene expression in the *scr-ftz* region. Communication between the *ftz* enhancers and the *scr* promoter could be blocked by SF1. However, this would not explain how the *scr* enhancers on the other side of the *ftz* promoter are capable of interacting with the *scr* promoter. One possible explanation is that there may be another insulator element further downstream of the ANT-C that interacts with SF1 to loop the chromatin, resulting in all *scr* enhancers being within proximity of the *scr* promoter. The interaction between SF1 and another insulator element may also isolate the *ftz* DNA elements into a separate loop, thereby preventing communication between the *ftz* and *scr* DNA elements. It has been speculated that insulators may engage in long-range interactions with each other to create loops that serve as distinct regulatory domains (Felsenfeld and Wallace, 2007). This would ensure that the enhancers within the *scr* and *ftz* regions do not contact the inappropriate genes.

1.4: Summary of purpose and hypothesis

Discovering the regulatory proteins of SF1 is one of the primary goals of this study. It is believed that insulators may be categorized in groups depending on what regulatory factors are involved (Mohan, 2007). Some regulatory factors may be needed for one type of insulator function, while others may be needed for another type of insulator function. The approach utilized to answer this question was to perform a genome-wide deficiency screen. The reverse

genetic screen is useful for discovering single or multiple genes that have a significant impact on the function of an insulator. This technique involves using deficiency lines containing deletions on the X, 2nd, or 3rd chromosomes of the fly genome. The deletions span thousands of genes long, making it more convenient than screening a smaller amount of genes. In addition to deficiency lines containing large deletions, there are also lines that contain smaller deletions within each of the regions covered by the larger deletions. This allows the region to be screened in the same fashion without having to separately screen thousands of genes. From this secondary deficiency screen, one or more potential SF1-modifying lines should be attained and genes within the region that the sub-deficiency line(s) covers will be considered for the next phase of the screen. We seek to find a deficiency line that significantly decreases the enhancer-blocking function of the SF1 insulator. The final phase of the deficiency screen involved single-gene RNAi knockdown. RNAi fly lines containing single-gene knockdowns will be crossed to transgenic flies containing a SF1 transgene. The transgenes used contained an iab5 enhancer, an RFP promoter, and a Gal4 driver. The Iab5-RFP transgenes are desirable for this part of the screen because they will allow us to obtain a quantitative result that is based on the amount of fluorescence present, rather than categorizing embryos based on qualitative results. In addition to having data that is cleaner and more credible than data from in situ staining, using the Iab5-RFP transgenic lines will require less time to acquire results. The ultimate objective is to find one or more single genes that negatively and exclusively affect SF1 insulator function. Knowing the identity of these regulatory factors will allow us to better understand the mechanisms by which SF1 functions and will contribute towards learning more about similarities and differences between SF1 and other insulators.

CHAPTER 2

INTERACTION BETWEEN SF1 AND LOCAL ELEMENTS

2.1: The insulator looping model

In an earlier study, the gypsy insulator was positioned in tandem with another gypsy insulator to determine if they would interact with each other. The result observed was strong PE-directed lacZ expression, which means that the two gypsy insulators cancelled out each other's enhancer-blocking activity (Cai and Shen, 2001). Similar results were obtained using different enhancer and promoter combinations (Cai and Shen, 2001). Another study found that the gypsy insulator was responsible for creating regulatory domains through interactions with other insulators (Geyer et al., 2003). One of the regions that the gypsy insulator was found to interact with is known as 1A-2, which is an element that was also shown to have enhancer-blocking activity in fly embryos (Geyer et al., 2003). The results from these studies support the idea that if an insulator boundary possesses the ability to block enhancer-promoter communication, and then it must interact with another insulator boundary and vice versa. Another insulator, scs′, has the same capability of interacting with other insulator boundaries through the use of the boundary element associated factor or BEAF (Hart et al., 1997). In order for insulators to be capable of binding to one another, they would require bound proteins that can form protein-protein interactions with each other.

According to the insulator-looping model, insulator boundaries interact with each other via bound regulatory proteins that form complexes with each other, which form a chromatin loop

(Wei et al., 2005). Each loop created would be a regulatory domain that is not influenced by other elements that are outside of the loop (Wei et al., 2005). The chromatin loop would also protect elements within the looped domain from the spread of heterochromatin, which would explain the purpose of chromatin loops. For instance, if an insulator blocks an enhancer from communicating with a promoter on the opposite side, then the looping model proclaims that the enhancer-blocking activity would be due to the enhancer and promoter being in separate regulatory domains. Results obtained from previous experiments support the looping model, including a study in which the gypsy, scs, and scs` insulators were positioned in pairs and flanked by cuticle and wing enhancers and the yellow promoter (Geyer et al., 2003). The results showed that even when spacer DNA was in between the paired insulators, an increase in yellow gene expression in the cuticle and wing was observed (Geyer et al., 2003). Though this model is the most popular insulator model, there are some drawbacks to it. Not all insulators follow the rules of the looping model.

A previous study involved testing whether various known insulators could interact with each other in numerous combinations (Majumder and Cai, 2003). The gypsy insulator, one of the insulators used in the previous study, was paired with various other boundary elements, including the SF1 boundary (Majumder and Cai, 2003). The findings were that pairing the gypsy boundary with other non-gypsy boundaries did not produce the expected cancellation effect, but instead increased enhancer-blocking activity (Majumder and Cai, 2003). It was speculated that this may be due in part to unique characteristics of different insulators, such as the type of insulator proteins bound to the insulator, the enhancer-blocking strength of the insulators, and the affinity of insulators to associate with one another (Majumder and Cai, 2003). Insulators fit into various classes in which each class is identified according to several criteria,

including, but not limited to the affinity to other boundary elements, the individual strength of the insulator, and the type of insulator proteins that bind to the insulator.

2.2: The discovery of SF1-interacting elements

A question that still persists about the SF1 boundary is which insulators are capable of interacting with SF1. The SF1 boundary does not exhibit the ability to interact with another SF1 boundary or with several other known insulators when paired with them. Based on the results from an earlier study, there is a speculation that an unknown insulator element interacts with SF1. (Majumder and Cai, 2003). Other insulators that were found not to interact with homologous insulators may also have other elements that interact with them. An effort has been made to search in the Antennapedia complex and the Bithorax complex for DNA elements that can interact with the SF1 boundary. In an earlier study, a technique known as chromatin conformation capture (3C) was employed in an effort to discover new elements that have a high affinity for binding to SF1. 3C is useful for discovering long-range interactions on a 3D scale, making this method ideal for scanning a large genomic region for interacting elements (Fullwood and Ruan, 2009).

This study led to the discovery of 13 SF1-interacting elements or SIEs. Most of the SIEs was found in the Ftz-Antennapedia region and are named R2-R10, while three others were found in the intergenic regions of the Antennapedia complex and are named LP1, DS1, and AU1. One SIE, called BU1 is located near the Abdominal-B-m promoter. It is speculated that these SIEs are located in places, which may create regulatory regions within the Scr region. The location of R2-R6 corresponds to the location of the T1 and PS2 early enhancers, whereas R7-R10's positions correspond to the PRE element, which is active at a later stage. Previous work

performed on testing the interaction strength of the SIEs shows that the R2-R4 and R7-R10 interact at a higher rate than the SIEs between R4 and R7. These results suggest that the interactions between SF1 and the SIEs may be responsible for establishing regulatory domains in the Ftz-Antennapedia region. In addition to R2-R10, the additional four SIEs located in the intergenic regions of the Antennapedia complex are also within 170kb of the SF1 boundary, with the exception of BU1, which is approximately 5Mb away in the Abd-B region. This information adds a more complex dimension to what was initially proposed. The presence of this number of SIEs could mean that there are various ways that the Ftz-Antennapedia complex is regulated. However, this information does not specify whether the SIEs function as insulator boundaries or if the interactions seen in the 3C results are tissue or stage specific.

In a separate experiment, a FISH analysis was performed to examine the nuclear localization as well as the relative position between SF1 and LP1 or AU1. The findings from the FISH analysis were that SF1 and the two tested SIEs colocalized at varying percentages. There was a significant percentage of colocalization between SF1 and AU1 compared to the AU1 and C140 control. SF1 and AU1 had 51% colocalization in the first thoracic segment (T1), whereas there was only 42% colocalization in the third thoracic segment (T3). When checking the overlapping trend of AU1 and LP1, the results showed that there was less overlap in the more posterior segments. This implies that these SIEs are dynamic and regulated, which means that they may play a role in regulating Hox genes. An additional test performed in an earlier experiment was to determine if the SIEs were capable of acting as individual insulator boundaries in an enhancer-blocking assay. The results showed that most of the SIEs were in fact able to block as insulators, but there were some SIEs that did not display any enhancer blocking activity. Both R2 and R6 showed little to no enhancer blocking in the assay, though they have

previously been found to interact strongly with SF1. One problem that these mixed results pose is that the results do not match the logic behind insulators because there are non-insulator elements that interact with the SF1 boundary. One possible explanation behind this phenomenon is that the SIEs' ability to interact with SF1 may rely on other factors besides the SIEs' insulator strength. The SIEs' ability to form DNA loops with SF1 may depend more on their proximity to the SF1 boundary. The question that needed to be answered was if positioning SF1 in a transgene with a non-insulating SIE would allow the SIE to function as an insulator. Another question that needed to be investigated was if pairing SF1 and a SIE with strong enhancer-blocking capability will cause the cancellation effect as observed in previous studies (Cai and Shen, 2001; Geyer et al., 2003).

2.3: Experimental design

To answer these questions, two sets of transgenic lines were constructed. One set is intended to address whether SF1 aids in improving the enhancer-blocking function of SIEs with weak or no individual insulator activity through interaction. An interaction with SF1 using this set of transgenic lines would result in the separation of the NEE enhancer and the lacZ promoter into different regulatory domains. The other set is designed to answer whether strong enhancer-blocking SIEs exhibit the cancellation effect when paired directly beside SF1. The expectation of this set of transgenic lines is that since the strong enhancer-blocking SIEs can interact with SF1, then pairing the two DNA elements together should result in the cancellation of both elements. It is believed that because the SIEs exhibited a strong affinity for the SF1 boundary, they should show evidence of such interaction in the enhancer-blocking assay.

To construct the Interaction constructs, several DNA fragments had to be secured for preparation of the transgene. The transgenes would require a Casper vector plasmid, containing the NEE enhancer positioned downstream of the white promoter as well as the H1 enhancer located downstream of NEE and upstream of the lacZ promoter. The NEE enhancer served as the blocked enhancer that is separated from the lacZ promoter by a SIE. The H1 enhancer was capable of contacting the lacZ promoter because there was no boundary between H1 and the lacZ promoter. To test the insulator strength of R2 and R6 in the presence of SF1, each SIE was cut using the NotI restriction enzyme and inserted into a NotI site within the Casper vector, which was located in between of the NEE enhancer and the lacZ promoter. The SF1 boundary was cut with EcoRI and inserted in an EcoRI site further upstream of NEE. The SF1 boundary had to be positioned in a manner that brought it within close range of the SIE, but without interfering with the NEE enhancer itself.

The construction of the Cancellation transgenes involved the same Casper vector, but involving different SIEs, with the SF1 insulator in a different position. Both the SF1 insulator and a strong enhancer-blocking SIE were inserted beside each other in the Casper vector. The upstream NEE enhancer and the downstream H1 enhancer and lacZ promoter flanked the pair of insulators. The two insulator boundaries separately displayed strong enhancer blocking effects in previous experiments. The strategy of constructing a cancellation construct is to position the insulators side by side with no elements in between of them. In previous studies, insulators that are paired closely beside each other are not capable of blocking enhancer-promoter communication. The speculation is that the paired interacting insulators are already within close proximity of each other, so interacting with each other does not separate enhancers and promoters from opposite sites into isolated regulatory regions. In an enhancer blocking assay,

cancellation between SF1 and the SIE would result in the expression of lacZ in the NEE and H1 stripes. On the other hand, if cancellation does not occur, then there would be little or no lacZ expression in the NEE stripe and lacZ expression in the H1 stripe. The cancellation constructs will help to determine if this concept applies to an SF1 and SIE insulator pair.

The Interaction and Cancellation constructs were designed through restriction digestion of DNA fragments, followed by ligation of the fragments into the Casper vector and transformation of E.coli culture using each construct. After the positive clones were identified through restriction digestion and PCR, the plasmid DNA concentration was increased using the Midi prep and the concentrated DNA plasmids were injected into the germ cells of w- flies to produce independent transgenic lines. The single lines were tested in an enhancer-blocking assay by scoring the amount of lacZ expression on the NEE stripe relative to the H1 stripe. Approximately 50 embryos were scored for each line and the scoring on all tested lines and the NbbH control was performed double blind. The data collected was quantified into a bar graph and analyzed according to the percentages of embryos that showed either strong NEE block or weak to no NEE block.

CHAPTER 3

DEFICIENCY AND SUB-DEFICIENCY SCREEN

3.1: Deficiency Screen of NbbH Line

To identify a factor that affects SF1 insulator activity, a transgenic fly line containing SF1b was used. SF1b is a component of the entire SF1 sequence that has been shown to possess the most insulator activity out of the remaining compartments of SF1. The transgenic line used is known as NbbH because it contains two SF1b sequences that are flanked by two enhancers, the neural ectoderm enhancer (NEE) and the Hairy 1 (H1) enhancer. The NEE enhancer directs expression in a horizontal stripe across the A-P axis of an early fly embryo, while the H1 enhancer directs expression in a vertical stripe at the anterior region of an early fly embryo. The NbbH construct also included two promoters, *lacZ* and *miniwhite*. The *lacZ* promoter and H1 enhancer are located downstream of the SF1b insulators, whereas the *miniwhite* promoter and the NEE enhancer are upstream of the insulators. Both the NEE and H1 enhancers are capable of contacting the *lacZ* and *miniwhite* promoter, however, NEE is blocked from interacting with the *lacZ* promoter. The NbbH line was utilized in a genetic screen, which also included a gene deficiency kit consisting of over 150 deficiency (df) fly lines. The flies in each df line contained a deletion in their genomes that spanned an average of 10kb in length. The deletions were located the X, second, or third chromosomes and the df lines were homozygous for the deletions. In the screen, female virgin flies from each df line were crossed to male flies from the NbbH transgenic line. Once the females began laying eggs, they were stored in 25^{o}C, where embryos

from the crosses were collected every two hours on apple juice agar plates with yeast. The collected embryos were then aged for two additional hours so that the embryos would be 2-4 hours old. After the aging period, the embryos were stored in 4ºC until fixation at the end of the day. Embryos from sub-deficiency lines were collected and fixed using the same methods as previously mentioned.

3.2: Embryo Fixation

To preserve the embryo tissue, the embryos had to be fixed. The 2-4 hour old embryos were transferred into embryo collection wells. The embryos had to be removed gently by using 0.01% triton-X detergent to loosen embryos from the plates they were collected on, followed by draining embryos from each df line into their respective well. The embryos were then dechorionated in 100% bleach for approximately 3 minutes, followed by washing with copious amounts of ddH_2O. Dechorionated embryos from each df line were then transferred into their own collection vial, which contained 3 ml of fixation buffer, 4 ml of heptane, and 1 ml of 37% formaldehyde solution. The fixation buffer used consisted of 1.3x PBS and 67mM EGTA pH 8.0. The embryos were shaken vigorously for 25 minutes. The bottom phase was removed and approximately 8 ml of methanol was added to each collection vial, followed by 1 minute of vigorous shaking. The top phase was removed and the embryos were rinsed twice more in methanol, followed by two rinses in ethanol. The embryos were stored in -20ºC.

3.3: Whole mount *In situ* hybridization

Approximately 50λ of packed embryos from each df line were transferred into 1.5-ml eppendorf tubes. The embryos were washed in 1 ml of 100% ethanol for 10 minutes. The washing steps involved aspirating the previous solution from the tubes and adding the next solution, followed by slowly rocking the tubes on a nutator platform. This process was repeated six times within 1 hour. The embryos were then washed in 1ml of 1:1 ethanol/xylenes solution for 30 minutes. The embryos were washed in 100% ethanol for 3 minutes. The ethanol washes continued four more times. The embryos were then washed in a 1:1 ethanol/PBTF solution for 5 minutes. The PBTF solution consists of PBT (1X PBS and 0.1% Tween) and 5% formaldehyde solution. The next wash was the post-fix wash in PBTF solution for 25 minutes. The PBTF solution was rinsed out by washing the embryos in PBT for 2 min, 5 times. Next, the embryos were washed in PBT with 4µg/ml non-predigested Proteinase K. The PBT and Proteinase K solution were quickly rinsed without rocking twice in PBT, followed by washes in PBT with rocking 4 times, for 2 minutes each time. The embryos were post-fixed in PBTF solution for 25 minutes. The embryos were then washed in PBT 5 times, for 2 minutes each time. The embryos were washed in 1:1 PBT/hybridization solution for 10 minutes. The hybridization solution consisted of 50% de-ionized formamide, 5X SSC, 100 µg/ml of sonicated, boiled SSpDNA, 50 µg/ml heparin, and 0.1% Tween. The embryos were then washed in hybridization solution for 2 minutes, followed by a one-hour incubation in a 55ºC water bath. The RNA probe was prepared by making a 1:10 dilution of the probe from the probe stock using hybridization solution. The 1:10 diluted probe was incubated in an 80ºC water bath for approximately 4 minutes. The hybridization solution in the tubes was aspirated down to equal volume just above the embryos and 10λ of the prepared RNA probe was added to each tube of embryos. The tubes were lightly

flicked to mix and the tubes were incubated in a 55ºC water bath for 18 hours. The probe was later drained from each tube and the embryos were washed in 1 ml of hybridization solution for 5 minutes. The embryos were next put through a series of washes in hybridization solution that was pre-warmed at 55ºC. The embryos were incubated in a 55ºC water bath and the hybridization solution was changed in each tube approximately every 15 minutes within 2 hours. The embryos were then washed once in 1:1 PBT/hybridization solution for 15 minutes, followed by five washes in PBT for 10 minutes each. An antibody solution was made, consisting of 1:2000 diluted anti-deoxygenin antibody in PBT. The tubes were drained of PBT and the embryos were rocked in 500λ of the diluted antibody solution for 3 hours. The antibody solution was drained from each tube and the embryos were washed four times in PBT for 15 minutes each. The embryos were later washed twice in staining buffer for 5 minutes each. The staining buffer was made up of 100mM sodium chloride, 50mM magnesium chloride, 100mM Tris pH 9.5, and 0.1% Tween. The embryos were drained of the staining buffer, rocked in the staining solution for 1 minute and stained for approximately 30 minutes to 1 hour. The staining solution consisted of NBT and x-phosphate solution mixed in ddH_2O. After the staining was complete, the embryos were washed twice in PBT for 10 minutes each. The embryos were then washed in 1:1 PBT/ethanol solution for 15 minutes. The embryos were then washed in 100% ethanol six times, for 10 minutes each. The embryos were quickly rinsed in xylenes, drained and mounted on slides in 300λ of permount. Each line was scored double blind by the assessment of the stripe intensity on the embryos from each line.

3.4: *White In situ* stain

To determine if the effect on *lacZ* expression observed was due to an alteration in the NEE enhancer, embryos from the candidate lines were stained *in situ* for *white* gene expression. The same methods for embryo collection, aging, and fixing were used as previously described. The same *in situ* hybridization procedure was used as mentioned; only using the *white* RNA probe. The NEE stripe intensity was scored double blind in comparison to the NEE stripe from the control, which is the same control as previously mentioned.

3.5: Insulator specificity tests

To determine whether the effects observed in the candidate lines are specific to the SF1 insulator, two additional transgenic lines were incorporated into the screen. Both transgenic lines contain the same enhancers and promoters as NbbH, but have a different insulator located between NEE and H1 enhancers. The additional transgenic line used contains Su(Hw) insulator. The Su(Hw) transgenic line was referred to as NSH.

3.6: Data analysis of NbbH Screen

The strength of SF1 insulator activity was measured by comparing the ratio of stripe intensities of *lacZ* RNA from the NEE and H1 stripes. An expression profile was created by scoring the embryos based on the intensity of the NEE stripe relative to the H1 stripe, which was treated as a control stripe to indicate which embryos were stained. The embryos were placed under one of five categories depending on the strength of enhancer block. The categories ranged from strongest block to weakest block. Approximately 50-100 embryos were scored for each line and bar graphs were constructed to illustrate the percentage of embryos that fall under each

category per line tested. Each line was compared directly with the data from the control cross, NbbH flies crossed to white$^-$ wild-type flies, for any significant changes in the insulator's ability to block NEE. Candidates from the deficiency screen were selected based on the degree of changes in the number of embryos with lighter NEE stripes. *White in situ* stain data was analyzed by comparing the stripe intensity between the candidate lines and the control. Several criteria were considered when selecting the best SF1-modifying candidate, which included the difference in the percentage of embryos with no block and full block in the *lacZ in situ* stain, the difference in the percentage of embryos with no block and full block in the *white in situ* stain, and the insulator specificity of the change in enhancer blocking activity.

CHAPTER 4

SINGLE-GENE RNAI SCREEN

4.1: SF1 RNAi Cross Scheme

A candidate from the sub-deficiency lines was selected for the next phase of the screen. The candidate line contained a deletion that spanned approximately 80 genes. Out of the 80 genes from the sub-deletion, 40 genes were selected for testing in the enhancer-blocking assay. To determine which gene within the deficiency could have an effect on SF1 insulator activity, each gene was tested using RNAi. RNAi lines for each gene were ordered from the VDRC stock center. The RNAi lines each contained an inducible UAS-RNAi construct against one protein-coding gene. When induced, hairpin RNA was expressed and cleaved by Dicer to form double stranded RNA (dsRNA), which was used to degrade the mRNA of the target gene. Prior to the RNAi screen, the components needed for induction of dsRNA had to be in the transgenic line used for this screen. That transgenic line used, known as ISR, contained a construct that consisted of an Iab5 enhancer, a downstream RFP promoter, and an SF1 insulator element between the enhancer and promoter. To ensure that the Gal4 driver was incorporated into a homozygous Iab5-RFP stock in the Iab5-RFP transgenic lines, several sequential crosses were set up. The Iab5-RFP lines currently contain the insulator construct (i.e.: Iab5-SF1-RFP) on the X chromosome. A fly line called [Ser/Gal], which contains both serrate and the Gal4 driver on the 3rd chromosome, was crossed to the Iab5-RFP line. Prior to this crossing, several crosses were set up to monitor the Iab5-RFP construct on X. The Ser/Gal line also contained the

mini*white* gene and flies of this line have dark red eye color. This would prevent the reliance on eye color to track the Iab5-RFP construct throughout the cross scheme. To distinguish the desired intermediate offspring, the FM7 balancer containing the gene for bar eye shape was used on the X chromosome. In the beginning cross, the homozygous Iab5-RFP lines and the FM7 line were crossed to the Ser/Gal line. The progeny selected from the Iab5-RFP cross contained Ser on the 3rd chromosome. In the FM7 cross, bar-eyed flies either with or without serrate wings were selected for the next cross. In the next series of crosses, the males and female F1 progeny from the previous Iab5-RFP cross were crossed together and homozygous serrate females were selected. The next FM7 cross involved crossing FM7 serrate flies with FM7 Gal flies (identified by lack of serrate wing and eye color) progeny from the first FM7 cross. This allowed Ser and Gal to recombine together in the next generation. Males with serrate wings and Gal4 were selected and crossed to homozygous Iab5-RFP serrate females. The desired progeny from this cross were Iab5-RFP/FM7 Ser/Gal4 females and Iab5-RFP Ser/Gal4 males. In the next cross, Iab5-RFP/FM7 Ser/Gal4 females and Iab5-RFP Ser/Gal4 males were crossed and the desired progeny from this cross were homozygous Iab5-RFP Ser/Gal4 females and Iab5-RFP Ser/Gal4 males. Crossing these progeny produced a perpetual stock of homozygous Iab5-RFP Ser/Gal4 flies. The completion of this stock allowed us to proceed with beginning the RNAi screen.

4.2: SF1 RNAi Screen

The goal of this experiment is to determine if there are any significant alterations in the insulator function of SF1. RNAi knockdown was utilized to target single genes and test for any significant effects of each single-gene knockdown on SF1 insulator function. 7-8 hour-old *Drosophila* embryos from the RNAi crosses were collected and split into two batches, a heat-

shocked batch and a batch without heat-shock. The batches of embryos designated for heat-shock were heat-shocked at 37°C for 30 minutes in a closed moist container 1.5 hours after being collected and aged in 25°C. The heat-shocked embryos were later aged at 25°C for 5 hours. The batch of embryos without heat-shock were collected and aged at 25°C for 7 hours. Both batches of embryos were dechorionated in 100% bleach for approximately 3 minutes. After rinsing in ddH_2O, the embryos were mounted on slides in 25% glycerol for imaging under a fluorescence microscope. The intensity of RFP fluorescence were calculated double blind and analyzed in comparison to control embryos from ISR flies crossed to w^- flies.

4.3: Data Analysis of SF1 RNAi Screen

The mounted embryos were scored according to the number of viewable Iab5 stripes that were fluorescing red under UV. The total number of embryos with one, two, or three fluorescing stripes was recorded for both the embryo batches with heat-shock and without heat-shock. Additional calculations made from each slide included the stripe percentage and the positive embryo percentage. The stripe percentage is the total number of stripes viewed from a slide of embryos out of the maximum possible number of stripes. The positive embryo percentage is the number of embryos viewed that had fluorescent Iab5 stripes out of the total number of embryos counted. Comparisons between each RNAi lines and the control were made for each line by calculating differences in the stripe percentage and the positive embryo percentage in both the heat-shocked and non heat-shocked batches. Similar comparisons were made between heat-shocked and non heat-shocked batches to quantify differences in stripe and positive embryo percentages for each RNAi line. The difference between changes in each test line verses the control was also quantified. The total values from all replicates were calculated for each line.

CHAPTER 5

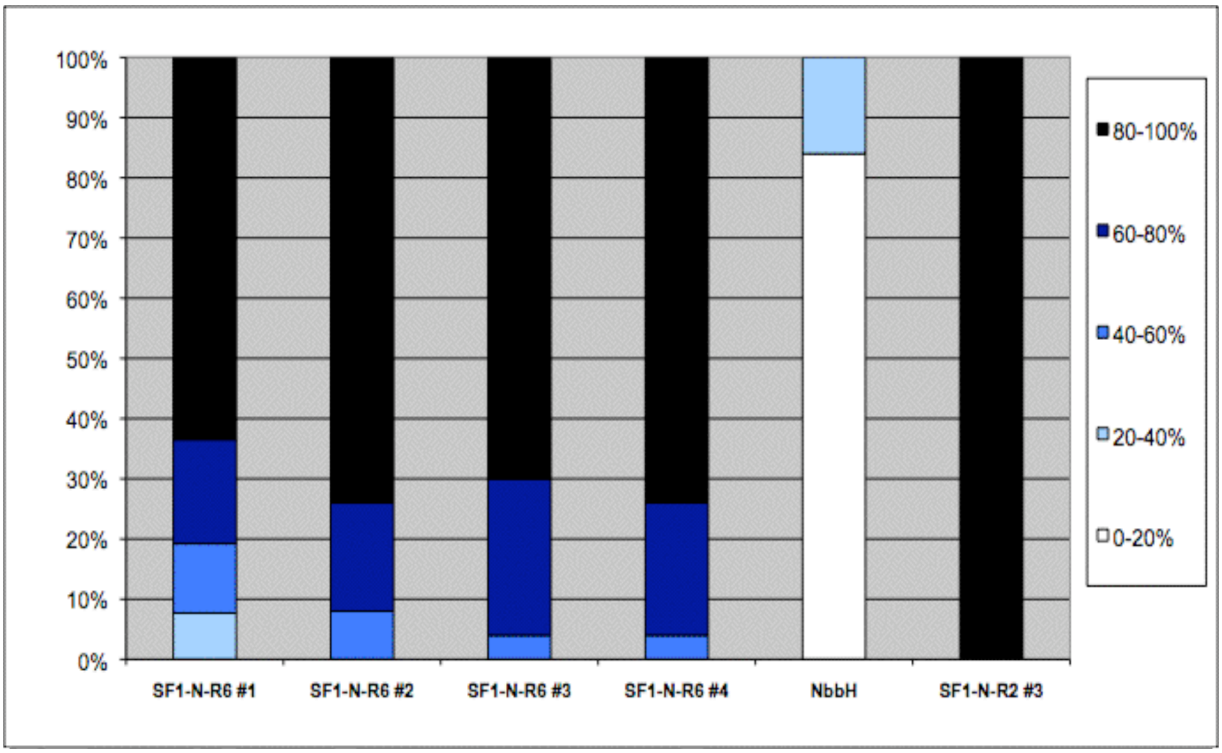
RESULTS/DISCUSSION

5.1: SF1 does not enhance R2 and R6 insulator activity

The SF1-N-R6 lines all display similar expression profiles, with the highest percentage of embryos having 80-100% NEE expression in every SF1-N-R6 line tested (Fig. 1). With the exception of SF1-N-R6 #1, the SF1-N-R6 lines had no embryos with strong or full block in the NEE stripe. The SF1-N-R2 line displayed full expression of lacZ on the NEE stripe in all embryos scored. The results of the interaction constructs tested indicate that the R2 and R6 SIEs' insulator functions were not enhanced in the presence of the SF1 boundary. When comparing the previous enhancer blocking assay results of R2 and R6 with the interaction test results, an increase in the number of embryos with full lacZ expression in the NEE stripe is seen in both R2 and R6 interaction lines. There is also a trend of having a much lower percentage of embryos with moderate or full lacZ block on the NEE stripe in the R2 and R6 interaction lines than in the N-R2-H or N-R6-H lines (Fig.1). This is more common when a significant decline in insulator function is seen in the enhancer blocking assay, but what is unexpected is R2 and R6's further decline in insulator activity when SF1 was positioned upstream of the NEE enhancer. The expectation was to either observe an increase in insulator block or no significant change and this is based off of previous enhancer blocking assays done in earlier studies. Interaction between these SIEs and SF1 occurred in previous 3C experiments, but what the results imply is that R2 and R6's interaction with SF1 does not support establishing isolated regulatory regions.

This could be due to the differences in insulator strength between the SIEs and SF1, which may not support advanced pairing capabilities as seen among other stronger insulators, such as the *gypsy* insulator (Geyer et al., 2003). One other possibility could be that R2 and R6 failed to interact with SF1 in this assay due to other elements that may have competed with SIEs for SF1. If there were other elements near where the transgene was inserted that SF1 had a stronger affinity for, then it would be possible for SF1 to choose an element besides the tested SIEs. The possibility of competing DNA elements is not as likely because similar results were obtained among several different SF1-N-R6 lines. Each SF1-N-R6 line has the same transgene in a different location in the genome. The results seen for these lines do not appear to be due to the position of the transgene because the data trend between the SF1-N-R6 lines are similar to one another. There is still much that is unknown about the SF1 insulator, including what preferences it has in a transgenic background versus the endogenous background. The data collected from the interaction tests show that the R2 and R6 SIEs cannot function as strong insulators, even when in the presence of the SF1 boundary.

Fig. 1: SF1 interaction *in situ* stain



The RNA in situ stain results of the interaction lines tested. The NbbH transgenic line was used as a control. Each line shows a percentage bar that is split into five categories that depend on the intensity of lacZ stain on the NEE stripe on scored embryos: white = 0-20%, light blue = 20-40%, blue = 40-60%, dark blue = 60-80%, black = 80-100%. Embryos from each line were scored double-blind.

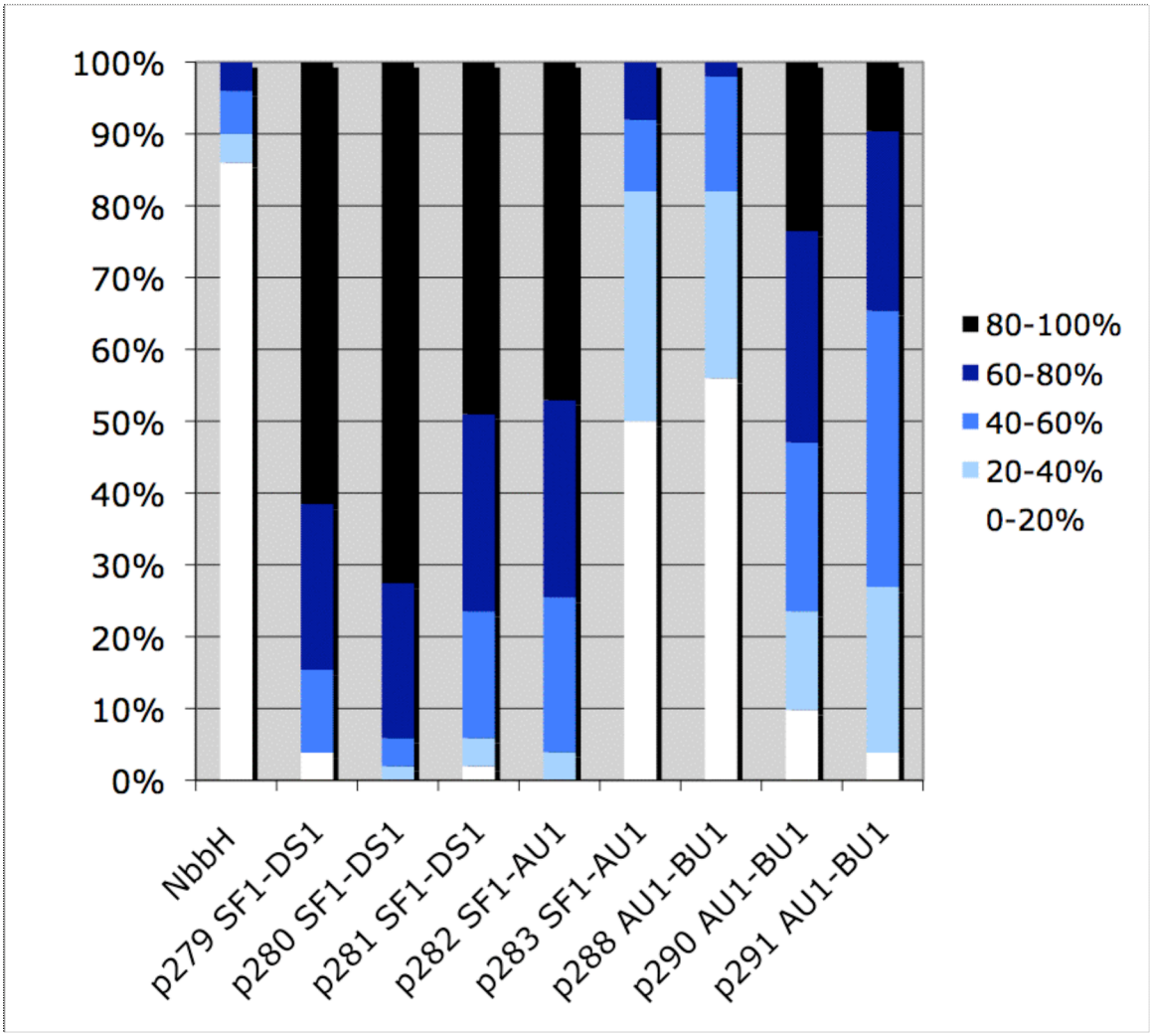
5.2: SF1 exhibits cancellation in tandem with AU1 and DS1

The results from the cancellation tests show that all of the SF1-DS1 lines tested have approximately 50% or more embryos with strong lacZ expression on the NEE stripe, in addition to approximately 5% or less of the embryos scored having little or no lacZ expression on the NEE stripe (Fig.2). The general profile trend of expression levels among the scored embryos is similar among all SF1-DS1 lines. The SF1-AU1 lines tested in this experiment yield results that

differed from one another. The p282 SF1-AU1 line had the majority of the population of scored embryos with little or no block of the NEE enhancer, with a minuscule amount of embryos having a moderately strong NEE block. On the other hand, the p283 SF1-AU1 line tested shows a totally different expression trend, with approximately 80% of the embryos scored having a full or moderately strong block of NEE. The three AU1-BU1 lines tested also show differing expression trends (Fig.2). The p288 AU1-BU1 line had an expression profile that most resembles the NbbH control line, which has most of the embryos scored in the moderately weak to no lacZ expression on the NEE stripe. The p290 AU1-BU1 line had a more even distribution of embryos among the categories of NEE stripe intensity, with approximately 50% of the embryos having moderate to strong NEE stripe intensity and the remaining amount of embryos having moderately weak to no NEE stripe intensity. The p291 AU1-BU1 line showed a trend that resembled the p290 line, but has a smaller percentage of embryos in the most extreme categories and the majority of the scored population in the intermediate categories. Cancellation appeared to have occurred in at least one of each of the SIE lines tested (Fig.2). The most direct results would be that of the SF1-DS1 lines, which all have significantly higher levels of lacZ expression on the NEE stripe as compared to the NbbH control. There appeared to be no significant difference in the data between the three SF1-DS1 lines, meaning that this result is due to an effect caused by pairing of SF1 with DS1. Regardless of where the transgene was inserted, SF1 cancelled out DS1's insulator activity. The SF1-AU1 lines were different from one another in results, however both lines showed an overall decline in enhancer blocking activity, which would suggest that SF1 and AU1 cancel with each other but that the degree of the cancellation effect observed in the transgenic background may be vary depending on the location of the transgene. The AU1-BU1 lines tested showed the most variation because all three lines showed

different expression profiles. The results from the AU1-BU1 lines suggests that AU1 and BU1 can cancel each other, but that the cancellation effect observed in the enhancer blocking assay may be affected by the location of the SIEs in the genome. Collectively, the results demonstrate SF1's ability to cancel with several DNA elements that can individually function as insulator boundaries. These results strengthens the claim that the newly discovered elements in the Antennapedia complex, including an element in the Bithorax complex, may interact with the SF1 boundary to form regulatory domains within the Antennapedia complex. The results from the AU1-BU1 lines may also explain how the scr and ftz genes are regulated. It is known that some enhancers of scr are located downstream of ftz, so the speculation was that a mechanism is put in place to ensure that the scr enhancers that are a long distance from the scr gene can contact scr instead of ftz. The AU1-BU1 results reflect the idea that there are boundary elements that interact and loop the chromatin, which brings the scr enhancers within closer proximity to their target gene. Likewise, the other SIEs in the Antennapedia complex are working with SF1 to form complex regulatory regions that establish proper gene regulation. Collectively, the cancellation test results suggest that SF1 can exhibit cancellation with other elements within the Antennapedia complex through interaction.

Fig. 2: SF1 cancellation *in situ* stain



The RNA in situ stain results of the cancellation lines tested. At least 2 independent lines were tested for each cancellation construct. The NbbH transgenic line was used as a control. Each line shows a percentage bar that is split into five categories that depend on the intensity of lacZ stain on the NEE stripe on scored embryos: white = 0-20%, light blue = 20-40%, blue = 40-60%, dark blue = 60-80%, black = 80-100%. Embryos from each line were scored double-blind.

5.3: Several deficiency lines affect the *lacZ* expression of NEE

In the deficiency screen, there appeared to be many lines that give a profile that differed from the NbbH w^- control. When the NbbH line is in the w^- wild-type background, approximately 75-80% of embryos had very little or no NEE expression and the remainder of the embryos showed moderate NEE expression. Some lines showed an increase in the number of embryos with little or no NEE expression (Figs. 3, 4, and 5). One example is line #7441, in which approximately 95% of embryos displayed a strong block. In general, lines such as the 7441 line also had fewer embryos with reduced enhancer block. Other lines, such as line #1910, had a significant increase in the number of embryos with a weak enhancer block or no enhancer block (Fig. 3). Likewise, the amount of embryos with stronger enhancer block was significantly reduced.

It was predicted that there would be very few regions that impact SF1 insulator activity, but throughout the deficiency screen, many potential modifiers were found. This came as a surprise because typically a genetic screen does not produce a large number of positives (Kalen et al, 2009). Approximately one fifth of the deficiency lines tested produced an effect on the insulator activity of SF1. This led to the speculation that many of the positive results observed were not due to a change in SF1 function, but in other factors. Because the screen initially involved testing lines with several hundred-gene deletions each, it was possible that other elements could be affected. The initial screen was more sensitive than expected, so to eliminate false positives, additional controls were incorporated in the deficiency screen. Two factors that needed to be taken into consideration included the function of the NEE enhancer and the *lacZ* promoter.

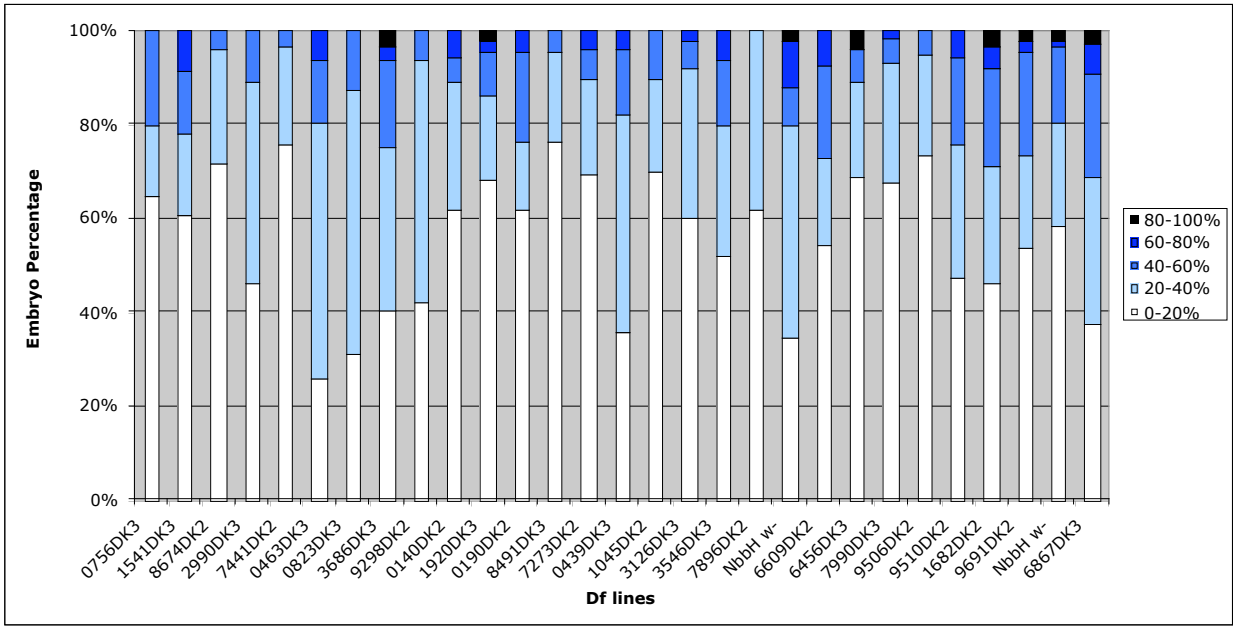
In past studies, the enhancer-blocking assay has been used to determine if novel DNA elements possess insulator properties. Previously, novel elements would be positioned in various ways to test whether they were capable of performing any insulator functions that were observed by insulators that were identified earlier. The enhancer-blocking assay can give an indication of insulator activity by comparing the levels of expression seen when the novel element is between the enhancer and promoter elements. One example of this would be the effect of SF1 on *miniwhite* expression in the *Drosophila* eye when SF1 blocks the CA enhancer from communicating with the *miniwhite* promoter in transgenic flies. The flies had eyes with lighter pigment than flies without SF1 in the transgene (Cai et al., 2009). Other studies have been performed that involved removing insulator elements from a transgene by crossing the transgenic line with flies containing *cre* recombinase. Insulator elements that are flanked by *cre* sites would be removed due to site-specific recombination. The product would be flies that contain all transgenic elements except for the *cre*-flanked element.

Insulator elements require regulatory elements to function as insulators. Because SF1 has possess insulator functions, there must be a protein or group of proteins that regulate it to ensure that the insulator functions of SF1 are active at the right time during *Drosophila* development. The expectation of this experiment is to use the enhancer-blocking assay as an indicator of insulator activity. From previous studies, it is understood that the disruption or removal of an insulator element that is blocking enhancers from interacting with promoters will result in more expression of the reporter gene. The removal of proteins that are responsible for SF1's insulator activity should also result in increased expression of the enhancer-deprived reporter gene.

5.4: Deficiency candidate lines show no change in NEE enhancer activity

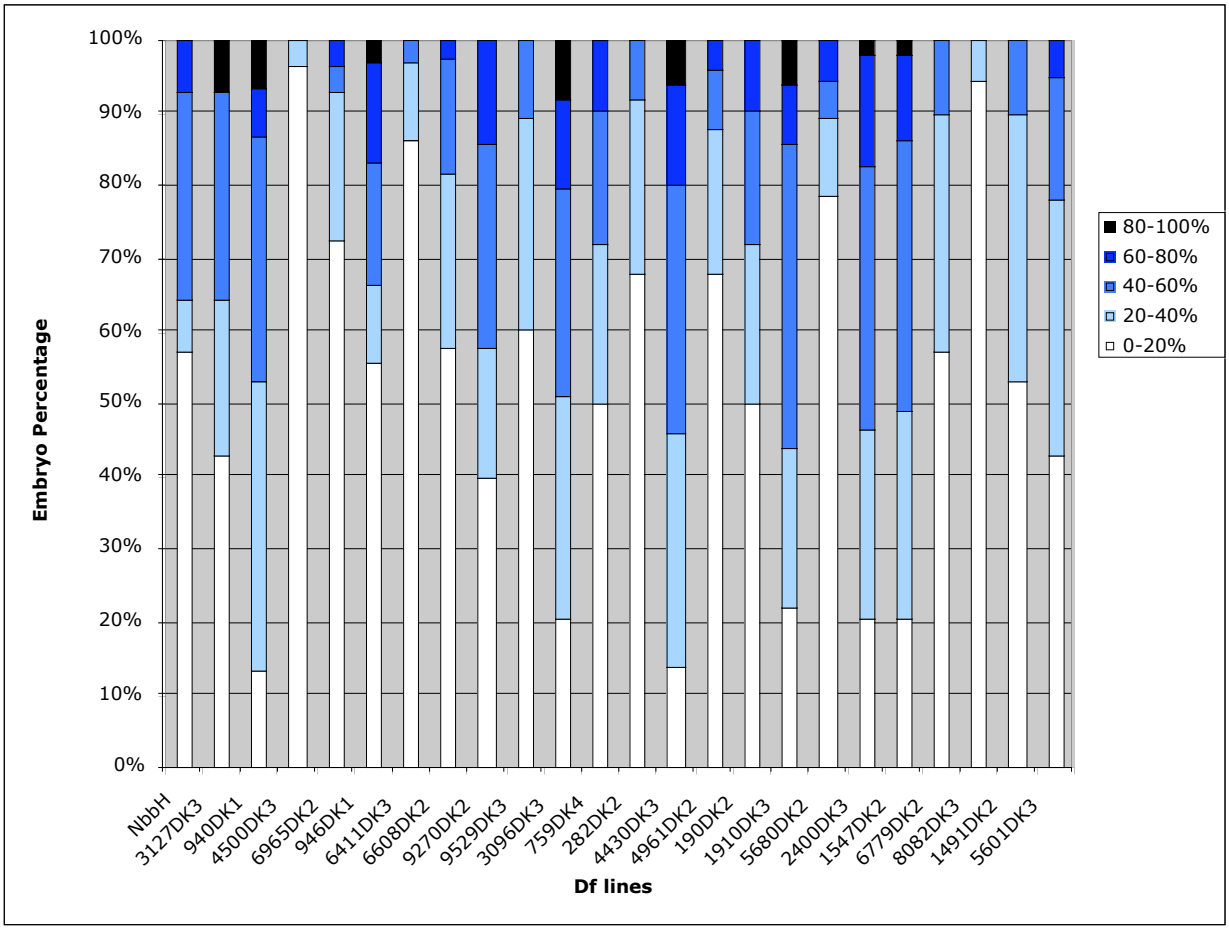
When the white gene expression was assessed, some of the candidate deficiency lines from the previous screen showed no significant change in NEE activity. Out of 13 candidate lines tested in the white in situ stain, 5 lines displayed a similar distribution of embryos with strong, moderate, and weak white expression along the NEE stripe as compared to the NbbH control (Fig. 6). The NbbH control displayed approximately 50% of embryos with moderate to high white expression. The expression of white should be higher than lacZ because the white promoter and the NEE enhancer are located upstream of the SF1 insulator. NEE can freely interact with the white promoter, but the H1 enhancer can't interact with the white promoter. The remaining lines tested had embryos with either more enhanced or suppressed white expression at the NEE stripe. One of the top SF1-suppressing candidates, line #5877, showed no significant differences in the percentage of embryos with little to no white expression as compared to the control. A few lines showed effects that were parallel to the effects seen in the lacZ in situ stain. The results of another SF1-suppressing candidate line, called line #5420, showed that there were significantly more embryos with more white expression compared to the control. A few other candidate lines displayed effects opposite to the lacZ stain results. For instance, line #7441 had one of the strongest SF1-enhancing effects observed in the preliminary part of the screen with approximately 3% of all embryos with little or no traceable amount of lacZ expression along the NEE stripe. However, the white in situ stain had approximately 85% of all embryos that contained moderate to high white expression along the NEE stripe.

Figure 3: Batches A and B of *lacZ in situ* stain



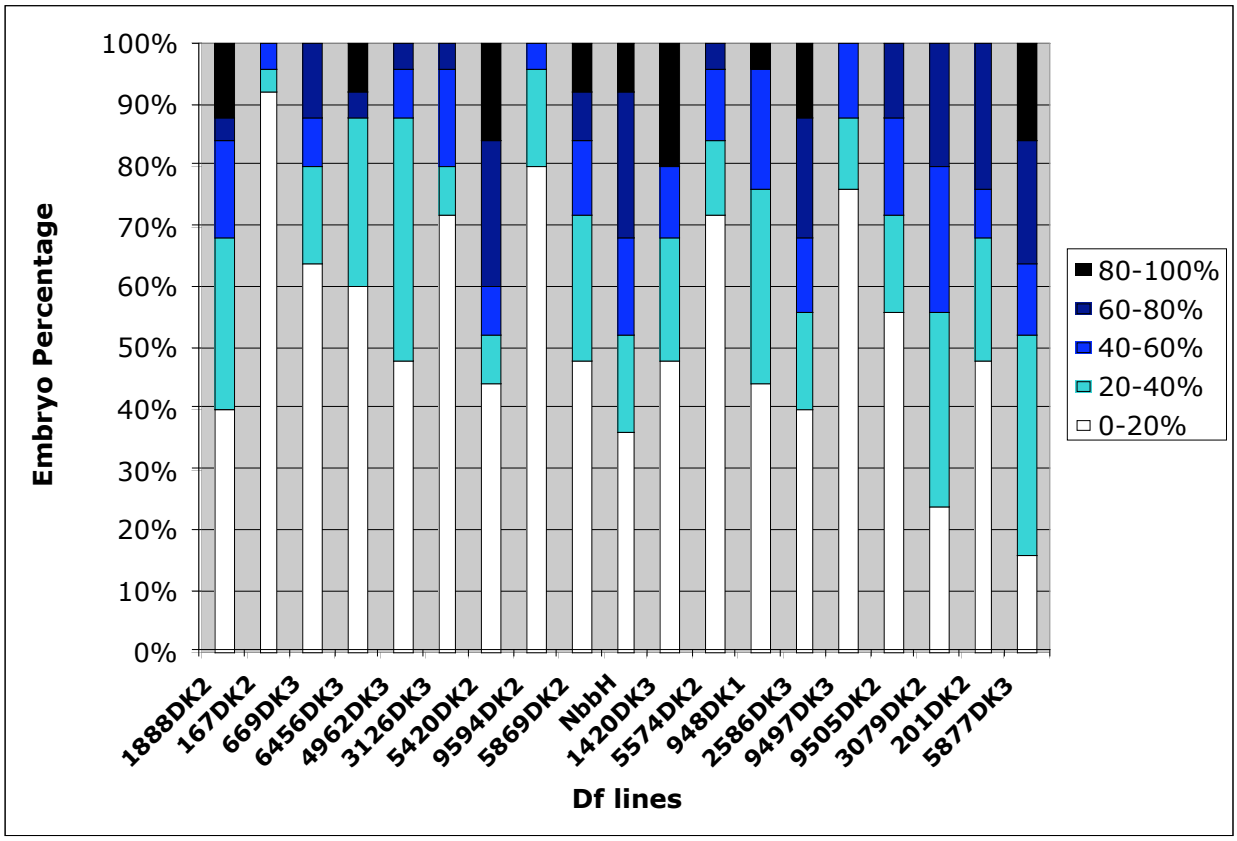
The RNA in situ stain results of the deficiency lines tested using the NbbH transgenic line. Each line shows a percentage bar that is split into five categories that depend on the intensity of lacZ stain on the NEE stripe on scored embryos: white = 0-20%, light blue = 20-40%, blue = 40-60%, dark blue = 60-80%, black = 80-100%. Embryos from each line were scored double-blind.

Figure 4: Batches C and D of *lacZ in situ* stain



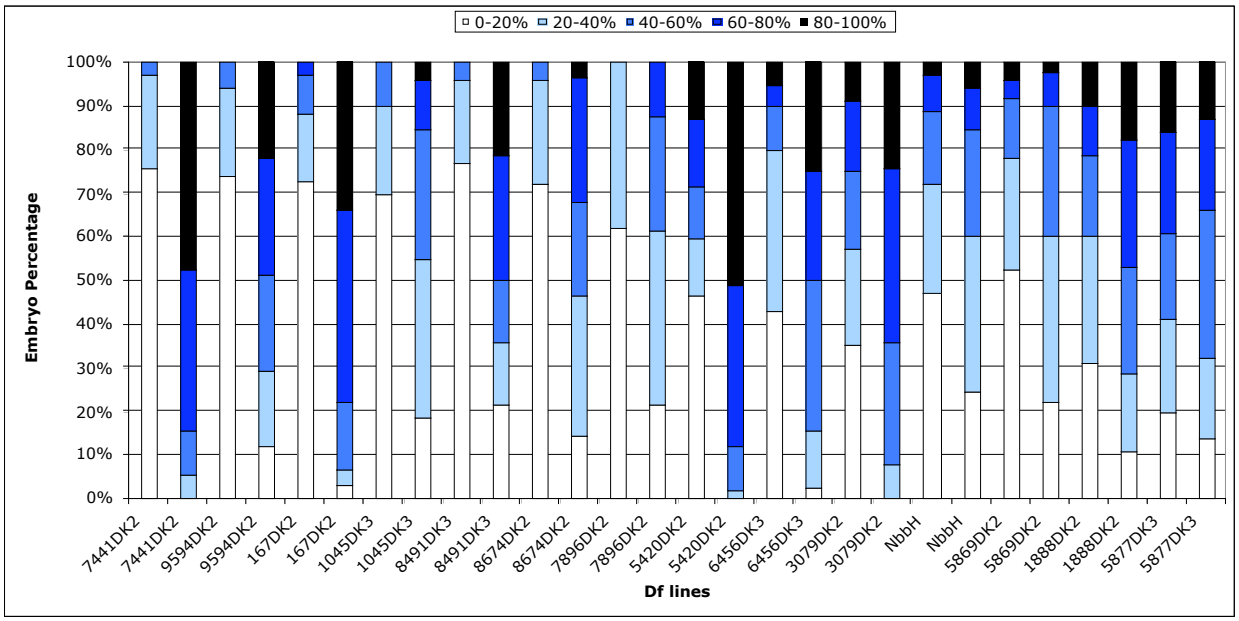
LacZ expression profile of additional deficiency lines tested with NbbH. Each line shows a percentage bar that is split into five categories that depend on the intensity of lacZ stain on the NEE stripe on scored embryos: white = 0-20%, light blue = 20-40%, blue = 40-60%, dark blue = 60-80%, black = 80-100%. Embryos from each line were scored double-blind.

Figure 5: Batch E of *lacZ in situ* stain



Additional lines tested for any effect on SF1 using NbbH. Each line shows a percentage bar that is split into five categories that depend on the intensity of lacZ stain on the NEE stripe on scored embryos: white = 0-20%, light blue = 20-40%, blue = 40-60%, dark blue = 60-80%, black = 80-100%. Embryos from each line were scored double-blind.

Figure 6: *LacZ* and *white in situ* stain of Extreme Modifiers



Expression profiles of both lacZ and white in situ stains using NbbH. From left to right, each line is listed in pairs with the lacZ stain results, followed by the white stain results. Each line shows a percentage bar that is split into five categories that depend on the intensity of lacZ stain on the NEE stripe on scored embryos: white = 0-20%, light blue = 20-40%, blue = 40-60%, dark blue = 60-80%, black = 80-100%. Embryos from each line were scored double-blind.

The results from the initial NbbH deficiency screen suggested that there were deficiency lines that produced genuine positive results. The entire result was seen through collectively analyzing the results of both the *lacZ* and *white in situ* stains. The *lacZ* stain allowed changes in the expression of the *lacZ* reporter gene in the NEE stripe to be assessed. This result could have been a consequence of reduced insulator activity by SF1. When the results of the *lacZ* and the *white* stain are combined, then what was observed was a profile of each deficiency line. The effects on the level of *lacZ* expression in the blocked NEE tissue region were observed in several

dozen lines. Out of the candidate deficiency lines, several lines showed no change in NEE enhancer activity as compared to the wild-type control. This proved that the effect on the *lacZ* expression observed was not due to a compromised NEE enhancer. Candidate lines that showed no change in NEE activity in the *white* stain were also tested for SF1 specificity.

One deficiency line that showed the most promise was line #5877. This line showed a strong SF1-supressing effect in the NbbH deficiency screen (Fig. 5). When calculating the ratio of scored embryos within each category of insulator block, line #5877 was observed having the highest percentage of embryos in the weak-block and no-block categories. In addition, line #5877 also had a significantly lower percentage of embryos with strong enhancer block. The *white* stain of line #5877 showed that the NEE enhancer activity was not significantly different from the wild-type control, which indicated that the difference in *lacZ* expression on the NEE stripe was not due to a change in NEE activity in line #5877 (Fig. 6). There was another line called line #7441 that appeared to be a genuine modifier of SF1. Line #7441 had an enhancing effect on the insulator function of SF1 and the NEE enhancer activity was surprisingly elevated compared to the wild-type control. This means that even with an increase in NEE enhancer activity, line # 7441 still showed a strong enhancer-block of NEE by SF1. Both lines 5877 and 7441 showed a significant effect on the SF1 insulator, but not the Su(Hw) insulator in a separate test. This indicated that within the deletions in these lines, there is a gene or smaller group of genes that are responsible for causing the SF1-modifying effect. The goal of the project was to find a gene or genes that are required for SF1 to function as an insulator, so line #5877 was pursued further in an effort to narrow the selection down to smaller gene deletions within this line's deleted gene region. The results obtained from the preliminary deficiency screen reveal that the in situ stain is sensitive, but false positives can be excluded using additional stains.

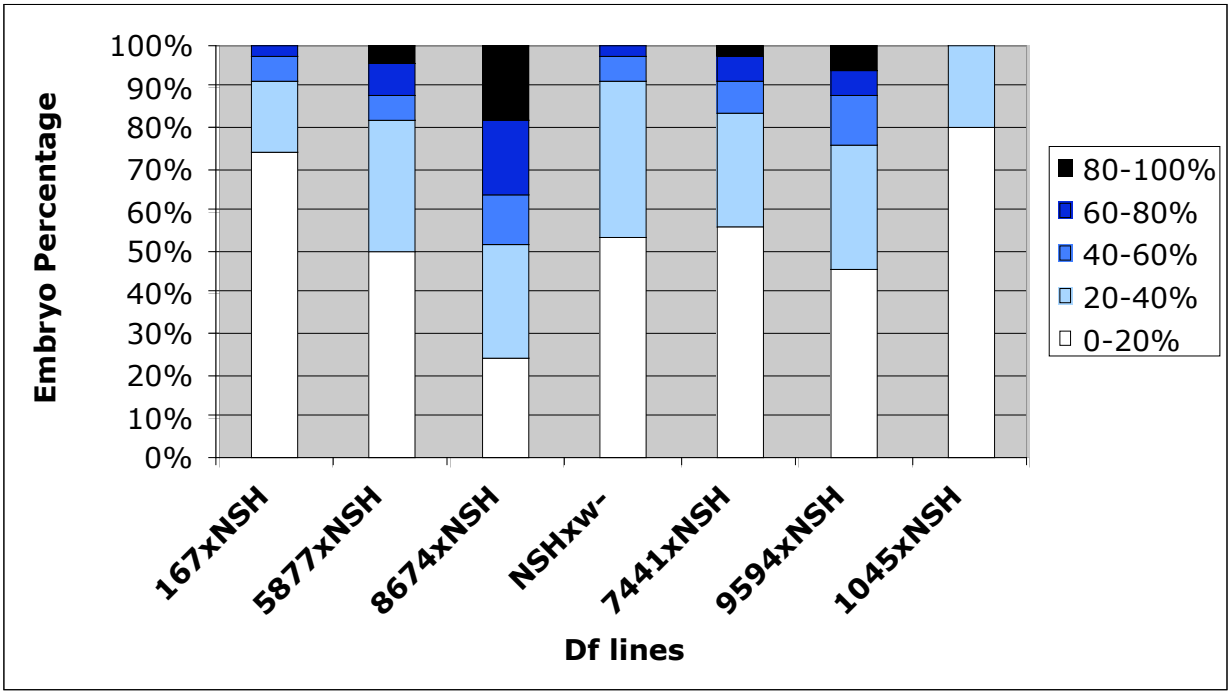
5.5: Candidate lines affect *lacZ* expression on NEE stripe using *gypsy* insulator line

Because the *gypsy* insulator displayed strong enhancer-blocking activity similar to that of SF1, it can be used to test for any changes in *lacZ* expression. Using a different insulator in the enhancer-blocking assay was important for several reasons. The effect that was observed in the earlier phase of the deficiency screen may not be specific to just the SF1 insulator. Because there are thousands of genes that were deleted in each deficiency line, the deficiency line may also affect the insulator activity of other insulators. The insulator specificity test would also reflect whether the effect observed was due to a compromise in the insulator activity or due to any change in the activity of other elements. Results from several candidate lines that were crossed to the NSH line containing the *gypsy* insulator showed varying effects on the *lacZ* expression. The NSH control line exhibited approximately 90% of all embryos with strong to moderate block (Fig. 7). Compared to the control, two candidate lines showed significant effects on *lacZ* expression, while three other candidate lines showed subtle differences in *lacZ* expression in embryos. Lines #167 and #5877 had approximately 90% and 80% of embryos with strong block, respectively. Lines #1045 and #8674, both of which were SF1-enhancing candidates, had approximately 100% and 52% of embryos with strong block, respectively (Fig.7). Based on the results, the effects observed were either similar to that seen in the deficiency screen using NbbH, opposite of the results from the NbbH deficiency screen, or negative. One explanation why there would be this variation of results would be that larger gene deletions can have differing effects because the proteins of the deleted genes may indirectly regulate how other proteins interact with the insulator elements. For instance, the *gypsy* insulator functions as an insulator with the aid of the Su(Hw) protein but the presence or absence of other proteins that interact with the Su(Hw) protein could result in a change in the frequency of the

Su(Hw) protein binding to the *gypsy* insulator. Another explanation could be that gene deletions that affected the function of one insulator could have had an opposite effect on a different insulator due to the difference in proteins that associate with each insulator. The Su(Hw) protein does not aid in SF1 insulator activity, but the regulatory proteins of both the SF1 and *gypsy* insulators may be indirectly influenced by common proteins.

Line #5877 showed a desired result in the insulator specificity test. The effect seen in line #5877 was subtle as compared to the NSH control line. A greater effect was seen in a different deficiency line, line #8674. Approximately 40% of embryos scored from line #8674 had weak or no enhancer block. On the other hand, approximately 10% of embryos scored from line #5877 showed elevated *lacZ* expression in the insulator-blocked NEE stripe. This suggests that the gene deletion of line #5877 does not affect the operation of the *gypsy* insulator and that the SF1-suppressing effect may be specific. This could not be completely verified due to the lack of transgenic lines containing other known insulators, but this result could mean that the gene deletion in line #5877 could exclusively affect SF1 activity.

Figure 7: SF1 specificity test: *LacZ in situ* stain of candidate deficiency lines



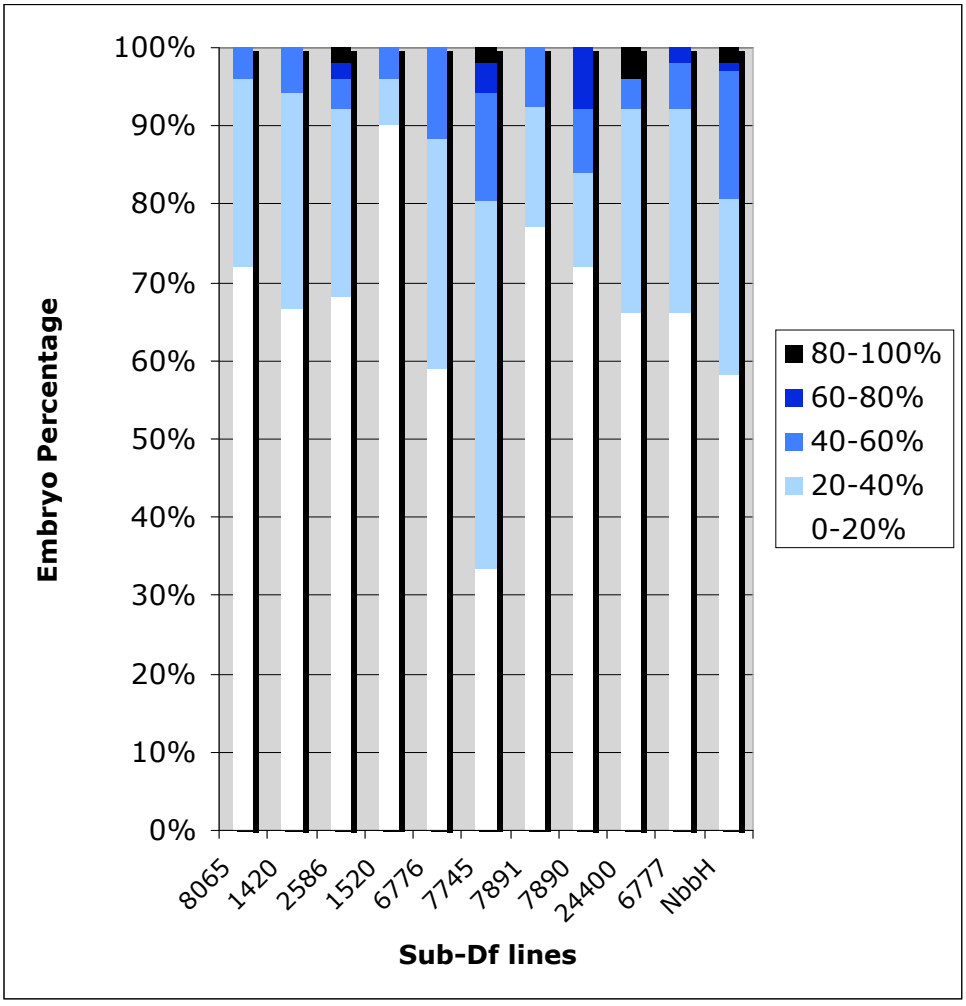
LacZ expression profiles of each candidate deficiency line tested using NSH. Each line shows a percentage bar that is split into five categories that depend on the intensity of lacZ stain on the NEE stripe on scored embryos: white = 0-20%, light blue = 20-40%, blue = 40-60%, dark blue = 60-80%, black = 80-100%. Embryos from each line were scored double-blind.

5.6: Sub deficiency line #7745 has a suppressing effect on SF1

The sub-deficiencies of line #5877 each had their own smaller gene deletions. Some lines had deleted gene regions that overlapped with that of other sub-deficiency lines. One of the sub-deficiency lines may contain the gene or genes that caused the SF1-modifying effect seen in the 5877 deficiency line in the preliminary screen. The *in situ* stain of the embryos from the sub-deficiency lines produced a profile of the distribution of embryos into five categories previously used in the preliminary screen.

The results from the sub-deficiency lines tested showed that the vast majority of the lines tested had an expression profile similar to the NbbH control. With the exception of one sub-deficiency line, line #1520, no significant differences were observed in the *lacZ* in situ staining results (Fig. 8). Surprisingly, the *in situ* staining of the sub-deficiency lines revealed that line #1520 had a SF1-modifying effect that was opposite to that seen in line #5877. Line #1520 had a significantly higher percentage of embryos with a strong enhancer block of the NEE stripe. Likewise, there was a line that had a similar effect on the SF1 insulator activity as the 5877 line. The sub-deficiency line, 7745, displayed a significant increase in NEE expression in the stained embryos that were scored for NEE block. The effect observed in line #7745 was not as prodigious as the effect observed in the 5877 deficiency line, but it was significant in comparison to the control as well as the majority of sub-deficiency lines tested that yielded negative results.

Figure 8: Sub-deficiency *lacZ in situ* stain



LacZ expression profiles of sub-deficiency lines from the 5877 deficiency. Each line shows a percentage bar that is split into five categories that depend on the intensity of lacZ stain on the NEE stripe on scored embryos: white = 0-20%, light blue = 20-40%, blue = 40-60%, dark blue = 60-80%, black = 80-100%. Embryos from each line were scored double-blind.

5.7: Single-gene RNAi knockdown affects SF1 insulator function

The expectation of this experiment is to pinpoint a single gene that is responsible for the effect on SF1 activity that has been observed through the deficiency and sub-deficiency screens. Embryos from the crosses between ISR and the RNAi lines had differences in the level of RFP expression in the Iab5 body region. The control cross, which consisted of ISR flies crossed to w^- flies, displayed embryos with little or no RFP expression, as indicated by both the intensity and the number of Iab5 stripes with RFP expression. Most ISR control embryos had one Iab5 stripe with RFP expression. The remaining percentage of embryos had two or three RFP-positive Iab5 stripes, but the intensity of the stripes were low. Most RNAi lines tested produced embryos with similar levels of RFP expression in the Iab5 regions. However, there were a small percentage of RNAi lines that displayed levels of RFP expression in the Iab5 region that significantly varied from the control.

Several criteria were analyzed to determine any significant changes in SF1 activity. Two primary values that were recorded were the percentage of Iab5 stripes present from a population of scored embryos and the percentage of embryos displaying fluorescence. The percentage of Iab5 stripes illustrated the amount of RFP expression and was calculated through adding together the number of stripes out of the maximum possible number of stripes. The percentage of fluorescent embryos showed how many embryos display the fluorescent stripes out of the total population of embryos collected for each line tested. These values were collected at both 25ºC and 37ºC and compared directly. The 25ºC temperature is one control that was utilized to observe any changes in stripe percentage or fluorescent embryo percentage. To maximize the amount of comparable data, additional comparisons were quantified for each line. The additional comparisons made include the change between each RNAi line and the control line at

25ºC, as well as the same comparison at 37ºC. The change in the 25ºC and 37ºC values of stripe and fluorescent embryo percentage was recorded and analyzed. The final value considered was the change in stripe and fluorescent embryo percentage in each RNAi line over the change of the same percentages in the control line. Collectively, the values collected created a profile that was used to identify any potential candidates (Table 2). Another interesting result was that several types of changes in RFP expression were observed when comparing the RNAi lines tested with the control. There appears to be several classes of RFP change between 25ºC and 37ºC embryos. Some lines displayed embryos that showed a relatively equal amount of RFP compared to the control and an increase in RFP when heat-shocked compared to the heat-shocked control (Fig. 9). However, other lines displayed varied levels of RFP expression at 25ºC, with some levels being higher or lower than the average RFP level of the control (Fig. 9). These various classes of change in RFP expression may be due to the leaky Gal-4 driver, which may result in varying levels of activity under 25ºC. Heat-shock is still needed to elevate the activity of the Gal-4 driver, so a potential positive line would still produce an increase in RFP expression.

Seven RNAi lines were selected based on fulfilling several criteria. The selected lines displayed a positive change in stripe percentage and in most of the selected lines, the change in the fluorescent embryo percentage is also positive when heat-shocked at 37ºC. A positive change indicates that the embryos from the RNAi line have a higher level of RFP expression than the control line. Another reason that these lines were selected was because they show a positive change when comparing 37ºC with 25ºC. Within these selected lines, there are two RNAi lines that have the largest positive change in stripe and fluorescent embryo percentages (Table 1). Collectively, the results suggest that the SF1 insulator function was reduced in some of the RNAi lines tested.

Table 1: RNAi screen results

Line#	Total 37ºC		Total 25ºC	
	stripe %	embryo%	stripe %	embryo%
wild type	22%	49%	25%	57%
100	11%	31%	27%	62%
wild type	21%	45%	21%	54%
8083	20%	45%	25%	56%
365	16%	35%	25%	59%
373	25%	56%	27%	58%
wild type	27%	52%	17%	43%
145	20%	52%	13%	27%
368	12%	28%	18%	43%
375	35%	61%	28%	54%
wild type	26%	49%	17%	44%
044	16%	36%	6%	13%
281	9%	23%	17%	36%
111	27%	61%	10%	25%
114	15%	34%	14%	35%
209	17%	39%	21%	47%
507	15%	38%	11%	29%
wild type	26%	53%	19%	49%
042	27%	63%	15%	42%
185	20%	47%	20%	54%
234	27%	50%	13%	31%
316	21%	50%	40%	72%
999	19%	39%	25%	57%
wild type	20%	49%	25%	51%
001	15%	35%	23%	49%
088	16%	39%	23%	55%
117	23%	51%	26%	55%
171	21%	50%	26%	57%
wild type	15%	35%	14%	39%
187	12%	30%	18%	44%
380	13%	35%	18%	43%

381	18%	42%	14%	34%
993	24%	52%	15%	36%
997	28%	58%	21%	48%
wt	24%	50%	32%	70%
110	21%	41%	28%	58%
269	18%	43%	24%	49%
287	20%	42%	23%	48%
683	26%	52%	21%	44%
935	27%	62%	25%	56%
wt	34%	62%	30%	60%
062	16%	34%	23%	50%
558	21%	44%	20%	48%
628	30%	53%	22%	55%
902	20%	41%	18%	45%
972	24%	48%	16%	38%
wt	38%	64%	29%	62%
367	19%	46%	16%	42%
553	27%	54%	15%	37%
892	21%	46%	21%	51%
958	18%	44%	21%	51%
wt	26%	51%	29%	64%
088	10%	21%	22%	52%
135	10%	20%	22%	47%
378	13%	27%	18%	45%
379	19%	36%	20%	45%
978	9%	21%	24%	52%
wt	13%	29%	15%	36%
098	9%	22%	12%	25%
144	5%	14%	14%	32%
992	8%	19%	13%	28%
wt	19%	39%	18%	39%
497	13%	28%	8%	-22%
498	7%	18%	8%	21%
511	7%	19%	11%	29%

List of RNAi lines that were tested for an effect on SF1 using the ISR transgenic line. The data is expressed in two values: the first value is the percentage of visible Iab5 stripes out of total possible stripes. The second is percentage of embryos with any expression. The data under the "Total 37ºC" column is from scored embryos that were heat-shocked at 37ºC and the data under

the “Total 25ºC” column is from scored embryos that remained at 25ºC for the entire aging duration.

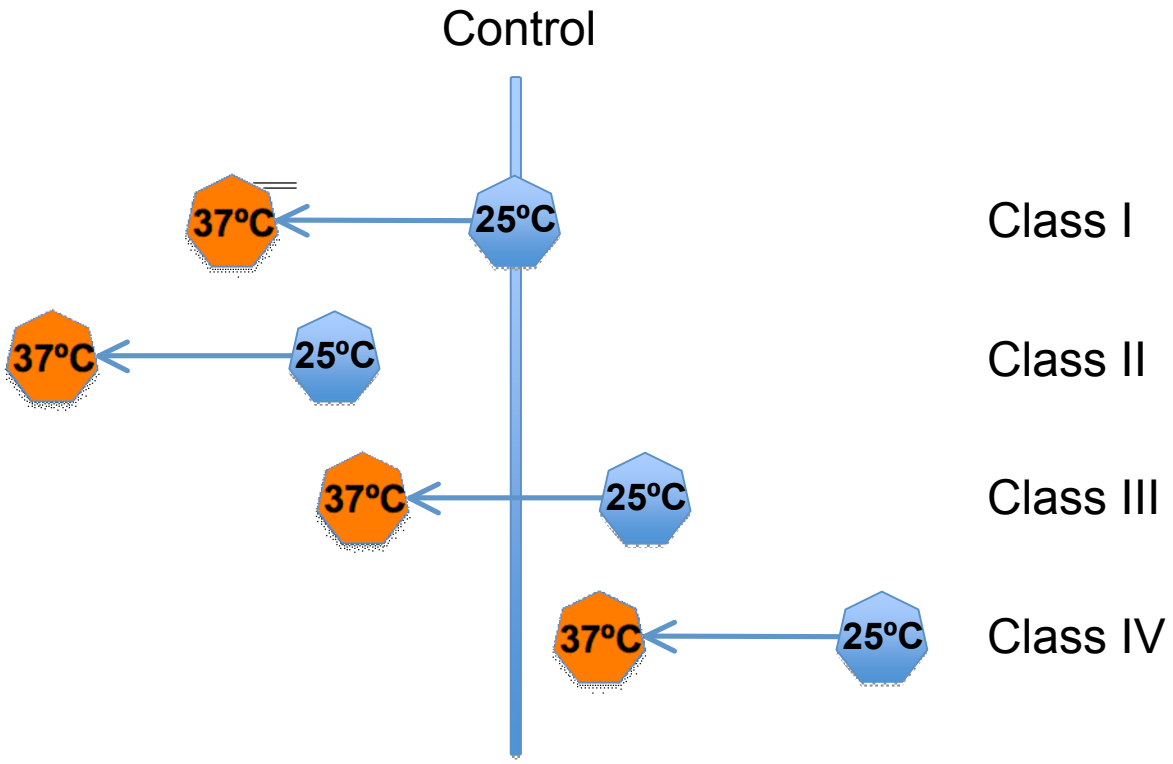
Table 2: RFP stripe assessment of RNAi candidate lines

CG number	∂ E/C @37ºC		∂ E/C @25ºC		37/25 set change		(Exp 37/25)/ (control 37/25)	
	stripe %	mbryos%	stripe %	mbryos %	∂ strp	∂ mbry	∂ strp	∂ mbry
8111	1%	12%	-7%	-19%	16%	36%	8%	31%
8042	1%	10%	-4%	-7%	12%	21%	5%	17%
42234	1%	-3%	-7%	-17%	14%	18%	8%	14%
13993	9%	17%	1%	-3%	9%	16%	8%	20%
13997	14%	23%	7%	9%	7%	10%	7%	15%
5683	1%	2%	-11%	-26%	4%	8%	12%	28%
7935	3%	11%	-7%	-14%	2%	6%	10%	26%

Candidate RNAi lines selected and RFP expression data on each line. The data is expressed in two values: the first value is the percentage of visible Iab5 stripes out of total possible stripes. The second is percentage of embryos with any expression. The pink columns show the change in each RNAi line over the control line at 37ºC. The green columns show change in experimental

over control at 25ºC. The white columns show the level of induction between 37ºC and 25ºC. The yellow columns combine the change in experimental over control over the heat shock.

Figure 9: Classes of RNAi lines



The diagram above displays the types of effects observed in the RNAi lines. The vertical line is the average RFP level relative to the control ISR line. Tthe left side of the vertical line represents a positive change in RFP expression compared to the control, while the right side of the vertical line represents a negative change in RFP expression compared to the control. The 25ºC and 37ºC icons represent the RFP expression levels of RNAi lines at 25ºC and 37ºC, respectively. The horizontal arrows represent represent the direction of change in RFP expression between 25ºC and 37ºC embryos.

5.8: Conclusion

Genetic screens can provide important information about DNA elements that are novel. Typically, the genes selected for testing in genetic screens are genes that have been extensively studied. This study involved screening through the fly genome for any modifiers of the SF1 insulator. Because there is not much information on SF1 besides its function, the screen had to be performed through gene deletions in random locations in the fly genome. Because there was no additional information known about what proteins may associate with the SF1 insulator, the reliance was on tracking the activity of the insulator using the enhancer-blocking assay. It seemed that the enhancer-blocking assay was sensitive because of the many positive results that were produced, but the incorporation of other tests led to the actual positives.

The genetic screen performed in this project has led to the discovery of several genes that may potentially be responsible for the SF1 element's ability to block enhancers from communicating with promoters. Most of the selected candidate genes are novel, with little or no information available about the function of the genes (Table 3). Two genes in particular that showed the greatest effect on SF1 activity are CG13993 and Vitelline membrane 26Aac (CG13997). CG13993 is suspected of having a function in protein folding (Luca et al, 2008). Vitelline membrane 26Aac also does not have much information that is known about it besides being a vitalline membrane protein-coding gene (Waring, 2010). It is possible that one of the candidate genes play a critical role in maintaining SF1's insulator function.

Additional tests will be required to ensure that the effect observed is not due to other factors, such as a change in the activity of the Iab5 enhancer or the RFP reporter gene. Also, to confirm any positives, RNAi may be performed on candidate genes *in vitro* using *Drosophila* S2 cells.

Previous insulator studies have been performed with the use of S2 cells to determine the state or function of insulators (Li, 2008; Mohan, 2007).

Table 3: Candidate genes of SF1-modification

Gene name	**Predicated Protein function**
CG8111	**novel**
CG8042	**novel**
Dbx	**transcription factor activity**
CG13993	**Protein folding**
Vitelline membrane 26Aac	**novel**
Adult enhancer factor 1	**transcription factor activity**
moleskin	**protein transmembrane transporter activity**

The list of candidate genes from the RNAi screen. The name or CG number of the gene and the predicted protein functions of each gene are listed. Predicted protein functions are based on indirect assay results from previous studies.

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