ANALYSIS OF CHROMATIN BOUNDARIES IN *DROSOPHILA*

by

SHARMILA ROY

(Under the Direction of Haini Cai)

ABSTRACT

SF1 is a chromatin boundary that helps to delineate the regulatory domains of the homeotic gene Sex comb reduced (Scr) and the non-homeotic gene fushi tarazu (ftz) in the Antennapedia Homeotic gene complex . SF1 and its subfragment Fb were shown to be a strong enhancer blocker in a transgenic embryonic enhancer blocking assay. It was also shown that in the adult eye SF1 was able to block the neighboring chromatin influences and subfragments a and c were strong CPE blockers. My work showed that SF1 and all its subfragments a, b and c are weak enhancer blockers in the adult eye. My work also confirmed that SF1 is a weak enhancer blocker when compared to suHw. Thus, SF1 is a developmental stage specific enhancer blocker. SuHw is a well studied chromatin boundary. We know that two suHw elements in tandem are unable to block an enhancer. SuHw is also a CPE blocker. My work shows that both the single and double suHw can act as CPE blockers. Hence, two suHw in tandem when flanking a gene can protect the gene from CPE.

Chromatin boundaries are formed by DNA elements and the protein factors that are involved in their function as boundaries. I conducted genetic screens to identify SF1 modifiers or protein factors. Deficiences and gene mutations were screened for their effect on SF1 enhancer blocking

function. The first deficiency screen of 50% of the genome was done using the enhancer blocking assay in the adult eye. Out of the 11 candidate deficiencies, 490DK2 on the 2nd chromosome was found to be specific for SF1 enhancer blocking function. I also conducted a deficiency screen for the 1st and 2nd chromosome with the SF1/bb embryonic enhancer blocking assay. 490DK2 was again a candidate modifier. I screened subdeletions in 490DK2 and found that there are both enhancer and suppressor of SF1/bb function in the 490DK2 region. After testing some of the P-inserted genes within this region, gene CG34380 is a strong candidate for enhancer of SF1 function, while genes CG34381 and CG13993 are strong candidates for suppressors of SF1 function.

INDEX WORDS: Drosophila, chromatin boundary, SF1, suHw, Hox gene, ANT-C, BX-C, Sex-comb reduced (Scr), transcription, Genetic screen

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS..iv

LIST OF TABLES vii

LIST OF FIGURES viii

CHAPTER

1 GENERAL INTRODUCTION 1

1.1: HOMEOTIC OR HOX GENE COMPLEX 1

1.2: CIS REGULATION OF TRANSCRIPTION..3

1.3: PROTEIN FACTORS ASSOCIATED WITH BOUNDARY ELEMENTS.......7

1.4: CHROMATIN BOUNDARY MECHANISM 9

1.5: FUNCTION OF SF1 BOUNDARY IN *SCR- FTZ* INTERGENIC REGION...13

1.6: REVERSE GENETICS FOR SF1 MODIFIERS.......................................17

2 DIVERSE TRANSCRIPTION INFLUENCES CAN BE INSULATED BY THE *DROSOPHILA* SF1 CHROMATIN BOUNDARY...25

2.1: INTRODUCTION...26

2.2: RESULTS 28

2.3: DISCUSSION 33

2.4: METHODS 36

3 MECHANISM OF CANCELLATION FOR SUHW BOUNDARY IN DROSOPHILA 45
3.1: INTRO 45
3.2: RESULTS 50
3.3: DISCUSSION 52
3.4: METHODS 53
4 MODIFIER SCREEN FOR SF1 BOUNDARY ACTIVITY IN DROSOPHILA HOMEOTIC GENE COMPLEX 59
4.1: INTRODUCTION 59
4.2: RESULTS 62
4.3: DISCUSSION 66
4.4: METHODS 70
5 SYNOPSIS 90
5.1: LESSONS FROM BX-C 90
5.2: REGULATION OF SCR-FTZ 92
5.3: SF1 TRANSACTING FACTORS 94
REFERENCES 100

LIST OF TABLES

Page

Table 3.1: Eye color distribution in suHw CPE block 56

Table 3.2: Compare eye color distribution with control 57

Table 4.1: The SF1 Deficiency lines for SF1 modifier candidates....................75

Table 4.2: The list of subdeficiencies in the 490DK2 region....................77

Table 4.3: The z test was performed for comparison of proportions....................85

Table 4.4: The list of candidate modifier genes to be tested by gene knockout....................85

LIST OF FIGURES

Page

Figure 1.1: Scr ftz genomic region 20

Figure 1.2: Enhancer blocking and CPE blocking models 20

Figure 1.3: Connection between boundary and transcription 22

Figure 1.4: Boundary elements facilitate formation of independent active and inactive chromatin domains in the nucleus 22

Figure 1.5: Structure of *Scr-Antp* gene region 24

Figure 2.1: The CPE-blocking activity of SF1 40

Figure 2.2: SF1 contains separate enhancer-blocking and CPE-blocking activities 42

Figure 2.3: SF1 is a weak enhancer blocker in the eye 44

Figure 2.4: SF1 has weaker enhancer-blocker in the eye than suHw 44

Figure 3.1: Compare suHw CPE blocking activity of 1x and 2x suHw 56

Figure 3.2: Compare the CPE blocking activity of suHw, 1x and 2x, to CA control 57

Figure 3.3: Compare the CPE blocking activity of a different enhancer blocker, SF1/Fb 58

Figure 4.1: The Deficiency screen using an adult eye-specific enhancer-blocking assay 74

Figure 4.2 : Deficiency screen using an embryonic enhancer-blocking assay 78

Figure 4.3: The 490 map 79

Figure 4.4: Analysis of 490 sub-deletions lacZ + white 81

Figure 4.5: NSH-LacZ 83

Figure 4.6: P-inactivation -LacZ 83

Figure 4.7: Wildtype Scr pattern....86

Figure 4.8: Scr pattern in P-inserted CG34381 88

Figure 4.9: Scr pattern in P-inserted CG34381 89

Figure 5: Models A and B for CG34381(GPCR) influence on SF1 function 98

CHAPTER 1

GENERAL INTRODUCTION

1.1: HOMEOTIC OR HOX GENE COMPLEX

The homeotic/ Hox genes are clusters of genes found in all bilaterians. The bilateria develop from three germ layers – the ectoderm, mesoderm and endoderm. Homeotic/ Hox genes are conserved in sequence and function among invertebrates and vertebrates (Duboule 1989). Homeotic genes are essential developmental genes as they are required for antero-posterior patterning of an organism. Genome sequences have revealed the enormous genomic diversity that exists among these organisms. For instance, genome sequencing of *Drosophila melanogaster* and *Mus musculus* has predicted ~13,600 and ~ 30,000 protein coding genes, respectively. Nevertheless, conservation of essential developmental phenomena among such diverse groups of organisms has established *Drosophila* as a powerful model organism for understanding developmental processes at a molecular level.

Hom/ Hox selector genes determine positional identity in the embryo. These positional values result in different developmental pattern in different embryos (Alberts 2002). Interestingly, Hom/ Hox genes exhibit spatial and temporal co-linearity. Temporal colinearity means that their order of temporal expression on the body correlates with the order of the genes on the chromosome. Spatial colinearity means that their expression and function along the body axis corresponds to the order of the genes on the body. The hox genes at the 3' end of the complex express at the anterior most end. The gene order on the chromosome corresponds to the order of expression domain in the body. Duplication and divergence of Hom/Hox genes is believed to have resulted in evolutionary diversity

among groups of organisms (Carroll 1995). Homeotic genes in *Drosophila* have been split into the Bithorax (BX-C) and the Antennapedia (ANT-C) gene complexes. Together they control the development of the head, thorax and abdomen. Antennapedia complex is approximately 500kb in length containing five homeotic selector genes for head and thorax developmental patterning. These are *labial (lab), proboscipedea (pb), deformed (Dfd), sex combs reduced (Scr)* and *antennapedia (Antp)* (Denell 1994). This region also contains several non-homeotic genes. Flies lacking the BX-C, only develop head and thoracic segments, since the abdominal segments transform into thoracic segments. The body segment identity is thus controlled by the hox genes (Lewis, 1978; Sánchez-Herrero et al., 1985). Bithorax complex is approximately 300kb long consisting of three homeotic genes- *Ultrabithorax* (*Ubx*), *Abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) (Lewis 1995). They control the identity of third thoracic and 1-8 abdominal segments. Hence, the genomic region is subdivided into nine autonomic domains abx/bx, bxd/pbx, and iab-2 through iab-8. For instance, domains of enhancers iab5 through iab8 activate *Abd-B* gene complex (Karch 1985; Celnikar 1990). Homeotic genes encode transcription factors active in specific segments. These factors regulate target genes determining the developmental patterning of the segment. The hox gene functions cell autonomously in the epidermis (Morata and García-Bellido 1976). For example, the lack of Ubx expression in a small number of cells in thoracic segment 3 (T3) will allow the T3 to transform into T2. Moreover, the hox genes are shown to express from early embryogenesis to late development (reviewed in Akam, 1987). Interestingly, there is a 180bp sequence within the homeotic gene that encodes 60 amino acid homeobox DNA binding domain called homeodomain (McGinnis, Garber et al. 1984).

1.2 : CIS REGULATION OF TRANSCRIPTION

The general principles of transcription can be applied in all metazoan systems (Arnosti 2002). In general, genes require a basal promoter surrounding the transcription start site (+1). RNA polymerase II along with over two dozen polypeptides comprises the basal transcriptional machinery. Their interaction with the promoter initiates transcription of a protein- coding gene. The promoter consists of a TATA sequence at -30, an Initiator sequence (Inr) at +1 and a Downstream promoter element (DPE) at +30. Not all promoters contain these elements (Arnosti 2003). Transcriptional regulation of many genetic systems including the Homeotic genes requires other cis-regulatory elements besides the promoter. The following are the different groups of elements.

Enhancers or Silencers: These are DNA elements that bind to sequence specific transcription factors in a positive (enhance) or negative manner (silence) (Arnosti 2003). Generally, they are distance and orientation independent. Specific interaction between the promoter and enhancer or silencer is crucial for transcriptional regulation. Two models have been proposed for such interaction. The Enhanceosome model, as exemplified by regulation of human *interferon beta* promoter, consists of a multicomponent complex including high mobility group protein HMG I(Y). HMG I(Y) binds the enhancer and promotes cooperative interaction among other factors of the enhanceosome. This facilitates recruitment of factors at the promoter to trigger transcription (Thanos and Maniatis 1995). Thus, transcription by enhanceosome assembly depends on the location of binding sites and specific factors bound to them. The Billboard model proposes that the enhancer acts like an information display system. It consists of less stringent

transcription factor binding sites so that both activating and repressing states can be displayed for interaction with basal transcriptional machinery. This might be the case for developmental genes undergoing evolutionary change (Arnosti 2003). Furthermore, developmental genes have multiple enhancers some of which are remote enhancers that determine specific expression in tissues. According to the Enhancer-facilitator hypothesis for activation of long range enhancer-promoter interaction, facilitator factors function between the enhancer and promoter to bring them in proximity (Dorsett 1999). In *Drosophila*, *trans* activitation by an enhancer can occur through a phenomenon called tranvection. It has been shown that body and wing enhancers from one allele can activate *yellow* promoter on a paired homolog (Chen, Huisinga et al. 2002). E.B.Lewis coined the term transvection while studying regulation of the Bithorax complex. He found that partial complementation of mutations that block successive steps in expression of Bithorax genes depend on transvection between paired homolog.

Maintenance elements: Packaging of eukaryotic DNA into higher order chromatin states is the next regulatory switch controlling transcription of genes. Hox gene expression can be divided into three phases- activation, establishment and maintenance (Deschamps, van den Akker et al. 1999). After initial transcriptional decision, the 'on' or 'off' states of transcription is maintained by the coordination of chromatin- remodeling complexes and histone modifying enzymes (Simon and Tamkun 2002). After the decay of segmentation regulators, Polycomb group (PcG) and Trithorax group (TrxG) proteins take over maintenance of Hom/ Hox expression pattern. The PcG and the TrxG proteins maintain the spatial pattern of Hom/ Hox genes. PcG proteins act as repressors and TrxG proteins act as activators of Homeotic gene function. TrxG and PcG proteins maintain the

transcriptionally active or repressed state of the gene by assembling chromatin complexes at the Trithorax Response Element (TRE) and Polycomb Response Element (PRE), respectively. PcG and TrxG proteins act synergistically and are dosage dependent (Pirrotta 1998). In *Drosophila*, two PcG complexes and two TrxG complexes have been characterized. PcG compexes are Polycomb repressive complex 1 (PRC1) and ESC- E(Z) complex. TrxG complexes are trithorax acetyltransferase complex 1 (TAC1) and Brahma (BRM) complex. PRC1 inhibits Chromatin remodeling complexes (SWI/SNF). ESC-E(Z) complex is linked to histone deacetylases which are involved is transcriptional repression. TAC1 complex has been linked to histone acetyltransferases and some BRM complex subunits are part of the SWI/ SNF complex (Mahmoudi and Verrijzer 2001). PRE and TRE sequences contain binding sites for proteins that are part of PcG/ TrxG complexes. It has also been found that some maintenance genes act as both activators and repressors of homeotic expression such as GAGA factor encoding gene (Bejarano and Busturia 2004). Pleiohomeotic (PHO) is a PcG protein while GAGA factor (GAF) and Zeste are TrxG proteins. It has been shown by *in vitro* experiments that GAF and Zeste increase repression by PRC1 (Mulholland, King et al. 2003). It has also been found that binding sites for PHO and GAF are present in some PREs . One example is the iab7 PRE in the Abd-B homeotic gene of the Bithorax complex (Mishra 2001). The MCP silencer in the Bithorax complex (Abd-B) is another PRE and requires PHO and GAF to maintain repression (Busturia, Lloyd et al. 2001). In transgenic *Drosophila*, a 800bp Mcp element acts as a silencer when paired with a PRE characterizing it as a pairing- sensitive silencer. It also allows long distance transregulatory interaction between enhancers and promoters (Muller 1999).

Boundary elements: As previously noted, chromatin states influence gene expression. Heterochromatin states are inactive condensed regions with hypoacetylated histones and few expressing genes. They are characterized by hypoacetylated lysine (K) in histones H3 and H4 and binding of heterochromatin protein 1 (HP1). This also involves increased methylation of H3 K9 and decreased methylation of H3 K4. Euchromatin states are active decondensed regions with hyperacetylated histones and are rich in expressing genes. They are associated with hyperacetylation of H3 K9 and K14 and methylation of H3 K4 (Alberts 2002). This difference in chromatin states is reflected in position effect variegation (PEV) whereby gene activity is affected by the surrounding chromatin state. Thus, it follows that domains of active and inactive states are formed to allow genes to function. Individual regulatory domains are maintained by formation of boundaries. Boundary elements can function in the following two ways.

a: <u>Enhancer blocker or insulator</u> that block nonspecific enhancer- promoter interactions when inserted between them. In the enhancer blocking assay the insulator is inserted between two enhancers and their expression patterns are observed. The insulator blocks interaction between the distal enhancer and promoter. The proximal enhancer- promoter interaction is unaffected.

b: <u>CPE blocker or Barriers</u> that protect the gene from surrounding positive and negative influences of the chromatin termed Chromosomal Position Effect (CPE) (Geyer 2003). In the barrier assay a transgene flanked by boundary elements protect it from CPE. Barriers also protect a gene from PRE mediated silencing when inserted between a promoter and PRE. Our studies with SF1 CPE blocking activity shows that the vertebrate barrier

activity and Drosophila SF1 CPE blocking activity might involve separate mechanisms (Majumder, Roy et al 2009).

Boundary elements are found in both invertebrates and vertebrates. Insulator elements were first discovered in *Drosophila*. These elements were called special chromatin structures (*scs and scs'*) and contained DNase I hypersensitive sites located at the boundaries of heat shock gene *hsp70* locus. Apart from boundary elements, the structural domains of chromatin organization are defined by scaffold and matrix attachment regions (SARs and MARs). These are AT-rich DNA sequences containing Topoisomerase II cleavage sites that mediate chromatin anchoring to nuclear matrix causing higher order domain formation. They remain attached to nuclear matrix after high salt or detergent treatment of nuclei (Kas and Laemmli 1992).

1.3: PROTEIN FACTORS ASSOCIATED WITH BOUNDARY ELEMENTS:

Considerable progress has been made in the characterization of eukaryotic boundary elements. The nature of protein counterparts of boundaries has also been studied. The following section discusses the characteristics of some of those boundary elements.

a. In ***Saccharomyces cerevisiae*** the boundary HMR tRNA [thr] is helped by factors RNA polymerase III complex, smc1 and 3, Med1cohesin. It works as a barrier blocking Sir-mediated silencing from the HMR silencer (Donze and Kamakaka 2001).

Another boundary STAR is known to associate with Tbf1, Reb1 (general transcription factors). These boundaries belong to X or Y' family of subtelomeric repeats, these are subtelomeric anti-silencing regions (STAR) that delimit telomeric silencing (barrier) (Fourel, Revardel et al. 1999).

b. *Drosophila melanogaster* has many known boundaries that have been well studied.

scs, scs' are insulators flanking the 87A7 *hsp70* locus. Upon heat shock, robust transcription produces decondensation called puff on the polytene chromosome. The insulators delimit the domain of decondensation. The protein factors involved are Zeste-white 5 (Zw5), boundary element- associated factor (BEAF) (Kellum and Schedl 1992; Kuhn 2004).

gypsy / Suppressor of Hairy-wing is a well known boundary. The boundary was identified within the *gypsy* retrotransposon. The 340 bp SUHw insulator has 12 binding sites for a zinc finger DNA binding protein, Suppressor of Hairy-wing SUHW. The insulator can directionally block distal enhancer from promoter. This also requires a BTB domain protein Mod (mdg4) which binds to SuHw protein. Endogenous *gypsy* insulator contains binding sites for CP190 (Geyer and Corces 1992; Gerasimova 1995; Pai, Lei et al. 2004).

Fab7, Fab8 and other Bithorax boundaries are well studied. In the Abd-B (Bithorax) complex, enhancer iab7 domain is flanked by insulators Fab7 and Fab8. A promoter targeting sequence (PTS) is found within iab7 that helps in overcoming blocking effect of insulators on distal enhancers. One known protein factor is GAGA (Fab7) (Hagstrom, Muller et al. 1996; Zhou 1999; Barges 2000).

Eve promoter (eve P) also acts as a boundary. eve P contains TATA element which is part of a strong promoter and GAGA sequence which mediates enhancer blocking. It facilitates activation by distal enhancers (Ohtsuki 1998).

Fa[swb] in chromosomal interband 3C6-7, protecting the Notch gene against CPE (Vazquez and Schedl 2000).

c. In ***Gallus gallus*** the well known boundary is called HS4 3'HS. CCCTC- binding factor or CTCF is required for enhancer blocking. Insulators prevent crosstalk. 5'HS4 insulator is located between *beta globin* locus control region (LCR) and upstream folate receptor enhancer. 3'HS insulator is located between *beta globin* LCR and downstream olfactory receptor enhancer. Within 5'HS4 a separable barrier element is sufficient for CPE protection (Chung, Whiteley et al. 1993; Recillas-Targa, Pikaart et al. 2002).

d. In ***Mus musculus*** differentially methylated domain / imprinting control region (DMD/ ICR) is very well studied. The protein factor CTCF (sensitive to CpG methylation) is associated with its function. This 2kb DMD/ ICR controls reciprocal imprinting of linked genes *H19* and insulin growth factor *Igf2*. It blocks access of *Igf2* to shared enhancers on the maternal chromosome.

e. In ***Homo sapiens*** 5'HS5 which is the Mammalian *beta globin* insulator element. CTCF is associated with its boundary function (Farrell 2002).

The examples given above elucidate the fact that boundary functions differ among organisms. Some boundaries have both insulator and barrier functions characterized within the same region whereas some boundary elements, especially in yeast, have been primarily characterized with barrier activity.

1.4: CHROMATIN BOUNDARY MECHANISM

Establishment of functional independence might involve formation of a physical block to cis environment, recruitment of specific regional interactions or formation of subnuclear active or silent domains (Capelson and Corces 2004). All these activities may involve

boundary elements. Proposed models, explaining the mechanisms of boundary function, are based on two broad concepts. Firstly, they might be involved in transcriptional regulation and secondly, they might be associated with structural chromatin organization. The "promoter decoy model" for instance, explains enhancer blocking activity of insulators by suggesting that insulators interact with activators bound to the enhancer thereby preventing enhancer – promoter interaction. This is supported by the evidence that eve promoter also contains GAGA sequence responsible for insulator activity (Ohtsuki 1998). However, the idea that the enhancer encounters the insulator while tracking for the promoter has not been proven. Moreover, existing models do not explain the directional effect of boundary on enhancer blocking (West 2002). On the other hand, a barrier may function as a passive physical block and in association with other factors, as an active element in delimiting spread of chromatin states.

Both, *scs/scs*'and *gypsy* insulators are found at borders of condensed and open chromatin. Chicken *beta globin* 5'HS4 is also located between open chromatin and heterochromatin. It has both insulator and barrier properties. Recent chromatin immunoprecipitation (ChIP) analysis of the transcriptionally activated *beta globin* locus has shown that boundary elements are present at the borders of heterochromatin marked by methylated H3 K9 and euchromatin marked by hyperacetylated H3 and H4 (Litt, Simpson et al. 2001; Litt, Simpson et al. 2001; Burgess-Beusse, Farrell et al. 2002). When transgene carrying cells were treated with deacetylase inhibitors their expression was comparable to the presence of insulators (Pikaart, Recillas-Targa et al. 1998). It has been observed that highly acetylated HS4 are required to maintain transgene expression (Mutskov, Farrell et

al. 2002). Thus, it is proposed that boundary elements may recruit histone modifying enzymes HATs to block spread of chromatin states. However, the barrier activity of HS4 does not depend on binding of CTCF. CTCF is a well known insulator protein. HS4 enhancer blocking activity requires CTCF. Hence, HS4 boundary contains barrier and insulator activities on separate fragments. In *Drosophila* the dCTCF is also associated with boundary function. In mammalian genome, the DNA methylation of the 3'-CG-5' (CpG dinucleotide) occurs. 75% of the CpG dinucleotide in human somatic cells is methylated. The unmethylated ones are mostly found in CpG islands (CGI). In the human brain, genome-wide study showed that un-methylated CGIs (U-CGI) are flanked by sites similar to zinc finger protein binding sites like Sp1 and CTCF binding sites. These sites are in the boundaries of these U-CGIs and the boundary core regions spread around 400bp upstream or downstream of the CGI. This could be associated with the prevention of methylation spread into U-CGIs (Fan et al., 2007).

Most boundaries are not characterized with insulator and barrier activities on separate fragments. Therefore, a mechanism that explains both insulator and barrier activities has been proposed. This involves formation of chromatin loops causing domains of structural and functional independence. Evidences from SuHw insulator studies support the "looping model". It was shown that two suHw insulators inserted between an enhancer and a promoter, cancel each other allowing enhancer-promoter interaction. It was also shown that enhancer blocking was stronger when the enhancer was flanked by two suHw insulators as compared to one suHw insulator inserted between the enhancer and promoter (Cai 2001). Genetic experiments had shown that SuHw and Mod (mdg4) proteins are required for *gypsy* insulator function (Geyer and Corces 1992) (Georgiev and

Kozycina 1996). Mod (mdg4) contains a BTB/POZ domain capable of self interaction. In Mod (mdg4) hypomorphic mutant both the boundary function and nuclear organization is disrupted (Gdula and Corces 1997). Recently, the insertion of GAGA factor binding sites between an enhancer and SuHw insulator also showed neutralization of insulator function. This depended on both GAF and Mod (mdg4) proteins and these proteins were shown to interact *in vivo* and *in vitro* (Melnikova, Juge et al. 2004). GAF is a DNA binding protein which also contains a BTB/ POZ domain like Mod (mdg4). *In situ* analysis of polytene chromosomes indicates localization of the SuHw and Mod (mdg4) proteins in several hundred endogenous sites (Gerasimova 1998). During interphase, in diploid nuclei these proteins aggregate forming insulator bodies and approximately 75% are close to the nuclear lamina (Gerasimova 2000). It was observed that sequences in the *gypsy* insulator have MAR activity (Nabirochkin, Ossokina et al. 1998). Matrix/scaffold attachment regions (MAR/ SAR) associate with nuclear matrix. Nuclear localization of *gypsy* insulator was supported by the analysis of two specific DNA sequences 4D and 7B on X chromosome about 3000 kb apart. In diploid nuclei they produced two separate fluorescent in situ hybridization signals. But, when these sequences contained *gypsy* insulator insertions (ct^6 fly strain) they associated into single nuclear localization. In the SuHw mutant background ct^6 strain produced two distinct signals similar to wildtype fly (Gerasimova 2000). However, another study has shown that suHw insulator which is a 340 bp part of the *gypsy* insulator does not particularly exhibit nuclear lamina localization. It has insulator activities even when localized inside the nucleus (Xu, Li et al. 2004). In recent data, we found that the single suHw flanking the white gene confers CPE protection. Double suHw flanking the transgene is also able to block CPE. Thus, in

this instance, cancellation did not occur and the barrier activity was not completely abolished. The insights into cancellation mechanism of suHw is discussed in third chapter.

The *scs* and *scs'* boundary elements were found to similarly interact supporting the looping model. They flank the heat shock locus *hsp70* 87A. The Zw5 zinc finger protein binds to *scs* and BEAF protein binds to *scs'*. These proteins were shown to interact both *in vivo* and *in vitro* (Blanton 2003). ChIP analysis showed that immunoprecipitate with BEAF antibodies was enriched in *scs'* as well as in *scs* (Zhao, Hart et al. 1995). This indicates there might be a physical link between the two boundary elements. However, in transgenic assays pairs of *scs/scs'* boundary elements did not show neutralization of insulator activity (Kuhn 2003; Majumder 2003). Thus, various molecular associations may be involved for the function of different boundary elements. Recent studies have shown that BEAF is linked to transcription in *Drosophila* (Jiang et al, 2009). They isolated DNA from chromatin immunoprecipitation and hybridized it to the genome tiling arrays. 32B BEAF has a more dominant role. More than 85% of the BEAF peaks are located within 300bp of annotated TSS. Most BEAF associated promoters are active which indicates that BEAF plays a role in facilitating transcription.

1.5: FUNCTION OF SF1 BOUNDARY IN *SCR- FTZ* INTERGENIC REGION

Fushi tarazu (ftz) is a pair-rule gene situated between the homeotic genes *Sex comb reduced (Scr)* to the 5' and *Antennapedia (Antp)* to the 3' in *Drosophila* ANT-C. *Ftz* and its enhancers constitute the Powell conserved region. Its position is conserved but the

orientation may vary among various species of *Drosophila* (Maier 1993). A 2.3 kb SF1 boundary element has been identified in the Antennapedia complex between the *Scr* promoter and the *ftz*-distal enhancer of *ftz* (Belozerov 2003). *Scr* is expressed in the labial and prothoracic segment of ectoderm, PS2 (parasegment 2) and PS3 of CNS and visceral mesoderm of anterior and posterior midgut (Mahaffey 1987; Gorman 1995; Rogers 1997). The complex pattern of expression of *Scr* depends on its enhancers which are proximal VM and distal T1 and PS2. *Scr* protein is first detected in parasegment 2 (PS2), determined by PS2 enhancer which is 30kb upstream of *Scr.* VM enhancer specifies *Scr* in anterior and posterior mesoderm. T1 is located ~25kb 5' to *Scr* promoter. Recently, a 450bp sequence was reported 5' to *Scr* that facilitates T1- *Scr* interaction. It is called Promoter-proximal tethering element (Calhoun 2002; Calhoun 2003). *Zebra* enhancer determines the seven stripe pattern of *ftz*. AE-1 is an intergenic enhancer that regulates *ftz* pattern essential for segmentation (Cai 2001). It has been shown to have high affinity for TATA containing Type I promoters (Ohtsuki 1998). AE-1 interacts with TATA containing *ftz* rather than TATA less *Scr.* Hence, specificity of AE-1 to *ftz* depends on promoter competition. However, the most outlying enhancer *ftz*-distal does not have such a promoter preference. In a transgenic assay, it strongly activated both TATA-less *white* promoter and TATA-containing *eve* promoter (Belozerov 2003). Thus, SF1 present between the *Scr* promoter and the *ftz* transcriptional unit might modulate enhancer – promoter specificity. In accordance with the looping model one might hypothesize that SF1 might require a partner SF2 between the *ftz* transcriptional unit and *Scr* distal enhancers. The recent data in our lab from the Chromatin Conformation Capture (3C) analysis conducted in the ftz- Antp region showed that some sequences interact at a

significantly higher frequency than others (Li Ph.D. thesis). For instance, EcoR1 fragments 36-39kb linear distance away from SF1 has higher interaction frequency with SF1 than the neighboring sequences. When this 3.2kb region (Peak 2) was tested as two subfragments in the embryonic enhancer blocking assay, the 3' subfragment contained stronger enhancer blocking activity than 5' subfragment. The SF1 interaction is not confined to the ftz-AntP region. Some sequences in the labial - Deformed homeotic region in ANT-C also showed interaction. Antp promoter also interacted with some regions in the ANT-C complex. Thus, according to the looping model, these interactions emphasize that multiple loops form to facilitate homeotic gene regulation. However, interaction between sequences are not merely boundary-boundary interactions. Understanding the significance of each loop and their tissue and developmental stage specificity is important. In recent data from 3C study in different developmental stage embryos we see important distictions. Early stage which is 4-8hr old embryos when Hox gene expression and body segmentation begins, and late stage which is 10-14 hours embryo when body segmentation has completed, were studied (Liu, thesis). Interestingly, the EcoRI fragment on the 5' edge of PRE close to Antp gene showed strong interaction with SF1 in both early and late stage. However, the frequency of significant interaction between EcoRI fragments between the PS2 enhancer and the PRE were different among early and late stage samples. Therefore, the SF1 and antP promoter interactions in the region provide clues to a complex mechanism for homeotic gene regulation.

SF1 boundary also acts as a barrier. *White* reporter gene flanked by SF1 is protected from chromosome position effect (CPE). In addition, SF1 blocks PRE mediated silencing by *Scr*-PRE which is also the property of a barrier (Belozerov *et al* unpublished). As noted

earlier, spatial patterning of homeotic genes is maintained by PRE/TRE. Interestingly, SF-1 boundary activity is observed throughout the life of *Drosophila melanogaster* (Belozerov 2003). SF1 is a developmental stage specific boundary that works as a strong embryonic enhancer blocker but a weak adult enhancer blocker. SF1 is able to block CPE in the adult eye (Majumder *et al* 2009).

2.3 kb SF1 boundary has been subdivided into three units, SF1 a-c. The 720 bp SF1a and 955bp SF1c subfragments show very little enhancer blocking activity whereas the 675bp SF1b is a strong enhancer blocker (Belozerov 2003). SF1b also has binding sites for GAGA factor. It was shown that SF1b3 subfragment is the minimal insulator and mutating its GAGAG/A sites disrupted its insulator activity. In the case of adult enhancer blocking in the eye, all subfragments are weak enhancer blocker. GAGA factor (GAF) is also required for Fab-7 insulator activity (Schweinsberg, Hagstrom et al. 2004). Fab-7 itself is a weak insulator but its enhancer blocking activity is increased when placed in tandem with SF-1b (Majumder 2003). In the transgenic barrier assay for CPE, when a P element carrying *white* reporter is randomly inserted in the genome, a normal distribution of eye color (light yellow to dark red) in the transgenic fly lines is observed. When the reporter is flanked by a boundary/ barrier a skewed distribution of eye color is observed. Using this assay system we observed SF1a and SF1c are better barriers than SF1b (Majumder et al, 2009) .

Protein factors required for SF1 boundary function are being studied. As noted above, GAGA binding sites are required for SF1 insulator activity. Recent investigations of trans-acting factors required in SF1 function involved the Yeast one hybrid screen. About six putative transfactors were isolated with SF1b3 as bait. Using a rational method some

candidates were selected for RNA interference studies in *Drosophila* S2 cells. SF1 and SF1b are active in transiently transfected cells. SF1b blocked metallothionein (MT) enhancer from interacting with eve promoter driving green fluorescent protein (GFP). RNAi knockout of GAGA factor showed recovery of GFP in these cells which is consistent with previous data. In addition, RNAi knockout of one of the candidates CG7022 had a significant effect on GFP recovery. It encodes a known protein *enhancer of bithorax* [*E(bx)*] which is part of the nucleosome remodeling factor (NURF) complex (V.Belozerov, PhD thesis). We also conducted a genetic screen for SF1 transfactors or modifiers.

1.6: REVERSE GENETICS FOR SF1 MODIFIERS

We have realized in the recent decade that a rather small number of signaling pathways regulate developmental choices. Moreover, there is a large diversity in the choices made by the cells receiving the signals. Recent studies have revealed that many components are determining the developmental decision making. There are networks of signaling components, many mechanisms of regulation and diverse effects. Reverse genetics and forward genetics have been developed to study invertebrate and vertebrate genes and their function. *Drosophila* is an important genetic model used in forward and reverse genetics.

Reverse genetics can be divided into two classes. Since the gene sequences are known, we can study the effects of mutating or knocking down or changing their expression . One part of reverse genetics consists of understanding effects of specific gene mutations. There are different methods of mutagenesis. One form of gene mutation would be the

transposable element mediated inactivation of a gene or insertional mutagenesis. The chemical mutagen EMS is often used for mutagenesis. The other form of mutation is the deletion of a genomic region, also called deficiency. The second class of reverse genetics involves changing the specific gene. This is achieved by using dsRNA to knock down a specific gene expression. The targeted gene replacement of a gene is another method. The strategies of reverse genetics used for screening for SF1 modifier genes involved screening deficiencies that changed SF1 or Fb enhancer blocking strength.The P-inactivated or P-inserted genes within the potential deficiencies are tested in enhancer blocking assay. The potential candidate genes from the screen that are tested and selected can then be knocked down to see their effect on the SF1 or Fb enhancer blocking function. This is discussed in the fourth chapter. I have also discussed the potential effect of a candidate gene CG34381 on SF1 and Scr gene expression.

Figure 1.1:

Schematic of the *Drosophila melanogaster* homeotic gene complex found on the third chromosome. Violet squares represent nonhomeotic genes. Color coding of the genes correspond to expression pattern on fly.

Figure 1. 2:

Boundaries (oval) have two functional categories. **A:** Insulator- blocking distal enhancer (filled circle) from activating a promoter; **B:** Barrier- a gene flanked by the boundary element is protected from surrounding chromatin state (blue circle is nucleosome in heterochromatin state).

Figure 1.1

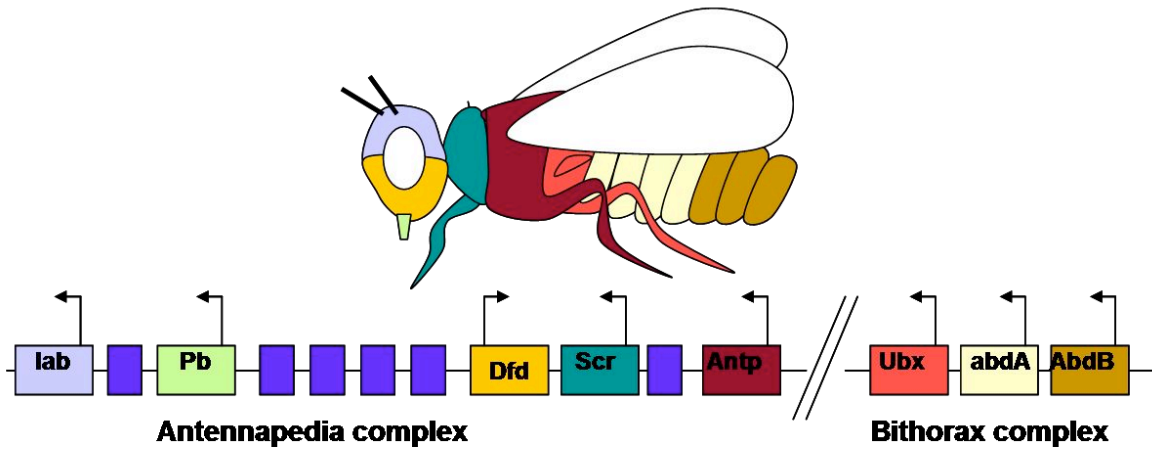
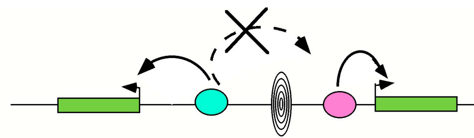


Figure 1.2

A. ENHANCER BLOCK



B. CPE BLOCK

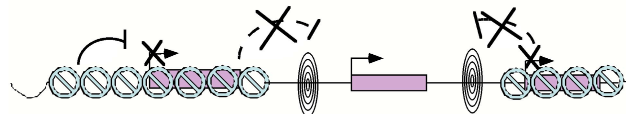


Figure 1.3: Connection between boundary and transcription.

Boundary element/ insulator protein block cis influences. Blue circle represents nucleosome in heterochromatin state and yellow circle represents nucleosome in euchromatin state. Spread of chromatin state may be blocked by interaction with transcriptional regulators (TR) or chromatin remodeling factors (CR activities) or by recruiting histone modifying enzymes (like HATs). Figure adapted from Capelson and Corces 2004.

Figure 1.4: Boundary elements facilitate formation of independent active and inactive chromatin domains in the nucleus.

Blue circle represents nucleosome in heterochromatin state and yellow circle represents nucleosome in euchromatin state. Insulator proteins (pink and brown filled ovals) interact with each other and with nuclear substrate (red) to form insulator bodies. Yellow open circles are nucleosomes. This causes chromatin to 'loop out'. Independent regulatory domain may result from physical separation of trascriptional regulatory elements. It may also be due to blocking of propagation of chromatin state. It may also occur as a result of interaction with nuclear bodies involved in positive or negative regulation. (Figure adapted from Capelson and Corces 2004).

Figure 1.3

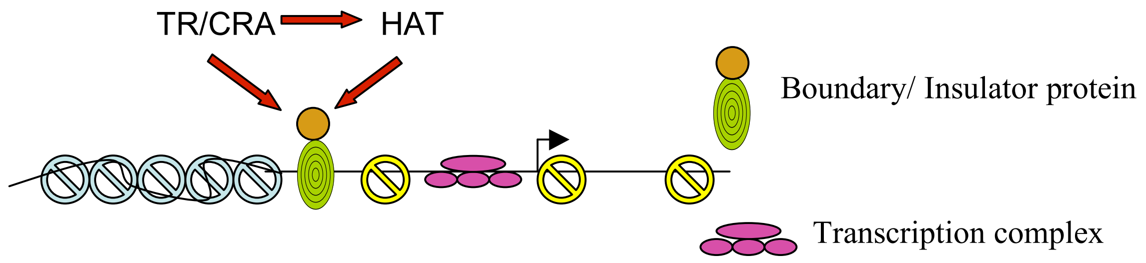


Figure 1.4

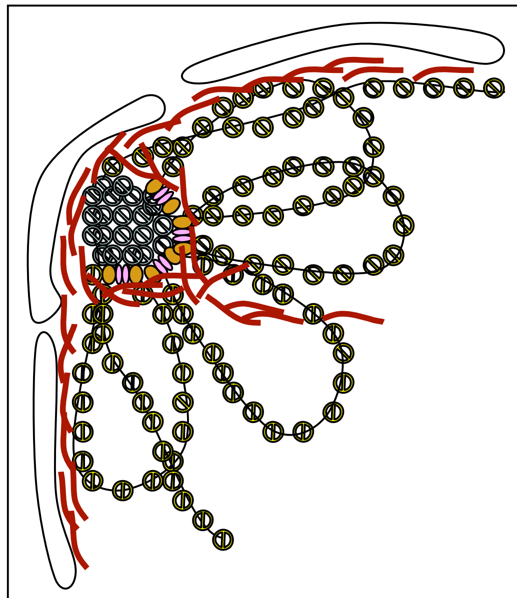
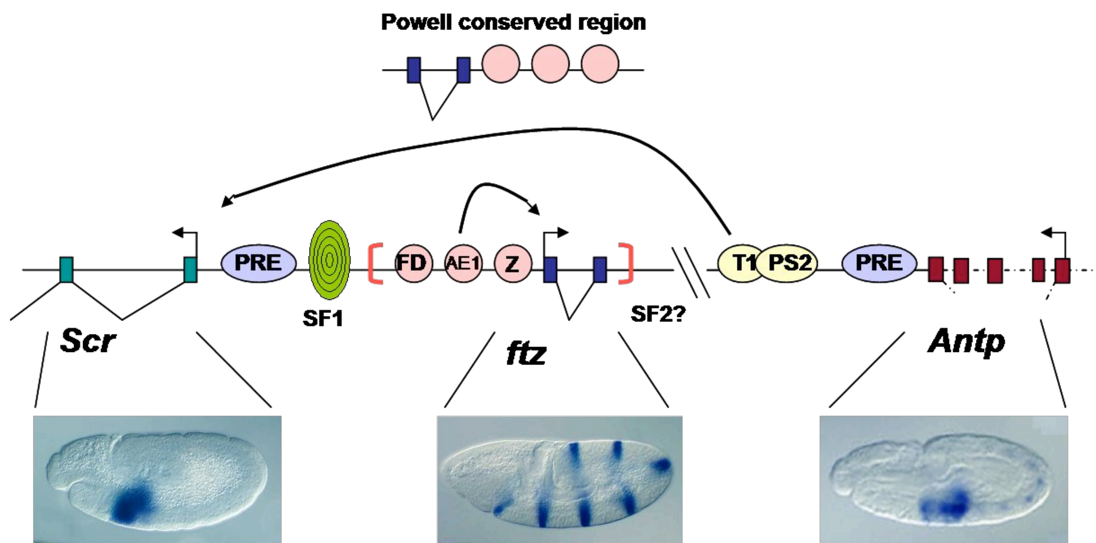


Figure 1.5

Structure of *Scr-Antp* homeotic complex in the ANT-C.

Red parentheses represent approximate limits of Powell Conserved Region. This region in conserved in many species of Drosophila. Embryo *in situ* pictures were taken from BDGP database. They correspond to *Scr, ftz* and *Antp* expression patterns. Concept adapted from V. Belozerov. Chromosome conformation capture was conducted in the lab for SF1 partner SF2.

Figure 1.5



CHAPTER 2

DIVERSE TRANSCRIPTION INFLUENCES CAN BE INSULATED BY THE *DROSOPHILA* SF1 CHROMATIN BOUNDARY

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Abstract:

Chromatin boundaries regulate gene expression by modulating enhancer-promoter interactions and insulating transcriptional influences from organized chromatin. However, mechanistic distinctions between these two aspects of boundary function are not well understood. Here we show that SF1, a chromatin boundary located in the *Drosophila* Antennapedia complex (ANT-C), can insulate the transgenic *miniwhite* reporter from both enhancing and silencing effects of surrounding genome, a phenomenon known as chromosomal position effect or CPE. We found that the CPE-blocking activity associates with different SF1 sub-regions from a previously characterized insulator that blocks enhancers in transgenic embryos, and is independent of GAF-binding sites essential for the embryonic insulator activity. We further provide evidence that the CPE-blocking activity cannot be attributed to an enhancer-blocking activity in the developing eye. Our results suggest that SF1 contains multiple non-overlapping activities that block diverse transcriptional influences from embryonic or adult enhancers, and from positive and negative chromatin structure. Such diverse insulating capabilities are consistent with the proposed roles of SF1 to functionally

separate fushi tarazu (*ftz*), a non-Hox gene, from the enhancers and the organized chromatin of the neighboring Hox genes.

2.1: INTRODUCTION

The expression of an integrated transgene in the genome of invertebrates and vertebrates is influenced by the surrounding chromatin. For example, *Drosophila* carrying the transgene *miniwhite* marker display a wide variety of eye colors depending on the transgene insertion site, a phenomenon referred to as chromosomal position effect (CPE), (Levis, Hazelrigg et al. 1985; Pirrotta, Steller et al. 1985). In vertebrates, integrated transgenes are often progressively silenced by the neighboring chromatin in an insertion-site-dependent fashion (Festenstein, Sharghi-Namini et al. 1999; Ayyanathan, Lechner et al. 2003). Some of the best-characterized boundary elements were initially identified by their ability to protect reporter transgenes. For example, the *Drosophila* scs and scs' elements were shown to protect *miniwhite* against CPE, resulting in more consistent and lighter eye colors (Kellum and Schedl 1991). The vertebrate β-globin cHS4 boundary can also protect reporter genes from the silencing effect of the genome (Chung, Whiteley et al. 1993).

Recent studies indicated that, in the β-globin boundary, the enhancer blocking function and the CPE blocking function (called barrier activity) has different cis elements and trans factors. The barrier recruits histone modification enzymes to establish centers of active chromatin (Mutskov, Farrell et al. 2002; Recillas-Targa, Pikaart et al. 2002). These results indicate that the two aspects of boundary function are mediated by distinct

mechanisms (West, Huang et al. 2004; Huang, Li et al. 2007). Although parallels have been drawn between the fly CPE-blocking activity and vertebrate barriers, separation of CPE-blocking and enhancer-blocking activities has not been reported in *Drosophila*. In particular, the *Drosophila* Gypsy suHw boundary appears to support both of its enhancer-blocking and CPE-blocking activities through the same DNA sequence and the same zinc finger protein SUHW (Roseman, Pirrotta et al. 1993). These observations are inconsistent with a common mechanism underlying all "position effects" in different organisms, and consequently, a common mechanism for all "barrier-like" activities.

To address these questions we have probed the CPE-blocking activity associated with SF1, a 2.3kb boundary element in the ANT-C. We have previously shown that SF1 contains a potent embryonic enhancer-blocking activity (Belozerov, Majumder et al. 2003). In this study, we show that SF1 can protect the *miniwhite* reporter from the influences of organized chromatin surrounding the transgene insertion site. Study by lab members Parimal Majumdar and Vladimir Belozerov also show that the DNA regions within SF1 that support CPE block is different from the element that mediates enhancer block; and that GAF sites (GAGA Factor binding sites), critical for the latter, is dispensable for the former (Figure 3, Majumder, Roy et al 2009). Importantly, my results provide evidence that the CPE-blocking activity cannot be attributed to a potential enhancer-blocking activity in the developing eye. Our findings suggest that the *Drosophila* SF1 boundary contains multiple non-overlapping activities that block enhancers or chromatin-mediated effects. These functional properties of SF1 may be important for the insulation of the non-homeotic *ftz* gene from neighboring enhancers and

repressive chromatin associated with homeotic genes. Our results also suggest the diverse mechanisms may underlie "chromosomal position effect", as well as the CPE blocking activities.

2.2: RESULTS

SF1 contains a CPE-blocking activity.

Transgenic flies carrying the *miniwhite* reporter exhibit wide range of eye colors depending on the site of transgene insertion (Figure 1A). Such variation, known as CPE, is attributed to the influences of the surrounding chromatin on an otherwise weak *miniwhite* promoter (Levis, O'Hare et al. 1984; Levis, Hazelrigg et al. 1985; Pirrotta, Steller et al. 1985). Since the effect of CPE on *miniwhite* expression has been evaluated by visual assessment of eye color in previous studies, we categorized the eye color of transgenic flies carrying *miniwhite* into 12 intensity levels, and used them to evaluate the CPE-blocking results shown in Figure 2.1. However, we also defined the eye color standard by measuring the absorbance (OD_{480}) of the eye pigment extracted from these flies (Figure 2.1 B, see methods). Our measurements indicate that the CPE-caused variation in the *miniwhite* expression, as measured by OD_{480}, can range up to 70 fold, and much of it is outside the sensitive range of the human eye.

To test the ability of SF1 to protect *miniwhite* from CPE, transgenic flies carrying the *miniwhite* reporter with or without the protection of flanking SF1 were assigned an eye color intensity level according to the color standard (Figure 2.1C). We found that fly lines carrying unprotected *miniwhite* exhibit a wide range of eye colors with a

comparable number of lines in each color category from 2.5 to 5.5 (Figure 2.1C-D). This is indicative of a strong CPE. In contrast, fly lines carrying *miniwhite* protected by the full-length SF1 (brown ovals, CA-SF1) display predominantly yellow to light orange eye colors (Figure 2.1C-D). Among 46 CA-SF1 lines, 78% exhibited eye colors between 3.0 and 3.5 (compared to 27% among unprotect CA lines) with few lines exhibiting extremely light or dark colors. In addition to decreasing eye color variation, shown by lower standard deviation (SD, Figure 2.1D), flanking SF1 also appeared to reduce the average eye color (MEAN, Figure 1D). Both such effects have been previously reported for the scs and suHw insulators and have been attributed to the insulation of primarily positive influences of the surrounding chromatin (Kellum and Schedl 1991; Roseman, Pirrotta et al. 1993). The average eye color in SF1 protected lines appears to be lighter than those in suHw and scs protected lines. This could be due to the slight variances in the assay parameters, such as the color standard, the inclusion of a yellow marker in the P-element in the previous studies, or to a potential repressive effect of SF1 (Roseman, Pirrotta et al. 1993). Average eye color for the suHw CPE protected eye colors is higher than SF1 (Figure 3.1). Taken together, our results indicate that SF1 contains a potent CPE-blocking activity.

Molecular dissection of the SF1 CPE-blocking activity.

To identify and characterize the CPE-blocking activity within SF1, the 2.4-kb full-length boundary was dissected into three fragments of comparable sizes (SF1a, SF1b and SF1c, Figure 2.2A), and tested them individually for CPE-blocking activity. For each SF1 sub-fragment, a large number of independent lines were scored for eye colors (N, Figure 2.2

A-B). Compared to the no insulator controls (No ins, CA in Figure 2.1C-D), SF1a- and SF1c-containing flies showed more lines with yellow and light-orange eyes, and/or fewer lines with dark-red eyes (Figure 2.2 B). This is indicative of CPE-blocking activity associated with these two elements, although both appeared significantly weaker than that of the full-length SF1. In contrast, the eye color variation among SF1b lines was similar to those of the CaSpeR control (Figure 2.2 B). Statistical analysis of sample groups indicates that SF1, SF1a and SF1c, but not SF1b, showed significant difference in the eye color distribution from the CaSpeR control (see P value in Figure 2.2A). Normally SF1 is not orientation dependent. However, in certain promoter combination they can be orientation dependent.

The lack of CPE-blocking activity in the SF1b region (>> <<) is somewhat unexpected because this element was previously found to exhibit about 80% of the enhancer-blocking activity of full-length SF1 in a transgenic embryo enhancer-blocking assay (for comparison see blue bars in Figure 2B, (Belozerov, Majumder et al. 2003). Interestingly when SF1b orientation was changed to tail to tail (<< >>), the barrier activity increased significantly (data not shown). In contrast, the SF1a and SF1c showed little insulator activity in the enhancer-blocking assay. This result suggests that the SF1 boundary may contain two potent and non-overlapping activities: one that blocks embryonic enhancers and the other insulates against positive and negative CPE in the developing eye.

Our previous study showed that mutations in GAF binding sites in the SF1b element abolished its enhancer-blocking activity (Farkas, Gausz et al. 1994; Belozerov, Majumder

et al. 2003). Therefore, our lab tested whether GAF sites are also required for the CPE-blocking activity. The two GAF sites were replaced in SF1c with unrelated sequences (Majumder, Roy, et al 2009). The mutations in the GAF sites in SF1c (SF1cGAKO) did not compromise the CPE-blocking activity. In fact, the proportion of SF1cGAKO lines with medium to light eye colors is slightly higher. This could be due to the loss of binding by GAF, which is also known to mediate transcription activation. These results suggest that the CPE-blocking activity in SF1c and the insulator activity in SF1b depend on distinct cis- and trans- components (Majumder, Roy, et al 2009).

In addition to GAF sites, SF1c also contains multiple TATA-like motifs in its central region. Similar AT-rich motifs and other promoter-like sequences are found in several other boundary elements, prompting the hypothesis that these cis-elements could serve as a sink of regulatory influences (Kuhn and Geyer 2003). To further define the cis-requirement of CPE-blocking activity in SF1c and to test whether TATA-motifs contribute to the CPE-blocking activity, the SF1c region was further dissected into three sub-fragments and tested each in three tandem copies in *miniwhite*-protection assay. The central region of SF1c (3c2) does not contain higher level of CPE-blocking activity than the neighboring regions, suggesting that GAF and TATA-like motifs do not contribute significantly to the CPE-blocking function of SF1c.

The CPE-blocking activity is distinct from a late eye enhancer-blocking activity in SF1

The *Drosophila* CPE has often been compared to the vertebrate position effect, which is the gradual silencing of integrated reporters by the genome or chromatin surrounding the

transgene. However, key differences exist between the two phenomena. First, the *Drosophila* CPE appears to be both negative and positive in nature, as shown by the decrease of both extreme light and extreme dark eye colors in boundary-protected transgenic lines. Second, the *Drosophila* CPE is more dramatically manifested in the behavior of the *miniwhite* reporter, which is also used in most boundary protection studies (Kellum and Schedl 1991; Roseman, Pirrotta et al. 1993). Other features, including a lack of time-dependence in the *Drosophila* CPE, also suggest mechanistic differences between the two effects. An alternative explanation for the *Drosophila* CPE suggests the action of eye-specific enhancers or silencers around transgene insertion sites. If this were true, the two non-overlapping activities in SF1 would represent two enhancer-blocking activities, one that functions in the embryo and the other in the developing eye.

To distinguish between these two mechanisms, we tested SF1 for its ability to block enhancers in the same tissue and developmental stage as in the CPE-blocking assay. We used the eye-specific glass multiple repeat (G5 and G2) and the *miniwhite* reporter to perform the blocking tests (Figure 2.3, (Moses and Rubin 1991; Ellis, O'Neill et al. 1993). As controls, we also made G5-*miniwhite* transgene with no insulator, or with the suHw insulator, which has been shown to contain enhancer-blocking activity in the adult eye (Figure 2.4). We found that the G5 enhancer can strongly activate *miniwhite* expression in majority of transgenic lines, resulting in an average eye color of 5.43, which corresponds to OD_{480} level of 53 (Figure 2.4, no insulator). The G5 enhancer was blocked weakly by SF1 and SF1 subfragments. The strength of block can be seen in the

difference of eye color when the SF1/subfragments are present and when they are deleted by cre-loxP (Figure 2.3). G2 is weaker enhancer (average 4.16 after cre, Figure 2.3), and the SF1 blocking is weak (average 3.51). The 340-bp suHw placed between G5 and *miniwhite* strongly reduced the average eye color and shifted the peak of eye color distribution to the lighter range, with an average eye color of 3.55 and OD_{480} level of 4 (Figure 2.4, suHw insulator). The 93% reduction in the OD_{480} level is consistent with the enhancer-blocking activity of suHw in the eye tissue. However, the 2.4-kb SF1 only weakly reduced average eye color, with an average eye color of 4.81 and OD_{480} level of 30 (Figure 4, SF1 insulator). This result suggest the SF1 contains much weaker or little enhancer-blocking activity in the adult eye, especially considering the 2.4-kb linear distance that separates the G5 enhancer from *miniwhite* due to the insertion of SF1. This is in strong contrast to the strong CPE-blocking activity SF1 exhibited in the same tissue. It is also in strong contrast to its potent activity in blocking diverse embryonic enhancers (Figure 2B, (Belozerov, Majumder et al. 2003). Taken together, our results do not support the hypothesis that an eye-specific enhancer-blocking activity is responsible for the CPE-blocking behavior of SF1. Our results also indicate that the ability of SF1 to block enhancers in the eye is weak, suggesting that the boundary element may be regulated in a stage-specific and/or tissue-specific fashion.

2.3: DISCUSSION

In this study we have characterized the CPE-blocking activity associated with the *Drosophila* SF1 boundary. Our results suggest that SF1 contains at least two non-overlapping boundary activities, a strong embryonic enhancer-blocking activity

associated with SF1b element, and strong CPE- blocking activities associated with SF1a and SF1c elements. Mutagenesis and dissection studies indicate that the CPE-blocking activity depends on different cis and trans components from the embryonic enhancer-blocking activity. We further showed that the CPE-blocking activity is unlikely to be attributed to a late stage enhancer-blocking activity in the developing eye.

Drosophila CPE, manifested predominantly by the enhancement or suppression of *miniwhite*, was thought to result from the active or repressive chromatin around the transgene insertion sites. CPE-blocking activity, therefore, has been compared to the vertebrate barrier activity and long used as a defining feature for chromatin boundaries in *Drosophila* (Kellum and Schedl 1991; Patton, Gomes et al. 1992). However, the ability of *Drosophila* boundaries to block both positive and negative CPE argues against a shared mechanism between these elements and the vertebrate barriers such as the β-globin barrier, which counter the progression of silent chromatin by establishing centers of active chromatin (Mutskov, Farrell et al. 2002; West, Huang et al. 2004).

An alternative explanation for the *Drosophila* CPE invokes the action of enhancers or silencers near the integrated transgenes. This model is consistent with the ability of boundaries to block both positive and negative effects. It also accommodates the fact that for some *Drosophila* boundaries the CPE-blocking activity depends on the same cis- and trans- components as the enhancer-blocking activity (Geyer and Corces 1992; Patton, Gomes et al. 1992; Roseman, Pirrotta et al. 1993; Gerasimova, Gdula et al. 1995; Mallin, Myung et al. 1998). However, this hypothesis would predict widespread presence of eye-

specific enhancers and silencers in the genome to account for the prevalence of the CPE effect.

Our analysis of the SF1 boundary provides the first evidence that the CPE-blocking activity can be separated from the enhancer-blocking activity, suggesting that these two insulating functions may be mediated through distinct mechanisms in *Drosophila*. It is possible that the CPE-blocking activities in *Drosophila* form structures that are transcriptionally "neutral", and able to insulate the weak *miniwhite* promoter from the effect of local chromatin. It is unclear, however, whether such local chromatin effect can compare, in range or strength, to that of constitutive heterochromatin, or whether such effect influences *Drosophila* gene promoters in general. A previous study showed that human MAR sequence could facilitate CPE blocking either arranged to flank the reporter or placed upstream in tandem copies (Namciu and Fournier 2004). This is distinct from the CPE-blocking behavior of Drosophila boundaries such as suHw and scs, further demonstrating the diverse mechanisms that could influence the regulation of the miniwhite reporter.

The SF1 boundary is located in the *Scr*-*ftz* genomic interval in the *Drosophila* ANT-C, which differs from other Hox clusters in that it contains both homeotic and non-homeotic genes. Proper regulation of these genes requires modulation of enhancer traffic as well as insulation of chromatin-mediated effects. The SF1 compound boundary fulfills both requirements: the SF1b element can restrict long-range enhancers from interfering with the *ftz* and *Scr* promoter (Belozerov, Majumder et al. 2003); and the SF1a and SF1c

elements may protect the non-Hox *ftz* gene from chromatin-mediated regulation, such as the PRE/TRE maintenance of the neighboring Hox genes. Separation and selective association of different types of boundary activities could determine the regulatory role of compound boundaries and provide flexibility in their function.

2.3: METHODS

Construction of CPE-blocking transgenes

The full-length SF1 and its sub-fragments SF1a-SF1c were generated by PCR using primers containing Not I site and cloned into pCRII/TOPO vector (Invitrogen). The resulting constructs were digested with Not I or Nsi I and the DNA inserts were gel extracted, purified and ligated into the respective sites flanking the *miniwhite* reporter in the pCaSpeR transformation vector. Site-directed mutagenesis of the two GAF sites in SF1c was performed using the single-stranded DNA method as described previously (Ip, Levine et al. 1992). The base substitution in the GAF sites was done using the following oligonucleotides: 5'ACAATGAACAGGATCCTGATGAATTA 3' and 5' GTTGTGATGCAGATCTGCTTACTTAG 3'.

Construction of enhancer-blocking transgenes

The G5 enhancers (provided by Jumin Zhou) were digested with Bam HI, purified, and ligated into the unique Bam HI site of the CaSpeR vector, resulting in the CA-G5 plasmids. G oligonucleotides were used to generate G2 by PCR. It was similarly inserted

in CaSpeR vector to form CA-G2 . SF1, SF1 subfragments or suHw insulators were inserted into the unique Not I site (converted from the original Eco RI site) in the CA-G5 and CA-G2 plasmids. Details of the embryonic enhancer-blocking assay in Figure 2 are described previously (Belozerov, Majumder et al. 2003).

P-element-mediated germline transformation

P-element mediated transformation was carried out as described previously (Rubin and Spradling 1982) (Cai, Zhang et al. 2001)(Rainbow Transgenic Inc, California). The $y^{1}w^{67c23}$ and w^{1118} *Drosophila* strains were used to generate transgenic lines. Eighteen or more independent lines were generated for all CPE-blocking tests. Five or more independent transgenic lines were obtained and characterized for each enhancer-blocking construct.

Eye color assessment and pigment measurement

The eye color of 5-7 days old heterozygous females was assigned and color level by visual assessment according to an 12-point scale of progressively darker color shown in Figure 1A, under 10x objective and intermediate illumination with NCL150 cold light source. Eye pigment was extracted from 20 7-day old flies of indicated eye color as described previously(Gindhart and Kaufman 1995): flies were homogenized in 100 µl AEA buffer (30% EtOH, 0.1% concentrated HCl) and brought to 1 ml by adding 900 µl AEA. The samples were then vortexed for 30 minutes and spun for 10 minutes in a microcentrifuge. Twenty microliters of 0.5% hydrogen peroxide was added to the

supernatant to oxidize the extracted pigment. The samples were mixed, spun and measured for absorbance at OD_{480} using Genova Life Analyzer spectrophotometer. Each OD_{480} reading was repeated tree times and the mean value was used to generate the chart in Figure 2.1B.

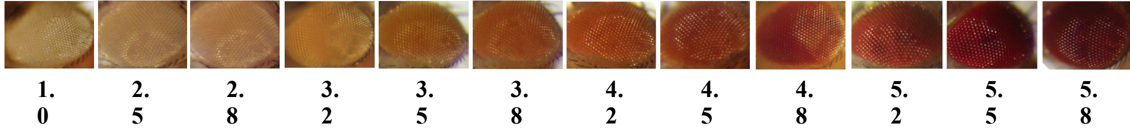
Statistical analysis

The P value in Figure 2.2A is calculated by Chi-square test using an on-line calculator from QuickCalcs (GraphPad Software, Inc., La Jolla, CA), where number of lines in each eye color category was compared to that of the control (CA). Using color distribution expected from CA transgenic lines, the probability (P) of observing eye color distribution as seen in CA-SF1 lines is < 0.0001 ($\chi^2 = 16.767$ with 1 degree of freedom). Similar calculation was done for CA-SF1a ($P< 0.0630$); CA-SF1b ($P< 0.9307$); and CA-SF1c ($P< 0.0190$). For eye enhancer-blocking assays in Figure 4A with no insulator, suHw or SF1, eye color of transgenic lines was scored according to the color standard in Figure 1A. Data compilation and statistical analyses, except otherwise indicated, were done using the Microsoft excel software.

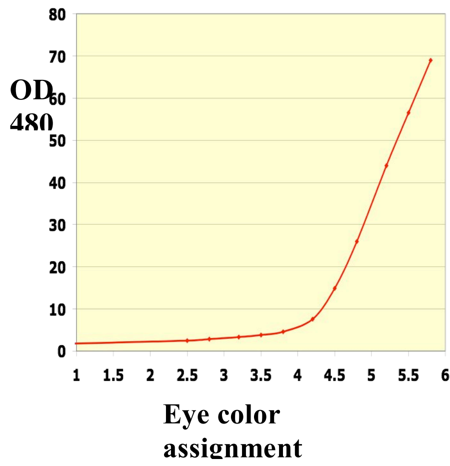
Figure. 2.1: The CPE-blocking activity of SF1

(A). Eye color intensity standard shown by eyes of w1118 and transgenic flies showing increasing eye color. The number below each eye indicates the color designation, with 1 being the parental strain w1118 and 5.8 being the darkest eye color observed. (B). OD480 absorbance of eye pigment extracted from flies in each eye color category (see methods). (C). Schematic representation of unprotected (CaSpeR, or CA) or SF1-flanked (CaSpeR-SF1, or CA-SF1) miniwhite randomly integrated into genome (brown bars). Arrows represent miniwhite promoter and ovals represent the SF1 boundary. (D). Bar graph showing eye color distribution of CA and CA-SF1 transgenic lines. Each independent line was assigned an eye color score according to chart in B. The Y-axis indicates the percentage of lines displaying eye color within the indicated range (shown in X-axis). The inset table provides sample number (N, in parentheses), eye color mean (MEAN) and standard deviation (SD) for CA and CA-SF1 transgenes.

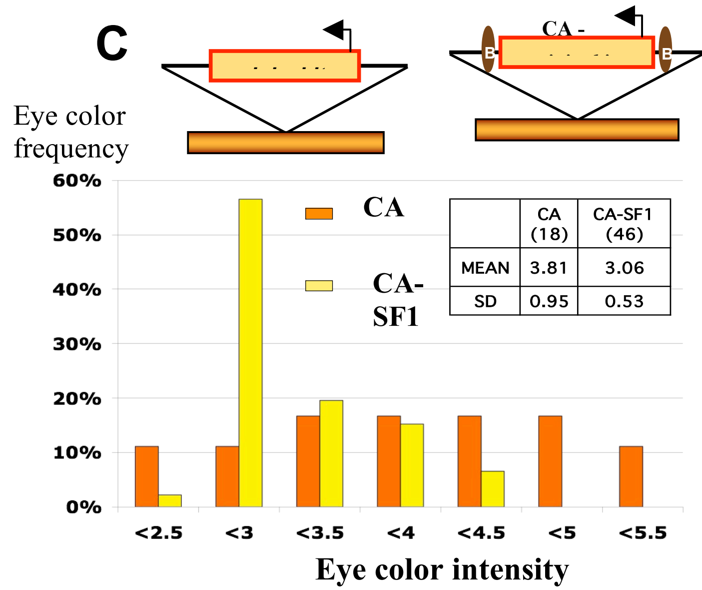
A



B



C



	CA (18)	CA-SF1 (46)
MEAN	3.81	3.06
SD	0.95	0.53

(Parimal Majumdar)

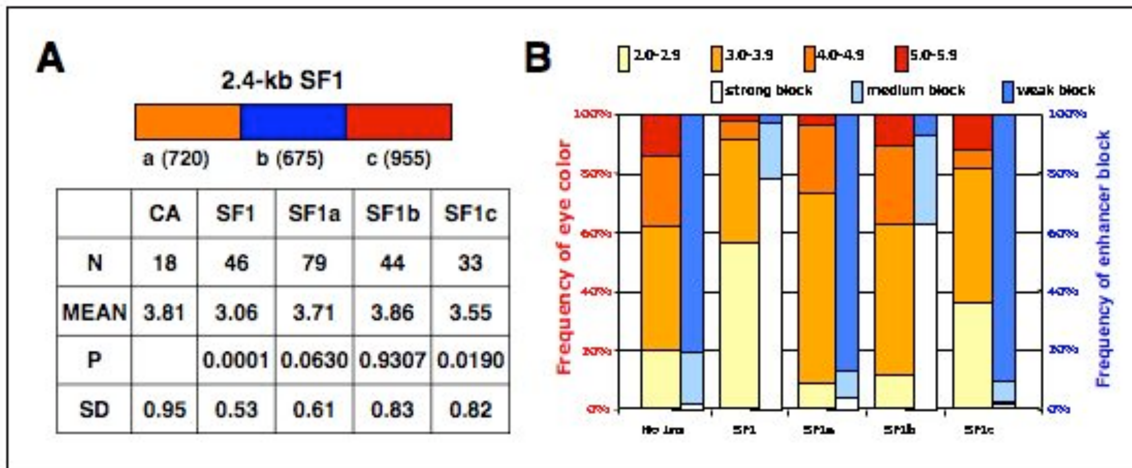
Figure 2.2: SF1 contains separate enhancer-blocking and CPE-blocking activities. This work was entirely done by Parimal Majumder and Vladimir Belozerov.

(A). Analysis of CPE-blocking activity in SF1 sub-fragments.

Top: A schematic of the three sub- fragments within the SF1 boundary, with size of each fragment in base pairs shown in parentheses. Bottom: A table summarizing the total numbers of lines (N), mean eye color (MEAN), standard deviation (SD), and the probability distribution (P) for each group against CA was calculated by chi-square test.

(B). Comparison of CPE- and enhancer-blocking activities in SF1 and its sub- fragments. The yellow-orange bar graph summarizes the CPE-blocking activity of no insulator (No ins, CA), SF1 and three SF1 sub-fragments using the assay outlined in Figure 2.1C. The percentage of lines displaying eye colors within the designated range, as shown on top of bar graph, is indicated on the left Y-axis. The white-blue bar graph summarizes the embryonic enhancer-blocking activity in the corresponding DNA elements. The percentage of embryos showing strong (70-100%), medium (30-70%) or weak (0-30%) block, as shown on top of bar graph, is indicated on the right Y-axis.

Figure 2.2



	CA	SF1	SF1a	SF1b	SF1c
N	18	46	79	44	33
MEAN	3.81	3.06	3.71	3.86	3.55
P		0.0001	0.0630	0.9307	0.0190
SD	0.95	0.53	0.61	0.83	0.82

(Parimal Majumder and Vladimir Belozerov)

Figure 2.3:

Enhancer blocking activity of SF1 and SF1 subfragments in adult eye.

Top: diagram of eye enhancer-blocking transgenes. The transgene containing G2 or G5 enhancer (red circle) and the miniwhite transgene (arrow), separated by insulator DNA (open oval), is randomly integrated in the chromosome (orange bar). The purple bars represent lines carrying G5-miniwhite with 2.4-kb SF1or SF1 subfragments insulators, respectively. The pink bars represent flies with G2 SF1 transgene. The cre bars represent lines after insulator is deleted.

Figure 2.4:

The SF1 contains little enhancer-blocking activity in the developing eye.

Top: diagram of eye enhancer-blocking transgenes. The transgene containing G5 enhancer (G, red circle) and the miniwhite transgene (arrow), separated by insert DNA (open oval), is randomly integrated in the chromosome (orange bar). Table: rows from top to bottom, number of lines in each eye color category for G5-miniwhite transgene containing no insert, 2.4-kb SF1 or 340-bp suHw insulators, respectively.

Figure 2.3

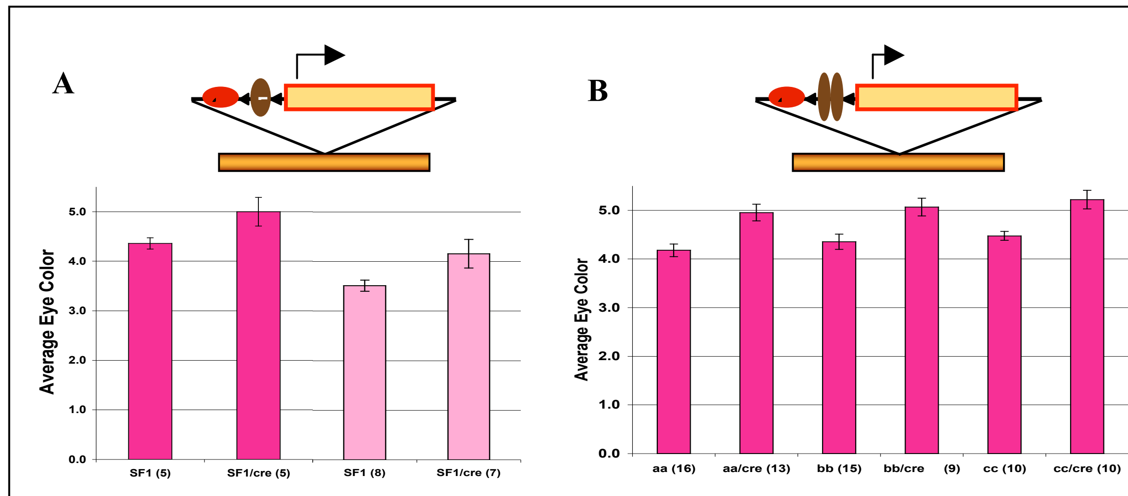
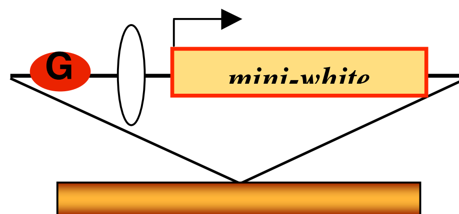


Figure 2.4



Insert	eye color										Average eye color
	2.8	3.2	3.5	3.8	4.2	4.5	4.8	5.2	5.5	5.8	
No insulator						1	2	4	13	6	5.43
2. 4kb SF1				2	1	13	8	9	2	2	4.81
340bp suHw	1	1	1	3	4	2					3.55

CHAPTER 3

MECHANISM OF CANCELLATION FOR SUHW IN DROSOPHILA

3.1: INTRODUCTION

The increase in genome size and the complexity of function made it necessary for the complex organism to regulate the function of genes through novel mechanisms. Without proper mechanisms there would be a marked increase in non-specific interactions between the enhancer and the promoter (Corces 1996). Boundary elements can establish separate chromatin and regulatory domains for the specific transcriptional regulation of the gene expression. Boundaries only block the distal enhancers but do not interfere with the proximal enhancer and promoter interaction. The gypsy retrotransposon contains a 340 base pair element that acts as an effective boundary element, called the suHw element (Spana 1988). The distal blocking activity however, does not significantly interfere with the transcriptional activation of the promoter influenced by the proximal enhancer. SuHw can also block silencers. The enhancer blocking activity of the suHw DNA element is also dependent on the trans-factors that bind to the boundary. The suHw element was found in the 5' untranslated region of the gypsy retrotransposon (Gdula et al 1996).

The gypsy insertion near genes causes mutations in certain tissues at specific developmental stage which indicates that the element blocks certain enhancer- promoter interaction related to that expression. A well studied effect of gypsy insertion is the effect of its insertion near the yellow gene. The yellow gene has a simple structure of gene regulation. There are several tissue specific enhancers that drive expression of the gene

for the cuticular pigmentation in the different stages of development. The yellow gene enhancers determining the pigmentation in the wings and body and the larval cuticle are on the 5' side of the gene. The insertion of the gypsy element between the distal wing and body enhancers, and the proximal larval cuticular enhancer can block the pigmentation of the wing and body cuticle but does not affect the larval cuticular pigmentation. The mutant phenotype is called the yellow mutant. These mutants have low pigmentation in the wings and body (Gdula et al 1996) . Another locus affected by suHw is the cut locus. This is an example of a more complex regulatory region that is affected by suHw insertion and it affects differently depending on the position of the insertion. The suHw can block long distance enhancer in this case. The suHw can block the wing margin specific enhancer from interacting with the cut locus.This enhancer is located 85 kb upstream. Several different insertions in this region between the enhancer and promoter have been shown to produce different cut phenotypes. When gypsy is inserted close to the promoter, it is embryonically lethal whereas when it is inserted in the middle of the region it is larval lethal. If the insertion is further away from the promoter, the adults are viable and their wing margins are affected (Dorsett 1993).

The suHw gene encodes a protein SUHW. When this gene is mutated the enhancer blocking activity of gypsy/ suHw elements is suppressed. The SUHW protein contains 12 zinc fingers that binds the suHw element. The interactions of SUHW with other proteins is necessary for the formation of boundary (Parkhurst and R. S. Coyne 1988). Apart from the zinc fingers in SUHW that bind the boundary element, the protein also contains other motifs that might also help in enhancer blocking (Gerasimova TI 1995) .

SUHW contains a leucine zipper domain. This domain is homologous to the helix 2 – leucine zipper that is found in the basic helix- loop- helix (bHLH) leucine zipper proteins. This domain is also essential for the enhancer blocking activity by the suHw. The protein also contains acidic domains located in the amino- and carboxy- terminal ends. When both the acidic domains are deleted, the SUHW protein does not allow the polar repression of distal enhancer. Thus, both the acidic domains are required for the polar repression of the enhancer (Harrison 1993). One such protein is modifier of midget 4, mod (mdg4), a BTB domain protein, that interacts with suHw. Mod (mdg4) mutations act as enhancer of position effect variegation. Since mod (mdg4) interacts with suHw, in the absence of mod (mdg4) suHw represses in both directions while in its presence suHw represses in one direction (Gerasimova TI 1995). The study using GAGA sites paired with suHw element showed that there is pairing between the GAF sites and suHw insulator. It was also shown that the pairing occurs in a Mod(mdg4) and GAF dependent manner (Melnikova et al 2004). The suHw boundary function probably depends on some higher eukaryotic transcription factors. The screen for mutations that decrease suHw insulator activity at the cut locus revealed 2 genes – scalloped (Sd), mastermind (mam), and a novel gene- Chip. Sd and mam were enhancer binding factors and Chip was an enhancer facilitator. The vertebrate nuclear LIM domain – binding protein and Chip are functional homologs. LIM domain contains two contiguous zinc fingers. Chip is a ubiquitous nuclear protein and is necessary for specific segementation expression pattern. Chip localizes at many sites on the polytene chromosomes. LIM domain proteins are involved in protein-protein interactions (Morcillo P 1997). Another protein that is involved in the enhancer blocking function of suHw is centrosomal protein CP190 (Pai

CY 2004). The gypsy insulator activity is impaired in CP190 mutants since CP190 interacts with both the suHw and mod(mdg4) (Bartkuhn M 2009).

The suHw element can block the enhancer promoter interaction when placed between the enhancer and promoter. The enhancer blocker disrupts the distal enhancer – promoter interaction and does not affect the proximal enhancer. The proximal enhancer is able to behave in its native way. The nature of enhancer blocking activity by suHw points to a mechanism that somehow blocks the distal enhancer machinery. The distal blocking activity however, does not significantly interfere with the transcriptional activation of the promoter influenced by the proximal enhancer. SuHw can also block silencers (Cai 1995). However, the enhancer blocking function is changed when rearrangement of the suHw occurs. When two suhw elements are introduced, the enhancer block is reduced. Different copy number of the element was used in the study. The yellow gene is the body pigment gene. Two enhancers used for the study are body cuticle and wing enhancers. When single suHw is placed between the enhancers and yellow promoter, the body and wing are yellow. When two suHw insulators are present between the enhancers and yellow promoter, expression of the body pigment is increased. Out of seven lines in which pigmentation is increased, three are wildtype dark pigmented flies. In the absence of the SUHW protein, the yellow body flies became dark pigmented ones. When single suHw flanked the two enhancers, the flies exhibited yellow body and wing pigmentation. In the absence of the SUHW protein, these flies became wildtype and dark pigmentation returned. From these results we see that the two suHw elements interact with each other and cannot block the enhancer (Cai HN 2001). This phenomenon was not found in the

case of two tandem SF1 elements blocking enhancer (Majumder P 2003). Our lab also showed that the enhancer blocking activity of SuHw, SF1, and other enhancer blockers can be observed in S2 cell culture studies. The SUHW and dCTCF dsRNA knockdown in S2 cells also showed the decrease in activity of the respective boundaries (Li M 2008).

A previous study has shown that when a single suHw is flanking a white gene, it blocks CPE. It blocks all surrounding chromatin influences and protects the gene from the negative influences of the chromatin. SUHW protein is involved in this function (Robin R.Roseman 1993) . Therefore, for the suHw boundary both of its enhancer-blocking and CPE-blocking activities are supported by the same zinc finger protein SuHw (Roseman, Pirrotta et al. 1993). It has also been shown that a weakened yellow promoter can be activated over a distance by suHw element (Golovnin A 2005). Therefore, suHw element may also have activating properties.

We wanted to test whether tandem suHw will cancel each other in the CPE blocking assay. Our broader question was to test whether the same mechanism governs enhancer blocking and CPE blocking activities. My study shows that the suHw boundary element flanking a miniwhite gene is able to block CPE activity. When 2x suHw is flanking the miniwhite gene the suHw elements can protect the gene from the influence of the surrounding CPE. In both cases the orientation of the suHw is head to head (> <). The distribution of eye color also points to partial cancellation of the suHw elements. There is also some activating influence of the suHw on the miniwhite gene.

3.2 : RESULTS

Effect of two suHw elements in tandem

It is a known fact that single suHw can block the distal enhancer while double suHw cannot block enhancers and allows insulator bypass (Ekaterina Muravyova 2001). Previously in our lab we tested the single versus double suHw enhancer blocking behavior in S2 cells. It was surprising to find that both single and double suHw are able to block the enhancer in the S2 cell culture studies . In this construct the Metallothionein enhancer (MT) is placed in between the RFP and GFP reporters. The boundary is inserted between the MT enhancer and the GFP reporter (MoLi 2008). It was observed that inserting two suHw in tandem in between the MT enhancer and the GFP reporter did not increase the GFP reporter expression when compared to the expression level in the single enhancer blocking assay (J. Liu thesis). Hence, in fly body double suHw cannot block an enhancer while double suHw in tandem in S2 cells can block enhancer. Thus, in S2 cells the suHw enhancer blocking mechanism does not completely mimic the suHw enhancer blocking mechanism in the fly body.

Since double suHw in tandem shows different enhancer blocking function depending on the site of study, we tested the CPE blocking activity of double suHw in the CPE blocking assay in the fly body. We tested the hypothesis that different boundary activities- the enhancer blocking activity and the CPE blocking activity have distinct properties. We flanked miniwhite with 1x and 2x suHw elements. 1x suHw flanking miniwhite can block CPE. 2x suHw flanking miniwhite can block CPE . The 2x suHw might stimulate miniwhite (Figure 3.1). According to our eye color standard the 1xsuHw CPE blocked flies show eye color range of 3.0 – 5.0. The eye color distribution is skewed

towards the dark red in the case of 2xsuHw 3.0 - 6.0. The distribution of the control unprotected miniwhite has a normal distribution of eye color, ranging between 2.0 – 6.0. Thus, 2x suHw behaves differently in CPE blocking as compared to enhancer blocking activity. We did a chi-square test comparing the CA eye color distribution in four groups 2.0-2.9, 3.0-3.9, 4.0-4.9 and 5.0-5.9 to the 1xsuHw and the 2xsuHw eye color distribution. CA-1xSu Chi-square test result shows Chi squared equals 173.942 with 3 degrees of freedom. The two-tailed P value is less than 0.0001. This difference is considered to be extremely statistically significant. CA-2xsuHw Chi squared equals 72.584 with 3 degrees of freedom. The two-tailed P value is less than 0.0001. This difference is also considered to be extremely statistically significant.

Comparison of CPE block activity

From the suHw CPE data we observe that the CPE block by suHw differs depending on the number of suHw element present . We also compared it to the CPE block by SF1 and subfragment Fb, which were previously observed in the lab. The eye color distribution for CPE block by SF1 has an eye color range of 2.0-5.0. The distribution for FbFb CPE block is between 2.0 and 5.9. But both have 70 percent or more fly lines in the range of 3.0-3.9 (Figure 3.3). The results show that frequency of flies in the eye color range 3.0-3.9 increases for the protected lines. The percentage of fly lines protected by SF1 and Fb in the range of 3.0-3.9 is significantly higher than suHw protected lines. In all CPE protected lines there is a concurrent fall in the extreme eye color range frequencies. The 2x suHw protected lines had an increased frequency of 5.0-5.9 eye color range when compared to SF1 or 1xsuHw. The 1xsuHw had greater frequency of 4.0-4.9 eye color

range when compared to SF1. SF1 and FbFb might be showing a silencing effect while 1xsuHw and 2xsuHw might be showing an activating effect.

3.3: DISCUSSION

suHw CPE blocking activity present in both 1xsuHw and 2xsuHw flanking

S2 enhancer blocking assay indicates that the properties of suHw insulator function are dependent on the system. We observe that there are non-overlapping mechanisms underlying enhancer blocking activity of suHw depending on the system being studied. The CPE protection assay of flanking 1x and 2x suHw shows that there are non-overlapping aspects in boundary functions. There may be distinct mechanisms of enhancer blocking and CPE blocking activities. Though the eye color distribution is significantly different in the 2xsuHw protected gene, there seems to be partial cancellation of the suHw activity. There is also some activating effect of suHw but the the eye color distribution seems to show a partial cancellation. This is suggested by the fact that in 2x suHw the percentage of eye color between 3.0 - 4.0 increased to 55 % from 39% in 1x suHw.

The distribution of eye color for CPE blocking activity is different for suHw and SF1.

Gypsy retrotransposon element is a well known boundary element in Drosophila. Supressor of Hairywing (suHw), a 340 bp element in the 5' untranslated region of gypsy acts as an effective boundary. It is known to protect a gene from the influence of CPE. When a white gene is inserted randomly in the genome the eye color of independent transgenic fly lines have a normal distribution- yellow to red. When the gene is flanked

by boundary elements like SF1 or suHw, that eye color distribution becomes narrow and skewed. We know that a single suHw is an enhancer blocker, but two tandem suHw elements cannot block (Cai 2001). This indicates a cancellation of two suHw elements. In the CPE protection experiments, we tested whether the ability to block CPE changed in the presence of one versus two suHw elements flanking the white gene. The data shows that 1x suHw flanked white gene has a narrow distribution of eye color while the 2x suHw is slightly skewed towards the red. However, none of the eye color distribution of suHw protected lines resemble the control. This supports the model that suHw elements flanking a gene, loop to interact allowing the gene to be exposed for activation (Cai HN 2001). Moreover, the looping of the flanking 2xsuHw brings together four suHw elements which would be able to stimulate gene expression to higher extent (Golovnin A 2005).

4.4: METHODS

Construction of CPE-blocking transgenes

The suHw R1 fragment was inserted into the R1 site in CaSpeR vector, resulting in the CA-suHw plasmids. Another suHw element was inserted into the Nsi site in CaSpeR vector. This is the 1xsuHw flanking miniwhite. For the 2xsuHw flanking miniwhite each 2x suHw was similarly inserted into the R1 and Nsi sites in CaSpeR vector.

P-element-mediated germline transformation

P-element mediated transformation was carried out as described previously (Rubin 1982; Cai 2001) (Rainbow Transgenic Inc, California). The w^{1118} *Drosophila* strains were used

to generate transgenic lines. Five or more independent transgenic lines were obtained and characterized for each enhancer-blocking construct.

Eye color assessment

The eye color of 5-7 days old heterozygous females was assigned and color level by visual assessment according to an 12-point scale of progressively darker color shown in Figure 1A, under 10x objective and intermediate illumination with NCL150 cold light source.

Statistical analysis

Data compilation and statistical analyses, except otherwise indicated, were done using the Microsoft excel software. GraphPad's web site was used to calculate the Chi-square test.

Figure 3.1: Eye color distribution suHw CPE

The suHw CPE constructs and the transgenic lines with eye color distribution.

The miniwhite gene is flanked by the suHw elements, single and double.

Table 3.1 shows that the eye color distribution is narrow in the case of 1xsuHw 3.0 - 5.0.

The eye color distribution is skewed towards the dark red in the case of 2xsuHw.

Figure 3.2:

The eye color distribution of CA control, CA flanked by 1xsuHw and CA flanked by 2xsuHw.

Table 3.2 Eye color distribution

The eye colors of the CA control is normally distributed.

The eye color for 1xsuHw carrying lines only ranges between 3.0 to 5.0: 39 in 3.0 - 4.0 and 61 in 4.0 – 5.0.

The eye color for 2xsuHw carrying lines ranges between 3.0 to 6.0: 55 in 3.0 – 4.0, 32 in 4.0 - 5.0 and 13 in 5.0 - 6.0.

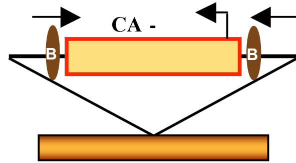
The eye color intensity standard is shown below the chart.

Figure 3.3: The comparison between suHw CPE and SF1 CPE activities.

The distribution of eye color an unprotected control and of CPE blocking activity of 1xsuHw, 2xsuHw, SF1 and FbFb. CA-SF1 CPE activity results in 75% of eye color in 3.0-3.9 range, while CA-1xsuHw CPE activity results in 52% of eye color in 3.0-3.9 range and 48% of eye color in 4.0-4.9 range.

Figure 3.1

CA-1xSuHw



CA-2xSuHw

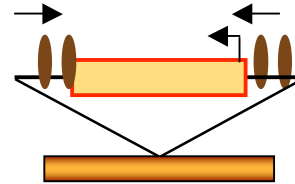


Table 3.1

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<tr><th>Eye color</th><th>Eye color range</th><th>Percentage</th><th>Eye color</th><th>Eye color range</th><th>Percentage</th></tr>
<tr><td>3.37</td><td rowspan="9">3 to 4</td><td rowspan="9">39</td><td>3.27</td><td rowspan="12">3 to 4</td><td rowspan="12">55</td></tr>
<tr><td>3.57</td><td>3.48</td></tr>
<tr><td>3.67</td><td>3.53</td></tr>
<tr><td>3.80</td><td>3.63</td></tr>
<tr><td>3.83</td><td>3.67</td></tr>
<tr><td>3.87</td><td>3.72</td></tr>
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<tr><td>3.97</td><td>3.83</td></tr>
<tr><td>4.0</td><td rowspan="14">4 to 5</td><td rowspan="14">61</td><td>3.92</td></tr>
<tr><td>4.0</td><td>3.93</td></tr>
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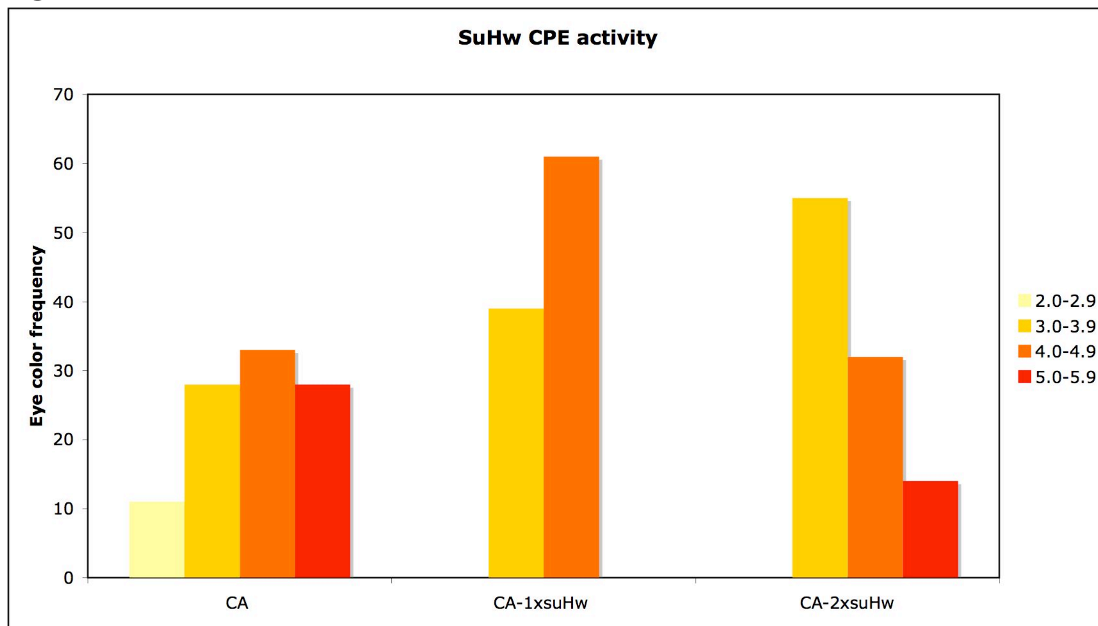
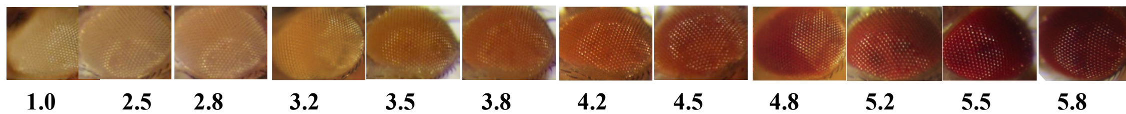
<tr><td>4.63</td><td>5.1</td></tr>
<tr><td>4.67</td><td></td></tr>
</table>

Table 3.2

Eye Color	CA	CA-1xsuHw	CA-2xsuHw
2.0-2.9	11	0	0
3.0-3.9	28	39	55
4.0-4.9	33	61	32
5.0-5.9	28	0	13

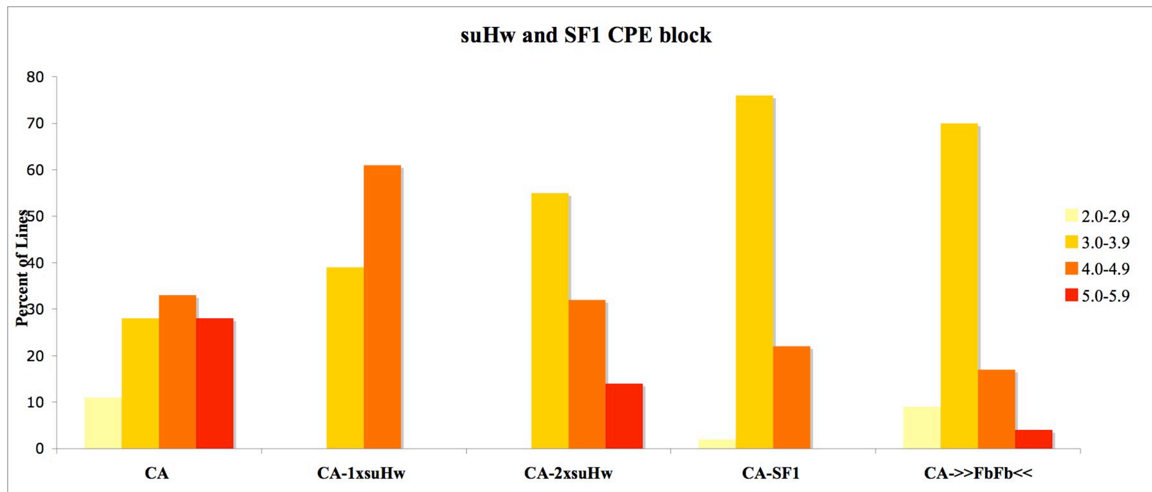
Figure 3.2





1.0 2.5 2.8 3.2 3.5 3.8 4.2 4.5 4.8 5.2 5.5 5.8

Figure 3.3



CHAPTER 4

MODIFIER SCREEN FOR SF1 BOUNDARY ACTIVITY IN DROSOPHILA HOMEOTIC GENE COMPLEX

4.1: INTRODUCTION

Globin locus - Looping mechanism and Modifiers

The vertebrate globin loci have been well studied especially the mammalian globin loci in human and mouse. The mammalian loci consists of the alpha (α) and the beta (β) globin loci. These loci are structurally different. The α globin locus is located in active chromatin containing housekeeping genes while the β-globin locus is in an inactive chromatin region containing the olfactory receptor genes. When the mature erythrocytes fill up with hemoglobin, the hemoglobin is formed due to the high expression of globin genes in the differentiating erythrocytes. Studies in the β-globin locus help us towards understanding the fact that inspite of being in the inactive chromatin region the β-globin genes are expressing at a high level in the differentiating erythrocytes.

The globin genes are arranged in the locus in the same order as they are expressed during development. This process is called 'switching' (Grosveld F 1993). The beta globin loci in humans have five genes ε, γG, γA, δ, β. The beta globin loci in mouse have five genes ε, γ, βh1 , βmaj, βmin. The Locus control region (LCR) controls the expression of globin genes. In these loci the genes on the left are expressed in the embryonic stages while the genes on the right are expressed in the adult stages. LCR can generate high expression of transgenes and are able to do so in a copy number dependendent but position of

integration independent manner (Dillon et al 1997). In anemic conditions like γδβ Thalassemia, the LCR has deletions (Van der Ploeg 1980). It was found that the LCR contained DNAse I hypersensitive (HS) sites (Tuac et al 1985) and their number varied among different species. LCR contains the enhancers (HS1-HS4 in human β-globin) and the globin genes in the locus compete for activation by the LCR. In globin expressing cells the expressing genes are in close proximity to the LCR as seen with 3C experiments (Dekker,Tolhuia et al 2002). Therefore, structurally the haemoglobin expressing chromatin is different from the non-expressing chromatin. The globin expressing chromatin forms a structure called the active chromatin hub (ACH) (Palstra et al 2003). The chromatin looping for specific regions is highly regulated. Both, the human β-globin HS5 and its ortholog chicken 5'HS4, have enhancer-blocking activities (Farrell et al 2002). These HS regions play a more structural role. Recently, it was shown that the LCR chromatin has a more open state in embryonic erythroblasts as compared to definitive erythroblasts. But in the tertiary chromatin level, chromatin is more tightly wound in embryonic erythroblasts as compared to definitive erythroblasts (Fang et al 2009). The following are some of the factors involved in looping.

CTCF: It is a CCCTC-binding factor. CTCF found in vertebrates is an insulator protein and is involved in enhancer – blocking function. For example enhancer blocking function in chicken 5'HS4 requires CTCF (Bell et al 1999). The CTCF forms a chromatin hub (Splinter et al 2006). CTCF and Cohesin are involved in chromatin loop formation (Stedman et al 2008). Drosophila d-CTCF is associated with many chromatin boundaries in Bithorax complex (Holohan et al 2007). GATA1 and FOG1: These factors interact and can have an activating or repressing effect during development (Vakoc et al 2005).

EKLF: It is Kruppel like zinc finger DNA binding protein. It is involved in loop formation (Drissen et al 2004). NF-E2: This consists of two DNA binding subunits (Forsberg 2000). LDB1: This is a non-DNA binding protein (Meier et al 2006).

Chromatin architecture regulates homeotic complex

The cis-regulatory elements regulate the expression of hox genes in two phases - initiation and maintenance (see Introduction). Many studies have been done in the bithorax complex. The initiation happens when the cascade of transcription factors interact with the cis-regulatory elements in the BX-C while the parasegments (PS) are determined (Busturia et al 2001, Barges et al 2000). The cis regulation occurs throughout the life of the organism. For each parasegment there is a complex set of regulators. The maintenance of the Hox gene regulation requires the Polycomb group (Pc-G) and the trithorax group (Trx-G) proteins. These bind the PREs and TREs, respectively. Pc-Gs are silencers while Trx-Gs are enhancers. Both the groups contain protein factors that are involved in histone interaction, movement and modification. GAGA Factor, for instance, is a Trx-G protein. Thus, a simple model suggests that Pc-Gs help to compact the chromatin while Trx-Gs open up the chromatin to allow activators to activate gene expression. Recent studies show that Pc-Gs create more dynamic chromatin states rather than maintaining a compact state throughout development (Shwartz and Pirrotta, 2008). Normally, the chromatin architecture determines the degree of gene activity. For instance, the heterochromatin has a silencing effect on the status of genes in that region. The chromatin state, in turn, is determined primarily by the presence or absence of methylation and acetylation markers. This marker profile can be hereditary as

exemplified in genomic imprinting. The functional aspect of developmental regulation is not confined to genetic expression profile but also involves regulation of the chromatin backbone. In recent studies our lab has shown that E(bx) and other NURF components of the chromatin remodelling complex can affect the SF1 chromatin boundary activity (Li M, Belozero V, Cai HN 2009).

My genetic screen using the Deficiency stocks showed that in a dosage sensitive screen we can find an abundance of modifiers that alter SF1 function . The goal of my screen was to identify SF1 modifiers that change the enhancer blocking function of SF1. When a mutation enhances the block, it is called enhancers of SF1 boundary function. When a mutation suppresses the block, it is called suppressors of SF1 boundary function. From my screens I found some candidate modifier genes like CG34381 and CG34380.

4.2: RESULTS

Deficiency screen in adult eye and embryos

I did a genetic screen to look for dominant modifiers that are responsible for SF1 enhancer blocking function. For this purpose we used the Bloomington Deficiency kit. Occasionally, chromosome breaks occuring in *Drosophila* causes elimination of intervening region during break repair. In such flies deficiency or deletion of specific chromosome region occurs producing mutant stocks. Thus, deficiency leads to loss of part of genome and the genes within that region. The fly containing the deficiency is crossed to a reporter fly which contains SF1 inserted between G5 eye enhancer and miniwhite, showing orange eye color (Figure 4.1). The deficiencies are generated through P-element excision, X-ray, etc. Many of the deficiency stocks also carried the eye color

marker. The eye color present along with the deficiency was masking the eye color of the reporter. Thus, we only tested 50% of the genome. We crossed the female flies to the deficiency males. We found 11 deficiencies that affected the reporter, when we tested 50% of the genome. When a specific deficiency changed the eye color of the reporter, we tested that deficiency with a non-SF1 boundary reporter. Out of the 11 deficiencies that were affecting SF1, one seemed to have specific effect on SF1 called 490DK2 (Table 4.1). We also tested the deficiencies on the 1st and 2nd chromosomes with embryonic enhancers blocked by SF1/bb (Figure 4.2) . Several deficiencies affected SF1function in the embryo, including the deficiency 490DK2 from the previous screen. Another, adult screen candidate 7441DK2 was also found among the candidates in the embryo screen. From my screen in embryos I also found new potential candidates like 3217DK1 among others.

In the SF1 modifier screen we used a reporter transgene (CaSF1GMR, Fig. 4.1) that carries a SF1 boundary element between the eye enhancer GMR and the miniwhite gene. Mutation in the SF1 modifier genes will cause changes in the phenotype of reporter gene, in this case the eye color. We generated several transgenic lines and used Line 4b that has intermediate eye color . When the 4b fly is crossed to CRE fly, CRE-mediated SF1 deletion causes the eye color to change in females from 3.8 to 4.5 after SF1 deletion (Figure 4.1).

We conducted the Bloomington deficiency kit screen (Figure 4.2) using the eye color reporter. Selected lines from the Bloomington deficiency kit were first used in an adult

eye enhancer-blocking screen (GMR-SF1-miniwhite Figure 2A). We covered about 50% of the genome for the 2nd and 3rd chromosome. From the deficiency screen we found 11 fly lines that produced changes in the eye color. To confirm that this was specifically due to the presence of SF1, we crossed the candidate deficiency flies to transgenic flies containing a non-SF1 insulator. One deficiency, 490 DK2, showed SF1-specific modification (Table 4.1). Because most of the overlapping deficiencies and sub-deletions within the Df490 DK2 interval (26B2-26E1) contain eye color markers, we adopted an embryonic enhancer-blocking transgene NbbH (Figure 4.2) for further analysis of these sub-deficiencies. The result is shown in Fig. 4.3. Green lines represent suppressor SF1/b activity, whereas red lines represent enhancers of SF1/b activity.

We also screened for deficiencies that modify SF1 activity in embryos. We used transgenic flies containing lacZ reporter driven by two tissue-specific enhancers NEE (N), active in the ventrolateral region and H (H1), active in a head stripe (Figure 4.2). When two copies of SF1b, a subfragment of SF1, are inserted between N and H, the horizontal NEE stripe is reduced due to the insulator block (Figure 4.2). If a deficiency deletes genes that are required for SF1 insulator activity, the N-directed lac Z expression will be altered.

Analysis of 490DK2 region

490DK2 covers more than a hundred genes. Its insertion/ breakpoint occurs from 25F3-26A1 to 26D3-11. Consequently, we tested some smaller deficiencies and deletions within this region (Table 4.2). Some candidate subdeficiencies were selected after couple

of rounds of *in situ* hybridization. These were 7499, 7799, 7501, 7502, 9185, 3365, 9272, 9341, 9182, 6338 (Figure 4. 3). The subdeficiencies 7499, 7501, 7502 and 7799 contain around 20 genes each. 9185 contains about 35 genes.

We measured the degree of N enhancer block using NbbH in 490-subdeletion background. In wt NbbH, 70% of embryos have 0-20% N-lacZ expression indicating strong block. 7799 deletion increases N-LacZ expression, with only 40% of embryos showing strong block. 7502 deletion further decreases N-lacZ expression with 90% strong block. Similarly,7501 and 9185 were found to have close to 90% strong block. 7499 produces 92% strong block. There is a small overlap between 7501 and 7502 deletions (Figures 4.3, 4.4).

We tested whether these deletions are causing changes in the NEE which in turn is changing the expression intensity. We used white probe to measure the intensity of NEE expression. We found that 7799 shows a weaker NEE - white intensity than control, but its NEE Lac Z pattern shows higher intensity than the control. This is a candidate for a strong suppressor of Fb. The 7501 is a strong enhancer because 7501 NEE-white shows slightly weaker intensity than control while the NEE-lacZ pattern shows that it is a strong enhancer of Fb (Figure 4.4).

We also tested for the specificity of these lines and found there were differences in NEE-lacZ intensity in embryos blocked by suHw. 7799 is a weak enhancer of suHw but it is a

suppressor of Fb. 7501 is a weak enhancer of suHw but a strong enhancer of Fb (Figure 4.5).

To identify SF1-modifier genes within Df490, P-insertion lines within the sub-deletions were tested for their effect on SF1/b activity in embryonic enhancer-blocking assay. Within the candidate subdeficiencies, I tested the P-inserted genes and found some candidate modifier genes that modified SF1 embryonic enhancer blocking assays. We identified both suppressors of insulator function that cause increased NEE driven-LacZ expression like CG34381 (line 23428), and enhancers of SF1 insulator function, such as CG34380 (line 18880). We are testing these genes by dsRNA knockdown (RNAi) in S2 cell culture enhancer blocking assay.

We further examined whether mutations in SF1 modifier candidates disrupt endogenous Scr , ftz and Abd-B expression. In situ hybridization with Scr probes indicate that several modifier candidates indeed alter the endogenous gene regulation. For further confirmation of SF1 specific modifier function we will study the effect of candidate gene knockdown in the S2 cell enhancer- blocking assay.

4.3: DISCUSSION

The heterochromatin and euchromatin junctions can silence active genes variably. This phenomenon is called position-effect variegation (PEV). Certain gene mutations suppress the variegation while certain mutations enhance the variegation. From the literature survey, we know that 490DK2 region contains genes with Suppressor of Variegation,

Su(Var) and Enhancer of Variegation, E(Var) functions. However, these functions have not been mapped to genes in this region. One example of Suppressor of Variegation is the gene Su(Var) 3-9. We know that a hallmark for heterochromatin state is Histone 3 Lys 9 dimethylation (H3K9me2). It has been shown that Su(Var) 3-9 is just one of the methyltransferases associated with H3K9me2 and there might be another (Jiro C. Yasuhara, 2008). Recent studies are revealing that there is a dynamic relationship between euchromatin and heterochromatin states. One example of an E(Var) is mod(mdg4). This interacts with suHw protein to form the suHw boundary as discussed in the previous chapter.

Both the embryonic SF1 modifier screen and the adult eye SF1 modifier screen showed that the 490DK2 deficiency was a suppressor of SF1 enhancer blocking function. The results showed that the deletion might have taken out a gene that was positively interacting with SF1 to form a boundary. However, when we studied the subdeletions we found that they varied in their effect on SF1 function. The subdeletions 7499, 7501, 7502 and 9185 were the smallest deletions to act as enhancers of SF1/bb enhancer blocking function. The subdeletion 7799 was able to reflect the effect of 490DK2 which showed suppression of the SF1/bb enhancer blocking function. When we observed the white expression we found that the expression of the white gene was not increased by NEE. Rather the NEE-white expression was weak. Out of the 30 genes that were screened with P-inserted lines, we selected six most strong enhancers and four most strong suppressors, when compared to the control. A challenge with P-inactivation lines is that they might be just P-insertions and not P-inactivations of the genes. However, very few showed any

significant change. The Z test was done to calculate the proportion change in each category when compared to the control (Table 4.3). We find that none show any significant change in the 40% -100% NEE intensity levels. Only two genes CG34381 and CG34380 show significant difference in the 0-40% NEE intensity range. The genes, in some cases, behaved differently from the subdeletion that covered them (Table 4.1). Thus, these genes are being tested in the S2 cell culture by knocking out the gene with dsRNA. We will observe the effect of gene knockout on the SF1 enhancer blocking assay in the S2 cell culture (Li M et al, 2008) . I have already observed the expression for these genes in the S2 cells.

The gene CG34380 has a molecular function described as a receptor signaling protein tyrosine kinase activity and is involved in processes like signal transduction and cell adhesion. This gene is found in subdeletion region 7501 and both the gene mutation and the subdeletion shows SF1 enhancer effect. The P-insertion for this gene is in the intron after the 4th exon. The gene CG34381 shows that its function is neuropeptide receptor activity and G-protein coupled receptor activity. This gene is present in the subdeletion region of 7799 and both are suppressors of SF1 boundary function. The P-insertion for this gene is in the 3rd exon. Another gene CG13993 acts as a suppressor of SF1 function but is present in the subdeletion region of 9185 which behaved as an enhancer of SF1 function. Two independent P-insertion lines of CG13993 have shown similar result. One insertion is in the 5'UTR. Its molecular function is to bind unfolded proteins. Hence, it is involved in protein folding.

The other genes among those 30 genes that show some change in the NEE expression are some enhancers and few suppressors of Fb function. The other potential enhancers of Fb function in the list are Follicle cell protein 26Ac (Fcp26Ac), CG9117, CG9497, chickadee (CG9553) and CG9171. The other potential suppressors of Fb function in the list are bchs (CG14001) and CG9098. The molecular and biological functions of Fcp26Ac and CG9497 are unknown. Some genes have little molecular information and no biological like CG9117 has hydrolase activity and CG9171 has N-acetyllactosaminide beta-1,6-N-acetylglucosaminyltransferase activity. The molecular function of gene chic is described as actin binding and is involved in many biological functions. The molecular function of gene bchs is described as zinc ion binding and it is involved in many biological functions. The molecular function of CG9098 is described as guanyl-nucleotide exchange factor activity and is involved in the small GTPase mediated signal transduction. There are other interesting genes in 490DK2 that we have not studied yet. For example, Kruppel homolog 1 and 2 (kr-h1 and kr-h2). These were not tested because of unavailability of P-inactivation lines in stock center during the initial test. Kr-h1 is known to be involved in transcription activity and is involved in the biological process of metamorphosis. Kr-h1 mutants fail to complete head eversion (Pecasse F 2000).

Our transgenic assays involved observing LacZ expression levels. We needed to observe the effect of these candidate gene mutations on the endogenous gene expression . SF1 helps to delineate the regulatory domains of the homeotic gene Sex comb reduced (Scr) and the non-homeotic gene fushi tarazu (ftz) in the Antennapedia Homeotic gene

complex. We used the Scr and the ftz probes to detect changes in the endogenous gene expression in mutant embryos. Scr is required for the labial and first thoracic segment development. We performed in situ hybridization with 5-12 hr embryos. We did not observe perceptible changes in ftz expression pattern. There was no homeotic transformation in Scr expression. We found that there was no clear absence of expression. However, we do find some overexpression in Scr. Moreover, we also found that some of these mutant backgrounds produced abnormal expression patterns. When compared to wildtype there was subtle differences in intensity and spread of the Scr expression pattern (Figures 4.7- 4.10).

These potential candidates for modifiers of SF1 function have generated mixed results. The results also indicate that the observations based on such a sensitive assay require more confirmation tests. Redundancy in the function of dominant modifiers can also cause difficulty in understanding the role of a particular gene mutation. There are high numbers of potential modifiers both from the adult modifier screen and the embryonic modifier screen. One might speculate that many players at all stages and aspects of the pathway of Scr homeotic regulation and segment identity can be revealed by conducting a dosage dependent screen. The screens have revealed that many deficiencies have varying amounts of effect on NEE lacZ expression.

4.4: METHODS

Costruction of enhancer-blocking transgenes:

The G5 enhancers (provided by Jumin Zhou) were digested with Bam HI, purified, and ligated into the unique Bam HI site of the CaSpeR vector, resulting in the CA-G5 plasmids. The full-length SF1 and its sub-fragments SF1a-SF1c were generated by PCR using primers containing Not I site and cloned into pCRII/TOPO vector (Invitrogen). The resulting constructs were digested with Not I and the DNA inserts were gel extracted, purified and ligated into the NotI sites between the GMR/G5 enhancer and the *miniwhite* reporter in the pCaSpeR transformation vector. Similarly, Fb Fb were digested with NotI from PBS2N and the DNA inserts were gel extracted, purified and ligated into the NotI sites between the N and H enhancers in the Ca lacZ NH transformation vector (NbbH) (Belozerov, 2003).

P-element-mediated germline transformation

P-element mediated transformation was carried out as described previously (Cai, 2001, Rubin, 1982). The w^{1118} *Drosophila* strains were used to generate transgenic lines. Five or more independent transgenic lines were obtained and characterized for each enhancer-blocking construct. NbbH line was generated previously in the lab.

Eye color assessment:

The eye color of 5-7 days old heterozygous females was assigned and color level by visual assessment according to a 12-point scale of progressively darker color shown in Figure , under 10x objective and intermediate illumination with NCL150 cold light source.

Collection and scoring of eyes and embryos:

The 4b virgins carrying SF1 enhancer blocking construct was crossed to the Deficiency males from the Bloomington stock. Then these 11 candidates were tested using the virgins of suHw enhancer blocking construct crossed to the Deficiency males. The eye color change was scored in females. For the embryonic enhancer screen about 100 NbbH females were crossed to 30 Deficiency males in collection bottles. 2-4hr embryos were collected. These were used for *in situ* hybridization with LacZ or white probes. Similar methods were followed for other reporters like those containing suHw and Fab8. Similar methods were followed for the P-inactivation lines crossed to NbbH reporter. All in situ hybridization embryos are scored double blind atleast 2 times by different individuals. The grading of the lines according to the NEE expression intensity was done by calculating the difference in the 0-20% expression level when compared to the control and the difference in the 80-100% expression level when compared to the control.

Figure 4.1:

The Deficiency screen using an adult eye-specific enhancer-blocking assay.

The SF1 boundary in between the GMR enhancer and the miniwhite reporter blocks the enhancer. The reporter line used is called 4b. The 4b females have an eye color of 3.8. When the SF1 is deleted using the Cre-LoxP the eye color changes to 4.5.

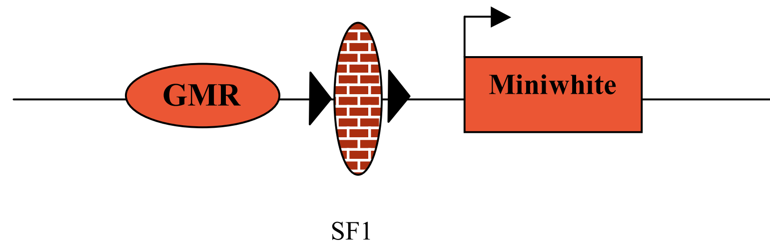
Table 4.1

The SF1 modifier candidate Deficiency lines:

A. The 4b screen candidates. The table shows the Line # and position. The eye color change for the 4b reporter female. Cy- 2nd chromosome Curly balancer. Non-Cy contains the deficiency.

B. The test for SF1 specificity using suHw transgenic females. 490DK2 is specific for SF1.

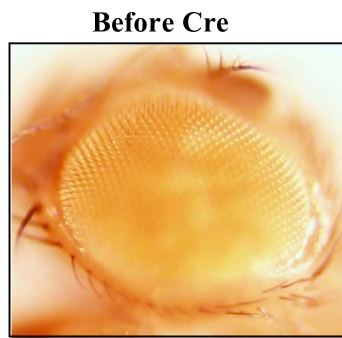
Figure 4.1.



SF1

Line 4b

Before Cre



eye color = 3.8

After Cre

eye color = 4.5-4.8

Table 4.1

line #	breakpoints/insertions	eye color change (4b cross)
6875	23C05-D01;23E02	Cy 3.8, nonCy 4.5
490	25F03-26A01;26D03-11	Cy 4.2, nonCy 5.8
6404	53E;53F11	Cy 3.8, nonCy 4.5
7414	54B1-2;54B7-10	Cy 3.8, nonCy 5.2
7441	54C8-D1;54E2-7	Cy 3.8, nonCy 4.2
7146	048E01-02;048E02-10	Cy 3.8, nonCy 4.2
6755	062E08;063B05-06	Ubx 3.5, nonUbx 5.2
6964	065E10-F01;065F02-06	Ubx 3.8, nonUbx 4.5
7080	085F01-02;086C07-08	Ubx 3.5, nonUbx 4.0
6471	067E03-07;068A02-06	Ser 3.5, nonSer 4.5
4431	089E01-F04;091B01-B02	Ubx 3.8, nonUbx 4.8

A.

	SF1 transgene	**suHw transgene**
6875	Cy 3.8, nonCy 4.5	Cy 4.2, nonCy 4.8
490	Cy 4.2, nonCy 5.8	all 4.2
6404	Cy 3.8, nonCy 4.5	Cy 4.2, nonCy 4.8
7414	Cy 3.8, nonCy 5.2	Cy4.2, nonCy4.8
7441	Cy 3.8, nonCy 4.2	Cy 4.2, nonCy 4.8
7146	Cy 3.8, nonCy 4.2	Cy4.2, nonCy4.5
6755	Ubx 3.5, nonUbx 5.2	Ubx 4.2, nonUbx 5.2
6964	Ubx 3.8, nonUbx 4.5	Ubx 4.2, nonUbx 4.5
7080	Ubx 3.5, nonUbx 4.0	Ubx 4.2, nonUbx 4.5
6471	Ser 3.5, nonSer 4.5	Ser 3.8, nonSer 4.5
4431	Ubx 3.8, nonUbx 4.8	Ubx 4.2, nonUbx 4.5

B.

Table 4.2 The deficiency and deletion stocks in the 490 DK2 region.

The breakpoint and insertion sites are shown.

Figure 4.2.

Deficiency screen using an embryonic enhancer-blocking assay

A. CaLacZNH construct. In situ hybridization with LacZ probe using 2-4hrs NH embryos. The horizontal N and the vertical H stripes are observed.

B. When FbFb is inserted between the NH enhancers, the in situ hybridization with LacZ probe has a weak to absent N horizontal stripe.

C. 490DK2 region contains an embryonic modifier of the SF1 function. In situ hybridization with LacZ probe showing transgenic NH pattern in 490 - NbbH embryo.

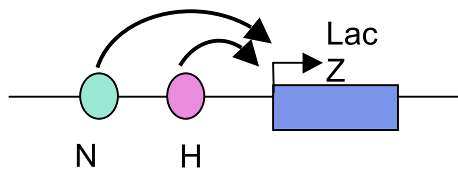
Figure 4.3: The map of deficiencies in 490 DK2 region .

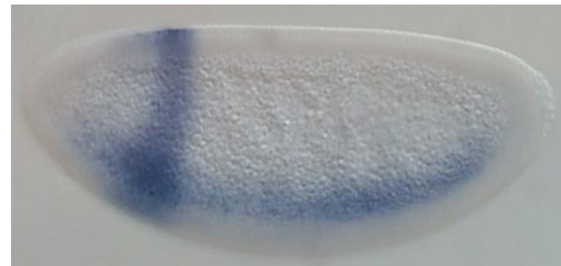
Smaller deletions are 7499, 9185, 7501, 7502 and 7799. Some larger deletions are 9341, 3365, 9182 and 9272. (See Table 4.2 for breakpoints/ insertions). Green colored bar represents suppressor of Fb function while red colored bar represents enhancer of Fb function. Black bar denotes Fb function not changed perceptible.

Table 4.2

STOCK	BREAKPOINT STARTS	BREAKPOINT ENDS
490	25F3-26A1	26D3-11
9272	25F5	26B5
9341	26B1	26D7
7499	25F2	25F4
7799	26A1	26A8
3365	25E1-2	26A7
9185	26B2	26B5
7501	26B9	26C1
7502	26C1	26D1
9613	26B10	26D7
6114	26A	26B5-6
9182	25F5	26B2
3788	25D6	25F4-5
1128	25D7-E1	26A8-9

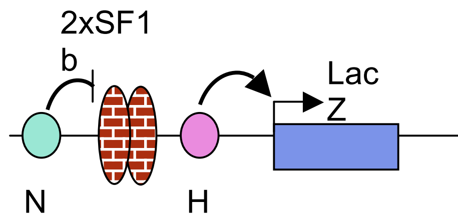
Figure 4.2





A.

in situ with lacZ probe



B

in situ with lacZ probe

C. 490, NbbH-LacZ

Figure 4.3

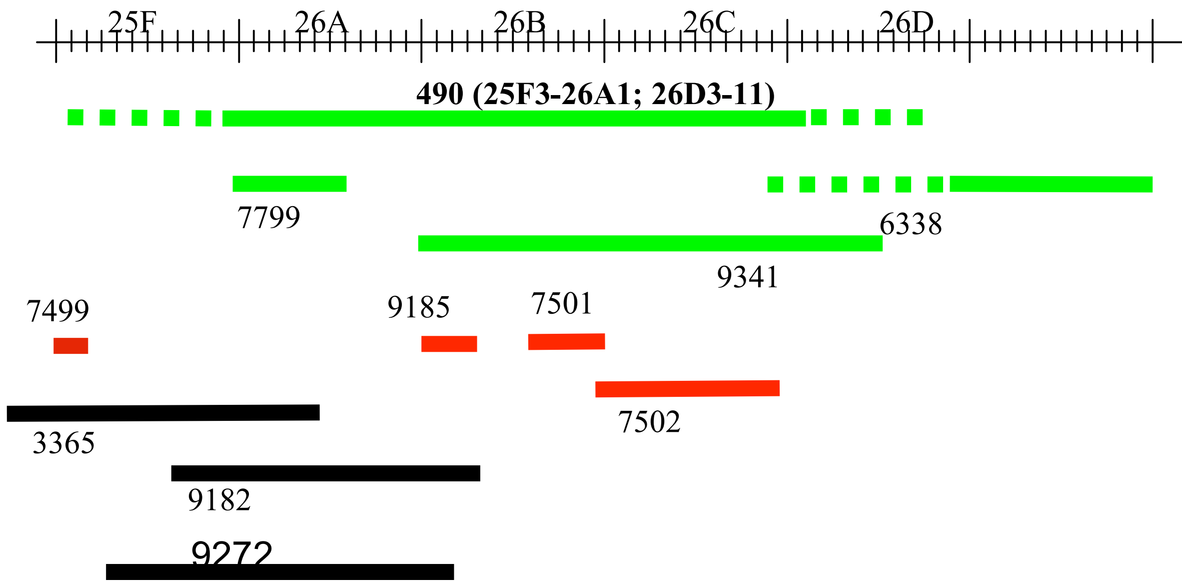


Figure 4.4

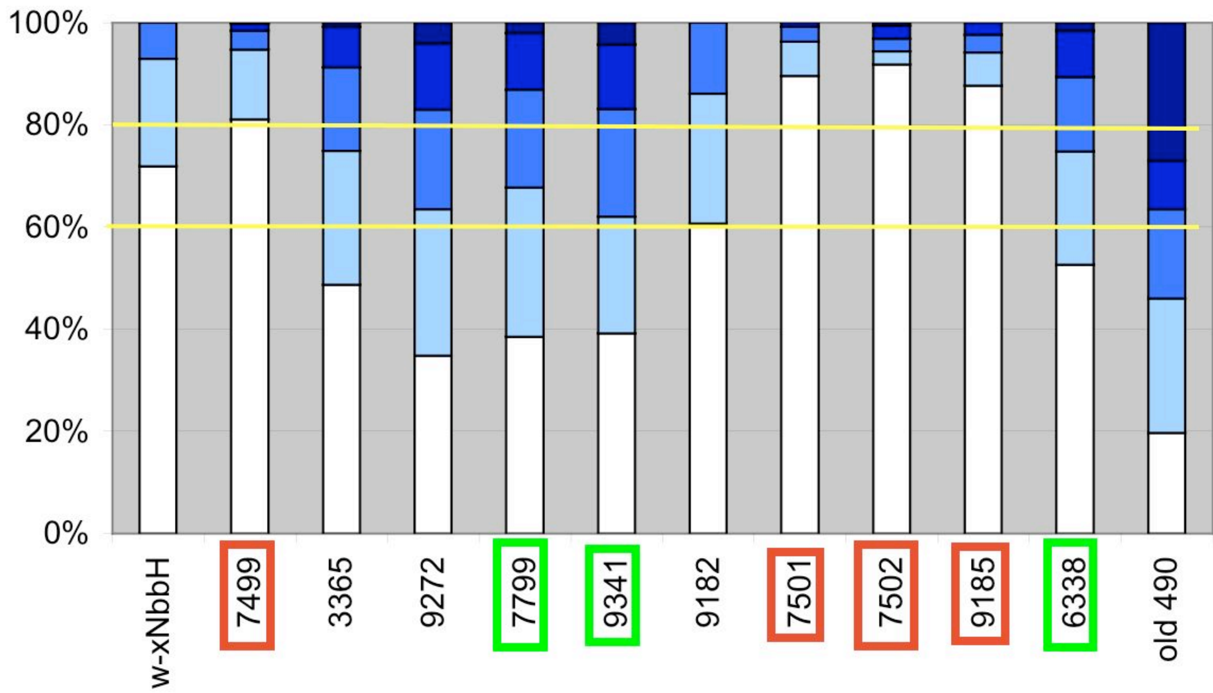
Analysis of 490 sub-deletions –

A. In situ hybridization with lacZ probe. The NEE lacZ expression intensity was measured in 5 categories ranging from 0-20% which represents strong block to 80-100% which represents no block. The x-axis shows the percentage of embryos with respective N LacZ intensity. In wt NbbH control, 70% of embryos have 0-20% N-lacZ expression indicating strong block. 7799 deletion increases N-LacZ expression, with only 40% of embryos showing strong block. 7502 deletion further decreases N-lacZ expression with 90% strong block. The yellow lines represent cut off for recognising potential candidates.

B. In situ hybridization with white probe. The NEE white expression intensity was measured in 5 categories ranging from 0-20% which represents low enhancer activity and low level of white expression, to 80-100% which represents high level of expression. The x-axis shows the percentage of embryos with respective N-white intensity.

We found that 7799 shows a weaker NEE - white intensity than control. This is a candidate for a strong suppressor of Fb. 7501 is a strong enhancer because 7501 NEE-white shows slightly weaker intensity than control.

Figure 4.4
A.



B.

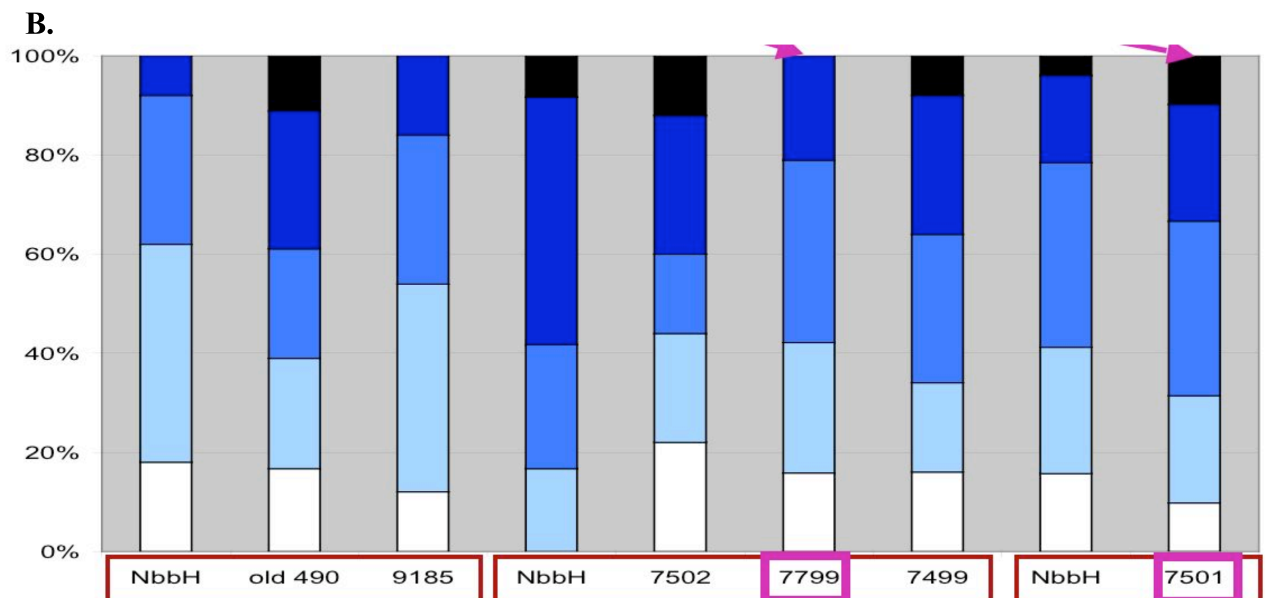


Figure 4.5: Test for the effect of deletions on suHw

In situ hybridization with lacZ probe. The NEE lacZ expression intensity was measured in 5 categories as mentioned above. The x-axis shows the percentage of embryos with respective N LacZ intensity. 7799 is a weak enhancer of suHw but it is a suppressor of Fb.

Figure 4.6: Screening P-inserted genes

The N-lacZ pattern in P-inserted mutant lines. In situ hybridization assay as described before. Two genes to be noted from this screen are CG34380 (in 7501, line # 18880 is enhancer of Fb) and CG34381 (in 7799, line # 23428 is suppressor of Fb). Both P-inactivation lines of CG13993 (line #s 14444 and 17182) show suppressor effect on Fb, though it is found within 9185 which is an enhancer of Fb.

Figure 4.5

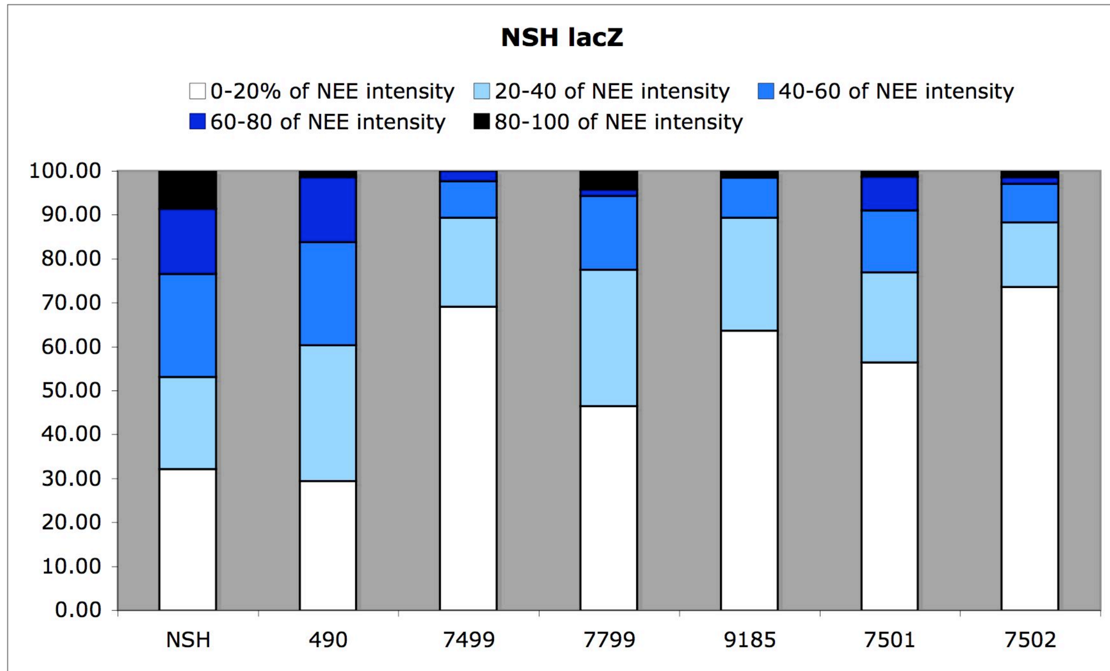


Figure 4.6 P-inserted lines

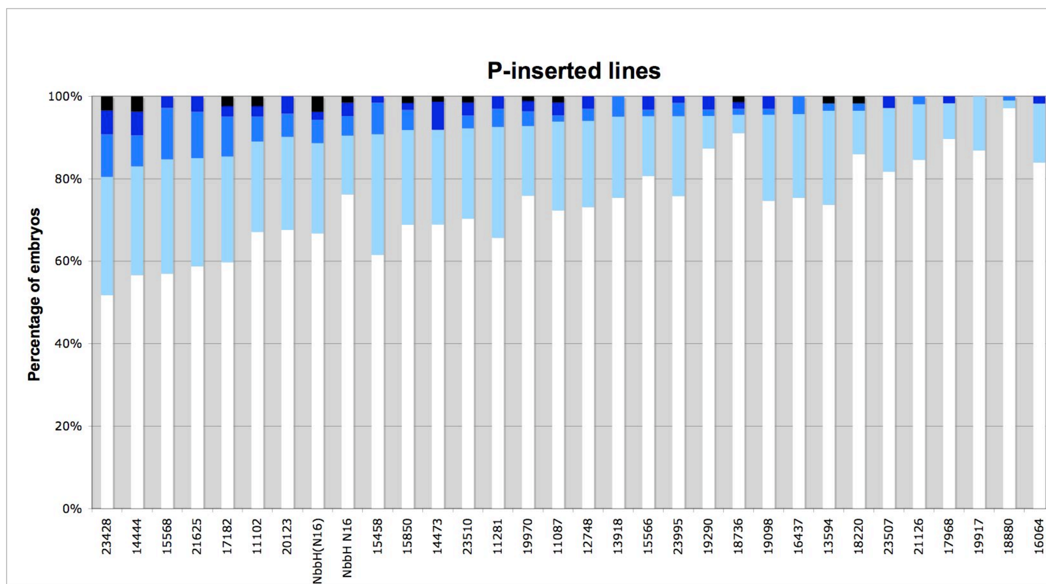


Table 4.3

The z test was performed for comparison of proportions, where the p value is <0.05. The results for suppressors (green) and enhancers (red) are shown. Each box contains the z score for the respective NEE intensity comparison with control. The significance values are shown in parentheses. The proportions that are significantly different from the control are marked with *.

Table 4.4

The list of candidate modifier genes that were selected as potential candidates. Genes denoted in bold are noteworthy. These are CG34380 (in 7501, red star for enhancer of Fb) and CG34381 (in 7799, green star for suppressor of Fb). Both P-inactivation lines of CG13993 show suppressor effect on Fb.

Figure 4.7

In situ hybridization with Scr probe in wildtype embryos. Scr is required for the labial and first thoracic segment development. Figures show embryo stage or hours post fertilization (hpf). Scr expression is observed in -

A. The germ band extension stage (5 hpf)

B. The germ band extension stage lateral view

C. The shortening of the germ band (9 hpf)

D. The dorsal closure and head involution (13-15 hpf)

Table 4.3

%NEE	CG34381 Z (sig)	CG13993 Z (sig)	bchs Z (sig)	CG9098 Z (sig)		
0-20%	3.047 **(.002)***	2.239 **(.025)***	2.345 **(0.019)***	2.193 **(0.028)***		
20-40%	2.086 **(.037)***	1.63 **(0.103)**	1.904 **(0.057)**	1.745 **(0.081)**		
	CG34380 Z (sig)	**CG9497 Z (sig)**	**Fcp26Ac Z (sig)**	**CG9117 Z (sig)**	**chicadee Z (sig)**	**CG9171 Z (sig)**
0-20%	3.076 **(.002)***	1.954 **(.05)**	0.994 **(0.32)**	1.315 **(0.188)**	1.532 **(0.126)**	1.05 **(0.294)**
20-40%	2.398 **(.016)***	0.972 **(0.33)**	1.956 **(0.845)**	0.127 **(0.89)**	0.189 **(0.85)**	0 **(1)**

Table 4.4

Subdeficiency	P-inactivation line	Gene affected
	Enhancers	
7799	23507	Follicle cell protein 26Ac
9185	21126	CG9117
7502	17968	CG9497
7799	19917	chicadee
7501*	18880	**CG34380**
9185	16064	CG9171
	Suppressors	
7799*	23428	**CG34381**
9185	14444, 17182	CG13993
7799	15568	bchs
9185	21625	CG9098

Figure 4.7

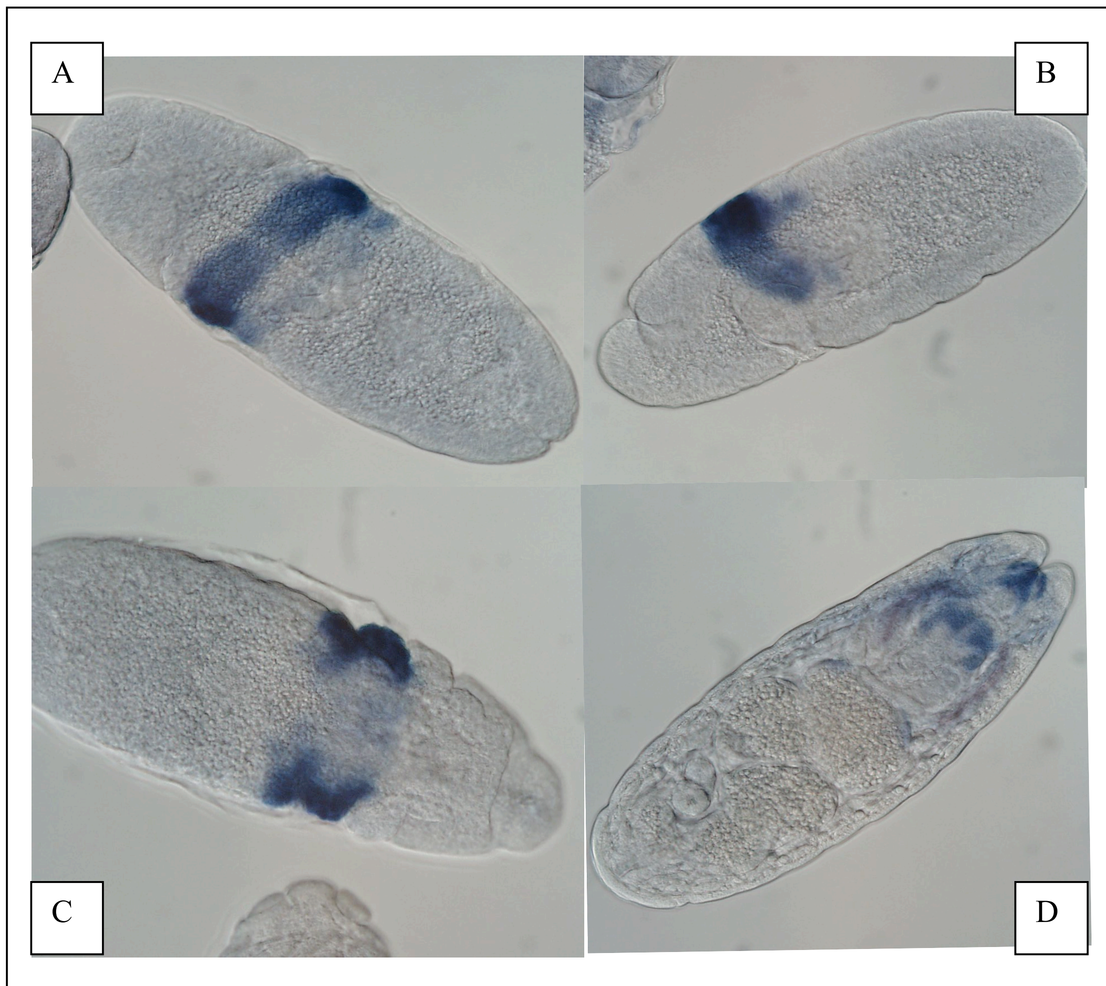


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Figure 4.8 : In situ hybridization with Scr probe in CG34381 embryos

At germ band extension stage (5hpf), Scr expression leaked into next segment. For comparison see wildtype embryo (arrow).

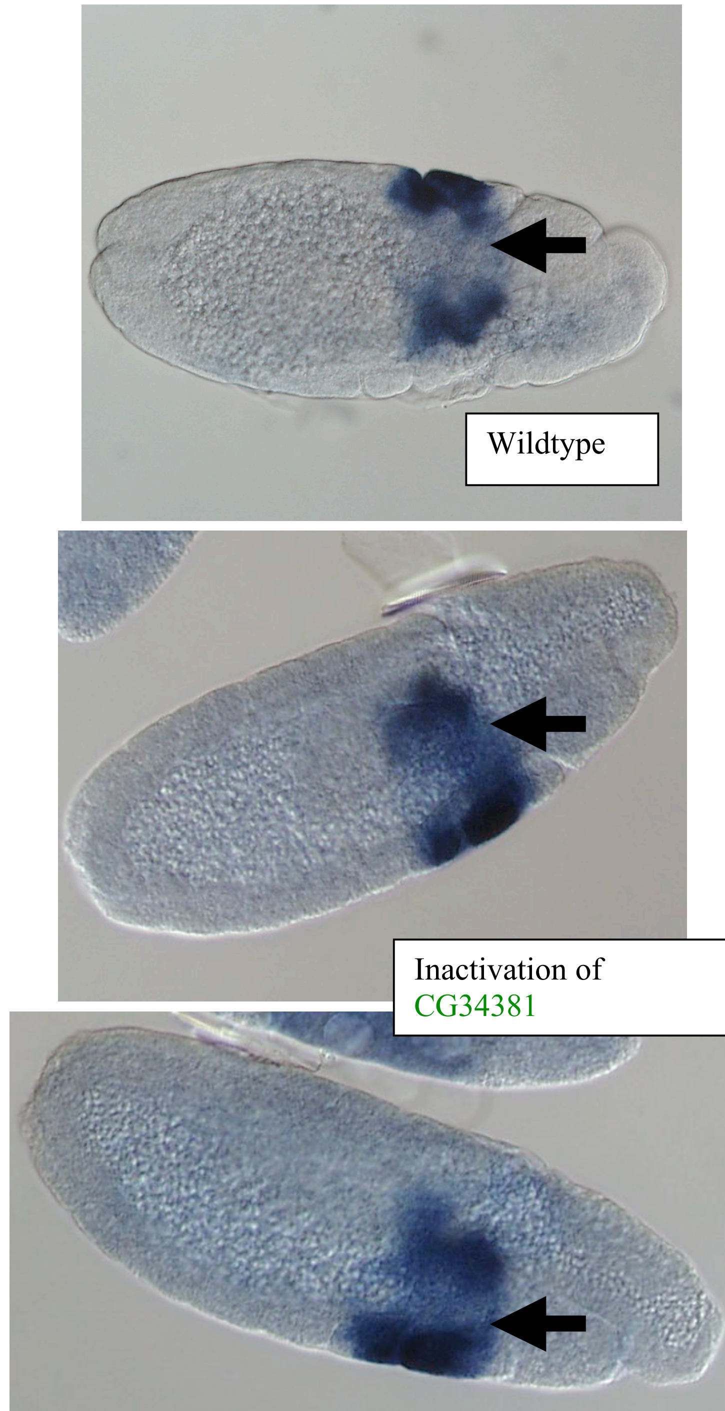
Figure 4.9:

In situ hybridization with Scr probe in CG34381 embryos

During the shortening of the germ band (9 hpf), Scr expression pattern in the first thoracic segment looks expanded. Compare to wildtype embryo (arrow).

Figure 4.8

Figure 4.9



CHAPTER 5

SYNOPSIS

5.1: LESSONS FROM REGULATION MECHANISMS IN BX-C REGION

We know that the loss-of-function mutations of Hox genes lead to the understanding of the regulation and determination of the body segment identity in *Drosophila* (Lewis, 1978). The BX-C region has been extensively studied in that respect. Initiators, maintenance elements, specific enhancers, tethering elements and chromatin boundaries are part of the cis regulatory network of this homeotic gene complex. In recent years many studies have been conducted to discover and elucidate the transacting factors involved in the BX-C homeotic gene regulation.

The initiation of homeotic gene expression occurs in the early embryogenesis when the gap and pair rule gene products activate the homeotic genes. It has been shown that the initiator elements in the BX-C region contain binding sites for the gap and pair-rule genes. Thus, these initiator elements activate the specific promoters in the early embryogenesis to determine the onset of parasegment and segment identity. When the gap and pair-rule gene products degrade, the expression driven by the initiator elements fade. The ability of the homeotic genes to maintain their expression pattern requires the function of the maintenance elements. These are the PRE and TRE elements (see Introduction). The Polycomb group and the Trithorax group of factors interact with the PREs and TREs. It was generally believed that the PRE maintains a silent chromatin state while the TRE maintains an active chromatin state. The recent evidence also shows that

PREs are dynamic regions involved in both silent and active chromatin configuration. The maintenance elements, however, do not contain any properties to drive gene expression in the segment and stage specific manner. Thus, the tissue specific enhancers are required for the regulation of transcription in these genes. These segment specific enhancers have been extensively studied in the BX-C region (Barges et al 2000). The expression pattern of homeotic genes determined by these enhancers is strictly confined to the parasegmental and segmental boundaries. But transgenic assays with some of these enhancers show patterns that are not similarly restricted and their expressions repeat along the AP axis. Thus, the precision in homeotic expression pattern requires the activity of the maintenance elements and other higher order regulatory domains. In recent years, it has been observed that tethering elements are regulatory elements that help in the formation of looped chromatin domains to allow higher order regulation. Studies from many labs including ours have shown that the chromatin boundaries are a very important part of the higher order homeotic gene regulatory domains. Chromatin boundaries in the BX-C are well studied both in transgenic and endogenous assays and in genomic studies. In mutational studies for the three well known boundaries Mcp, Fab7 and Fab8 it is observed that these boundary mutations lead to dominant gain-of-function phenotype (Barges et al 2000, Karch et al 1994, Mihaly et al 1998). For example, Fab7 mutational studies in combination with mutational studies of the enhancers iab6 and iab7 , and AbdB gene shows that Fab7 and the enhancers are functionally linked. In a Fab7 mutant the iab6 and iab7 domains are fused. This allows iab6 to act as an initiator of the pattern while iab7 determines the identity of the segment (Gyurkovics et al 1990). Thus, Fab7 separates the domains of iab 6 and iab7. The other part of this regulation is the

association of Fab7 boundary with AbdB promoter (Cleard et al 2006). This Fab7-AbdB interaction is found in the anterior tissues where AbdB is silent. This association is not found in the posterior tissue (Iampietro et al 2008, Maeda and Karch 2009 review).

Recent studies with sequence comparison has shown that there are several boundaries in the BX-C region apart from the known Mcp, Fab6, Fab7 and Fab8. These were postulated as boundaries due to the presence of dCTCF binding sites (Holohan et al 2007, Mohan et al 2007). However, Fab 7 boundary activity does not depend on dCTCF. The dCTCF binding sites are also found near the AbdB promoter.

5.2: THE REGULATION OF THE SCR –FTZ REGION OF ANT-C

We know that the Hox complex has a developmental ground state of thoracic segment T2. For loss-of-function mutations in the BX-C region, the homeotic transformation converts posterior segments towards the anterior segments. However, the loss-of-function mutations in the most of the homeotic genes in ANT-C region lead to posterior transformations. Sex comb reduced (Scr) is different from other homeotic genes in ANT-C. The polarity of transformation in Scr mutants is altered. T1 is transformed T2 which is posterior transformation but the labial segment is partly transformed into maxillary segment anterior to the labial. It has been shown that Scr overexpression converts T2 and T3 towards anterior T1. Sometimes homeotic gene products have highly specific functions. For example, Scr by itself can direct the formation of salivary glands through a network of genes. Scr is found in prothoracic (ectoderm) region and in mesoderm of midgut. Scr is expressed in a band of cells in the subesophageal region of the ventral

ganglion (Glicksman and Brower, 1988). It is expressed in the CNS in parasegments 2 and 3 (Goran and Kaufman 1995).

The ftz regulatory domain is embedded within the Scr regulatory domain. The chromatin boundary SF1, present between the genes Scr and ftz could help to separate the Scr promoter from the ftz enhancers. The boundary would also help to protect the ftz gene from the silencing by Scr PRE. In the anterior half of parasegment 2 there are cells that have expression of Scr off but have ftz on. This means that during embryogenesis in those cells the ftz regulatory domain is independent and active. In recent studies by our lab (Mo Li thesis) it has been observed by Chromosome Conformation Capture (3C) experiments that SF1 interacts with boundary like elements between the ftz regulatory domain and the Scr distal enhancer and PRE. This interaction between SF1 and SF1 partner might allow looping of the ftz regulatory domain separating it from the influence of spread of homeotic silencing. In our lab, previous lab members have assayed SF1 and SF1 subfragments for their boundary functions. They showed SF1 had non-overlapping boundary activities. SF1 and SF1b had strong embryonic enhancer blocking activity while SF1, SF1a and SF1c had strong CPE blocking activity in the adult eye. To differentiate between these two activities I tested the enhancer blocking activity in the same stage and tissue as the CPE blocking activity. We observed that the SF1 has a weak enhancer blocking ability in the adult eye. I found that the SF1 enhancer blocking activity is weaker than the suHw enhancer blocking activity in the adult eye. Thus, we reported that SF1 is a developmental stage specific enhancer blocker (Majumder, Roy et al 2009). This provides new insights into understanding the role of SF1 in between Scr and ftz. Perhaps during embryogenesis a stronger SF1 enhancer blocking activity is required, to

establish the separation of the active ftz regulatory domain from the surrounding inactive Scr regulatory domain. However, in the adult stage SF1 might no longer require the high enhancer blocking ability since the specific function of the enhancers in that region have already been established. But, SF1 CPE blocking activity might be present in the adult stages because SF1 could play a role in delineating looped domains in this complex regulatory region. Therefore, SF1 plays an important part in the dynamic process of chromatin domain organization.

When we compare the mechanism of SF1 CPE blocking activity to the suHw CPE blocking activity we observe some variations. For instance, both SF1 and suHw have strong CPE blocking activity but the eye color distribution is markedly different between them. SF1 CPE blocking activity produces 70% lines with 3.0-4.0 eye color range while SuHw CPE blocking activity produces 61% lines with 4.0-5.0 eye color range. Therefore, SF1 might have a repressive effect while suHw might have an activating effect. Although two suHw elements in tandem cancel each other in an enhancer blocking assay, 2x suHw flanking a gene can still block CPE activity. The eye color distribution for 2x suHw CPE blocking activity is significantly different from the unprotected control, but there could be partial cancellation. The activating effect of SuHw activity alone cannot explain the eye color distribution pattern (Figure 3.2).

5.3: SF1 BOUNDARY TRANS-ACTING FACTORS

Previous studies with SF1 has shown that SF1 contains GAGA factor binding sites. The enhancer blocking function of SF1 depends on the GAGA factor but not the CPE blocking function (Majumder, Roy et al 2009). Interestingly, both Fab 7 and SF1

boundary activity require GAGA factor which belongs to the trithorax group of factors. The recent studies in our lab have shown that boundary activities also depend on the components of the Nucleosome Remodelling Factors (Li, Belozerov and Cai, in press). Mutations in the nurf 301/ebx and iswi affect the SF1 boundary function. Double knockdown of these two factors resulted in marked decrease in SF1b enhancer blocking function in S2 cells.

We conducted a genetic screen in order to identify factors that are involved in the SF1 or SF1b enhancer blocking function. We screened for the effect of haplo-insufficiency of genes on the SF1 function. From two independent deficiency screens we found that the deficiency 490 produced suppression of SF1 boundary activity. And within this region we found several subdeletions that affected the SF1 activity. Subdeletion like 7799 suppressed SF1b function. However, subdeletions like 7501, 7502, 9185 produced enhancement of SF1b insulator activity. The preponderance of potential candidates for SF1 modifiers in the 490 region also points to certain known challenges in this form of screen. Some subdeletions are generated by P-element deletion by Exelexis and they sometimes have second site deletions. In that case some of these candidates might be false negatives. Also the changes caused by the subdeletions might be due to alterations in chromatin organization which points to cis- alterations rather than effects of trans-factors. However, the abundance of modifiers from this screen can also be explained biologically. As we have seen from my previous discussion that SF1 boundary, like other boundaries, is involved in the formation of higher chromatin structures. Their mechanism of function is also dictated by the regional regulatory network. In recent years many studies have shown that signaling pathways in disease states affect the chromatin

architecture and not just the gene transcription. Since this is a dosage sensitive screen, we could potentially observe the effect of various factors involved in signaling pathways that ultimately affect chromatin organization.

The screen of the P-inactivated lines showed that there are several mutant genes that affect the SF1 boundary function. A challenge with P-inactivation lines is that they might be just P-insertions and not P-inactivations of the genes. However, very few showed any significant change (Table 4.3). Another challenge for observing effects of these mutants is the possibility of ectopic expression. Since we tested for boundary specific and SF1 specific effects, we found that 7799 is a good potential candidate for suppressor of SF1 function. We also found that P-inactivated CG34381 within 7799 is a potential suppressor of SF1 boundary activity. The P-insertion is in the 3rd exon of CG34381. No data on expression pattern is available. CG34381 is described as a G-protein coupled receptor (GPCR) and a neuropeptide receptor. These receptors are the initial step in any signaling cascade and are the first to receive external signals. One can speculate that this receptor signaling might ultimately affect SF1 chromatin boundary formation and regulation of chromatin domains (Model A). Studies have shown that GPCR signaling is involved in chromatin modification. Stimulation of a certain GPCR induced the translocation of β-arrestin 1 to the nucleus. This facilitated the recruitment of histone acetyltransferase which in turn activated gene transcription (Kang et al 2005). We found that in the mutant CG34381 the endogenous Scr gene expression pattern is affected. There is a spread of the Scr expression pattern. This might be due to the fact that SF1 enhancer blocking function is reduced in these lines. This might lead to the higher expression of Scr due to the activation by non-specific enhancers (Model B).

In the future this project will require confirmation of the SF1 modifier genes by gene knock-out studies. Gene knockout in the fly body would be a more substantial confirmation of the gene activity than dsRNA mediated knock-down in S2 cells. Besides, the other potential candidate like CG34380 could be pursued since it is a significantly strong enhancer. Also more P-inactivation screen can be conducted. For instance, Kruppel H1 can be tested and further confirmation steps can be followed as above. The rescue experiments for the gene mutants can also be performed for modifier candidate genes. Though deficiency screens cover large genomic regions, the method of screening is bulky and inconclusive. Thus, observing mutant phenotypes of genes is very necessary. These screens have revealed a huge number of modifier candidates. The major part of the future work would predominantly deal with confirming and understanding the function of the candidate genes.

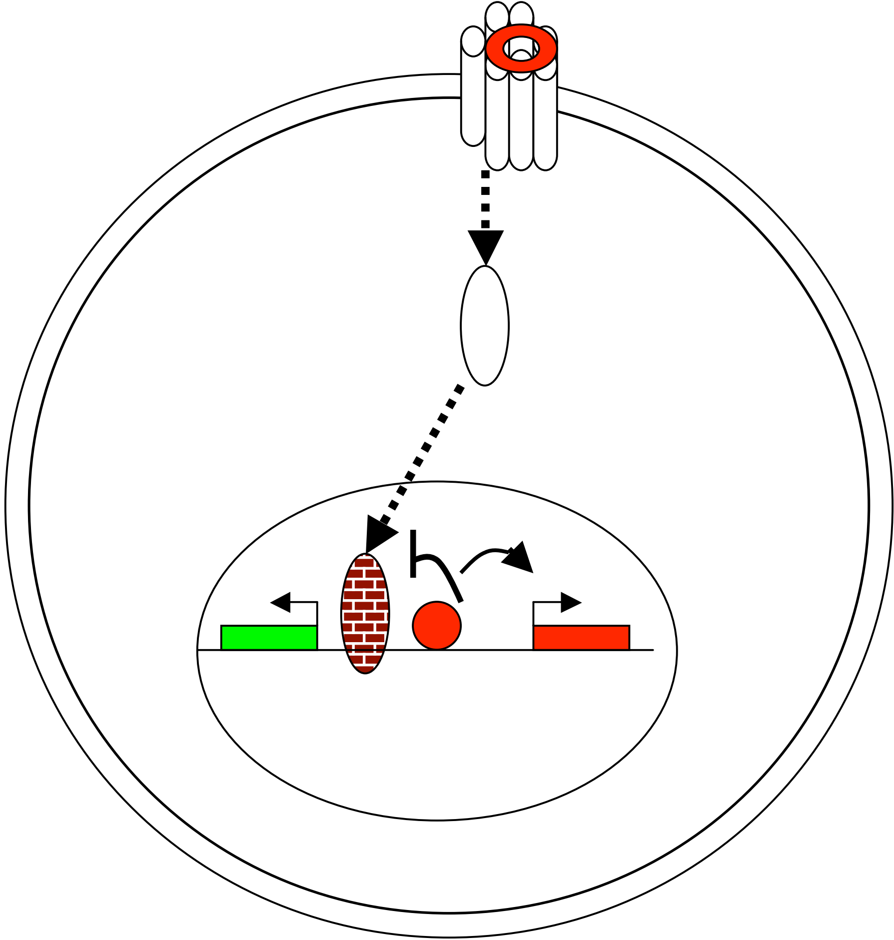
MODEL:

The following model shows a schematic of the candidate modifier gene (CG34381) function leading to the regulation of SF1 chromatin boundary activity.

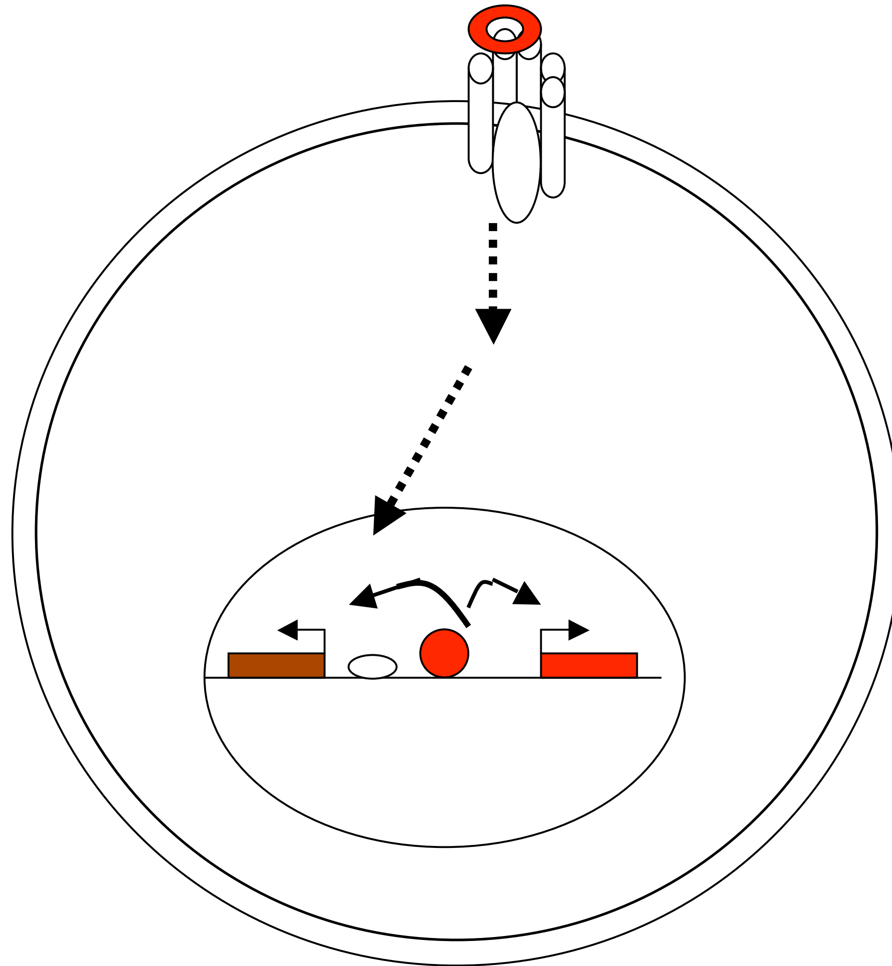
A. Simple diagram to show the activation (red circle) of CG34381(GPCR) (white cylinder) which leads to the signaling cascade that finally leads to strengthening of the SF1 boundary (oval brick pattern) and Scr (green box) and ftz (red box) genes have independent regulatory domains.
B. In CG34381 (GPCR) mutant the GPCR does not activate the downstream factors which leads to weakening of the SF1 boundary leading to non-specific activation

of Scr (brown box) reflecting the spread of Scr expression in the CG34381 mutants.

Model A



Model B



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