DEVELOPMENT OF A REPORTER GENE ASSAY TO IDENTIFY CONTROL ELEMENTS REQUIRED FOR DOSAGE COMPENSATION IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Dosage compensation (equalisation of X-linked gene products) occurs in *Drosophila melanogaster* by a two-fold transcriptional increase of X-linked gene expression in the male. This involves the binding of four proteins, MSL-1, MSL-2, MSL-3 and MLE (collectively known as the MSLs), to hundreds of sites along the length of the male X. The MSLs are thought to recruit MOF, a histone acetyl transferase, which facilitates the increase in transcriptional activity of X-linked genes. The DNA sequences required to target the MSL complex to the X chromosome (known as dosage compensation regulatory elements, or DCREs) remain elusive, despite numerous attempts over the last ten years to identify them. DCREs are thought to be present at multiple sites along the length of the X chromosome, as antibodies to the MSLs bind to hundreds of sites along the X, and autosomal genes transduced to the X usually become dosage compensated.

The first objective of this study was to develop a reporter gene assay to screen for DCREs that would minimise problems previously encountered. A construct consisting of the constitutive *armadillo* promoter fused to the *lacZ* reporter gene (called *arm-lacZ*) was flanked by insulator elements which block the repressive effects of the autosomal chromatin environment. Fragments of X-linked DNA were inserted upstream of the *armadillo* promoter with the premise that males carrying one copy of an autosomal insertion of this construct would express twice the level of β-galactosidase as females. Transgenic flies carrying autosomal insertions of X-linked fragments plus *arm-lacZ* were generated and one dose males and females were assayed for β-galactosidase activity using a spectrophotometric assay. In all cases, males and females expressed the same level of *lacZ*. This suggests that no DCREs that could confer dosage compensation onto *arm-lacZ* were present in the X-linked fragments. *arm-lacZ* is capable of being dosage compensated as males and females carrying one copy of an X-linked insertion of *arm-lacZ* produce a 2:1 male to female ratio. This implies that DCREs of the 'strength' required to dosage compensate *arm-lacZ* are rarer than previously thought.

A second method of dosage compensation that is independent of the MSLs is thought to occur in *Drosophila*. The X-linked gene *runt* is dosage compensated in the absence

of the MSLs. It is possible that *runt* is sex specifically regulated by the female specific *Sex lethal* protein (*Sxl*). *Sxl* down-regulates *msl-2* in females by binding to $(U)_8$ or $A(U)_7$ sequences in the *msl-2* 5' and 3' untranslated regions (UTRs) of the mRNA. *runt* mRNA contains three *Sxl* binding sites in its 3'UTR, as do 20 other X-linked genes. The second objective of this project was to determine if *Sxl* could down regulate a gene in females, purely by the addition of three *Sxl* binding sites to the 3'UTR. *Sxl* binding sites were inserted into the 3'UTR of *arm-lacZ* in the form of a 40 bp synthetic linker containing three of the sites, and also as a 170 bp fragment from the *runt* 3'UTR. β-galactosidase assays of flies carrying the *Sxl* binding sites from *runt* showed that males expressed an average of 1.31 to 1.46 times the level of *lacZ* than females. This shows that *Sxl* can down-regulate a gene if there are *Sxl* binding sites in its 3'UTR, however, to achieve two-fold regulation, additional factors may be required, or topologically, the sites may not have been in the right position in the 3'UTR for optimal activity of *Sxl*. Flies carrying the synthetic linker expressed the same level of β-galactosidase in both sexes which suggests that either additional elements within the 3'UTR are required, or that the spacing between the sites is critical for the action of *Sxl*.

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ABBREVIATIONS

ß	beta
Δ	delta
λ	lambda
bp	base pairs
°C	degrees Celsius
dNTP	dinucleotide triphosphate
DNase	deoxyribonuclease
DNA	deoxyribonucleic acid
F	female
g	gram
kb	kilobase pairs
mRNA	messenger ribonucleic acid
µ	micro
m	milli
M	male
M	molar
min	minute
nm	nanometer
PCR	polymerase chain reaction
RNase	ribonuclease
RNA	ribonucleic acid
rpm	revolutions per minute
UV	ultra violet
U	units
UTR	untranslated region
V	volts
v/v	volume per volume
w/v	weight per volume

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1. INTRODUCTION

1.1 OVERVIEW OF DOSAGE COMPENSATION

Dosage compensation is the process by which the expression of X-linked genes is regulated to equalise the level of expression from the single male X chromosome with that of the two female X chromosomes. Different organisms have evolved unique mechanisms of dosage compensation. In mammals, one of the two female X chromosomes is randomly inactivated early in development to equal expression of the single male X (Lyon, 1961). In *Caenorhabditis elegans*, gene expression of the two female X chromosomes is down regulated to equal expression of the single male X chromosome. (Hsu and Meyer, 1993). Dosage compensation does not exist in all animals. Both birds and butterflies, in which the female is the heterogametic sex (ZW), do not appear to exhibit dosage compensation (Baverstock, *et. al.* 1982; Johnson and Turner, 1979).

1.2 DOSAGE COMPENSATION IN DROSOPHILA

1.2.1 Dosage Compensation Involves Transcriptional Up Regulation.

In *Drosophila*, dosage compensation is achieved by transcriptionally up regulating genes on the male X chromosome to equal expression from the two female X chromosomes. Early support for this came from Offermann's (1936) observation that in polytene chromosome spreads, the male X chromosome appears much wider and more diffuse than that of the female, indicating an increase in gene expression. Subsequently, Mukherjee and Beerman (1965) demonstrated that incorporation of ^{3}H uridine into nascent salivary gland transcripts was significantly higher in the single male X chromosome than one female X.

1.2.2 Dosage Compensation Requires *trans*-acting Proteins

It can be rationalised that a mutation in a gene responsible for dosage compensation would result in male lethality due to a dramatic decrease in male X-linked gene expression. Belote and Lucchesi (1980a) systematically screened for genes on the 2nd and 3rd chromosomes required for dosage compensation by ethyl methanesulfonate (EMS) mutagenesis. Three new male-lethal recessive genes *male-specific lethal one* (*msl-1*), *male-specific lethal two* (*msl-2*) and *maleless (mle)* were discovered. Male-lethal mutants of *mle* had previously been isolated from natural populations of *D. melanogaster* (Fukunaga *et al.*, 1975; Golubovsky and Ivanov, 1972). These three genes, plus the subsequently found *male-specific lethal three* (*msl-3*) (Lucchesi, 1982) were collectively named the *male-specific lethals* or *msls*. Males mutant in any of these genes die as late larvae or early pupae but there is no discernible negative effect on females (Belote and Lucchesi, 1980b; Belote, 1983).

All four *msls* are required to give the diffuse chromatin appearance characteristic of the male X (Belote and Lucchesi, 1980a). Males homozygous for *mle*, *msl-1* or *msl-2* show reduced levels of X linked enzymes, whereas autosomal enzymes are unaffected (Belote and Lucchesi, 1980a).

The MSL proteins bind to hundreds of site along the entire length of the male X chromosome (Kuroda *et al.*, 1991; Palmer *et al.*, 1993). Co-immunolocalisation experiments show that the MSLs are present at the same sites on the X chromosome (Bone *et al.*, 1994) and the wild type banding of each MSL protein is dependent on the presence of the other three MSLs (Bashaw and Baker, 1995; Gorman *et al.*, 1993; Hilfiker *et al.*, 1994; Kelley *et al.*, 1995 and Palmer *et al.*, 1994;) suggesting they form a multicomponent complex. Furthermore, MSL-1 and MSL-2 co-immunoprecipitate *in vivo* (Kelley *et al.*, 1995), providing direct evidence for a physical interaction.

1.3 THE MALE-SPECIFIC LETHALS

The *msl* genes have all recently been cloned. The MSL-1 protein does not contain any motifs characteristic of DNA binding proteins (Johnson and McKnight, 1989). It does, however, contain two highly acidic regions in the N-terminus consisting of aspartate and glutamate clusters which show homology to proteins involved in transcription and chromatin modelling (Palmer *et al.*, 1993). These acidic regions could provide a structure for interaction with histones to mediate nucleosome assembly or release to promote changes in chromatin structure and transcription (Turner *et al.*, 1992). MSL-1 protein is present in *mle* and *msl-3* mutants, but is drastically reduced in *msl-2* mutants, suggesting that MSL-2 may positively regulate the level of MSL-1. The absence of MSL-2 in females could prevent the stable accumulation of MSL-1 (Palmer *et al.*, 1994). MSL-1 contains regions rich in proline, serine, threonine and glutamate, (PEST sequences) which are associated with rapidly degraded protein (Palmer *et al.*, 1993).

MLE contains motifs characteristic of DEAH RNA helicase proteins (Schwer and Guthrie, 1989). 50% amino acid identity is shared between MLE and human RNA helicase A (RHA) which mediates complex formation between CBP and RNA polymerase II (Nakajima *et al.*, 1997). After recruitment of the CBP complex, RHA is proposed to unwind promoter DNA to allow access of transcriptional activators (Nakajima *et al.*, 1997). As the bovine homolog of MLE is a DNA helicase (Zhang *et al.*, 1994) the possibility exists that MLE too may possess DNA helicase activity. Preliminary *in vitro* experiments by Nakajima and Montminy (unpublished data cited by Nakajima *et al.*, 1997) have revealed an association between MLE and a 250 kDa CBP with histone acetylase activity. These results together provide evidence that MLE may be involved in initiation of transcription.

Treatment with ribonuclease strips MLE from polytene chromosomes, but does not affect binding of MSL-1 and MSL-2 (Richter *et al.*, 1996). MLE could potentially interact with processed RNA or with nascent transcripts to increase the rate of transcription by increasing the rate of elongation. Alternatively, MLE could catalyse the movement of the MSL complex along nascent RNA (Henikoff and Meneely, 1993). Bashaw and Baker (1996) argue that MLE cannot interact exclusively with

nascent transcripts as it remains associated with the male X throughout mitosis (Lavender *et al.,* 1994). In an *msl-1*, *msl-2* or *msl-3* mutant background, MLE does not bind the X chromosome but MLE protein is still present, indicating that it requires the other MSLs for binding, but not for regulation of its expression (Gorman *et al.,* 1993).

MSL-3 contains two chromatin organisation modifier (chromo) domains which are 30-50 aa regions that are conserved in several eukaryotic chromatin binding proteins such as Polycomb and Drosophila heterochromatin protein 1 (HP1). Chromodomains has been implicated in delivering positive and negative transcriptional regulators to their chromatin targets (Koonin *et al.,* 1995).

MSL-2 contains a RING finger (Zhou *et al.,* 1995; Kelley *et al.,* 1995, Bashaw and Baker, 1995) which is a C_3HC_4 zinc finger common to a large group of proteins that is thought to be a DNA binding domain via which the MSLs may be targeted to the X chromosome (Lovering *et al.,* 1993).

1.4 HISTONE ACETYLATION AND DOSAGE COMPENSATION.

A specific isoform of histone H4 acetylated at lysine 16 (H4Ac16), is also predominantly associated with the male X chromosome. (Turner *et al.,* 1992). Its distribution along the X is strikingly similar to that of the MSLs, and the presence of H4Ac16 is dependent on the wild type binding of all four MSLs (Bone *et al.,* 1994). This suggests that the mechanism of dosage compensation involves histone acetylation through association with the MSL proteins.

1.4.1 Histone Acetylation and Transcriptional Activity

Histone acetylation has been linked with transcriptional activation and cell cycle transit (reviewed by Turner, 1991). Neutralisation of positive charges by acetylation of specific amino acids is thought to alter interactions between the DNA and histones which may allow access of transcription factors to their DNA targets. The acetylation of ε- amino groups of lysine residues of core histones is most strongly correlated with transcriptional activity. The most highly acetylated form of histone H4 in the

nucleosome core possesses the highest affinity for the transcription factors USF and Gal4-AH (Vettese-Dadey *et al.*, 1996).

The *Saccharomyces cerevisiae* mating type (MAT) locus (Johnson *et al.*, 1990) is presumed to be controlled by histone acetylation. When lysine 16 is mutated to an arginine, which retains the positive charge, regulation of the MAT locus is unaffected. However, when lysine 16 is mutated to a neutral amino acid such as glutamine (a change which mimics acetylation), derepression of the locus occurs (Hecht *et al.*, 1995).

A direct mechanistic relationship between histone acetylation and gene activation was revealed after the cloning of a histone acetyl transferase (HAT) from yeast (Brownell *et al.*, 1996) HAT A shares a striking 60% sequence similarity with Gcn5 (Georgakopolous *et al.*, 1995). Gcn5p is putative transcriptional adaptor which interacts with enhancer binding factors (Horiuchi *et al.*, 1995) and has been implicated in forming a bridge between upstream activating sequences and basal transcriptional machinery in yeast (Guarente *et al.*, 1995). Gcn5p also displays HAT activity in vitro (Brownell *et al.*, 1996).

The transcriptional co-activator p300/CBP has also been identified as a HAT. When the oncogenic protein EIA binds to p300/CBP, normal gene expression is altered and cell proliferation occurs (Ogryszko *et al.*, 1996). Misdirected histone acetylation caused by a chromosomal translocation of p300/CBP has been associated with the uncontrolled cell growth of acute myeloid leukaemia (Borrow *et al.*, 1996).

1.4.2 MOF - A Putative Histone Acetyl Transferase

A HAT that is crucial for dosage compensation has been discovered. A fifth male lethal gene, MOF (males absent on the first) was isolated from an EMS screen for mutations causing male specific lethality. Males mutant for *mof* die as third instar larvae and association of MSL-1, MSL-2 and MLE with the male X is reduced and H4Ac16 is absent (Hilfiker *et al.*, 1997).

The 827 amino acid protein contains a 250 amino acid signature motif for the acetyl coenzyme A binding site which is common to many acetyl transferases including

histone acetyltransferase I of yeast (Kleff *et al.,* 1995) and p300 CBP-associated factor P/CAF (Yang *et al.,* 1995). The *mof* mutation results from substitution of a highly conserved glycine residue with an aspartate. Mutation of the corresponding glycine residue in the human spermidine/spermine acetyl transferase abolishes enzyme activity (Lu *et al.,* 1996) providing strong evidence that the wild type MOF protein displays histone acetyl transferase activity.

Given the link between histone acetylation and transcription, it is tempting to speculate that acetylation of histone H4 at lysine 16 by MOF leads to hypertranscription of the male X.

1.5 NON CODING RNAS ARE INVOLVED IN DOSAGE COMPENSATION

The mutants *roX1* and *roX2* were serendipitously isolated in an enhancer detector screen for mushroom body expression of *lacZ* in the brain (Amrein and Axel, 1997; Meller *et al.,* 1997). Both of the genes are X-linked and lack significant open reading frames and their expression is restricted to the male nucleus, suggesting they may encode non-coding RNAs (Amrein and Axel, 1997; Meller *et al.,* 1997). The expression of *roX1* and *roX2* is dependent on the dosage compensation system as neither are expressed in flies mutant for any of the *msls*. Furthermore, expression of *msl-2* in females induces expression of both *roX* RNAs (Amrein and Axel, 1997; Meller *et al.,* 1997). *In situ* hybridisation of *roX1* probes to male third instar larval salivary glands reveals a subcellular localisation of *roX1* RNA identical to that of *msl-2* which binds to the X chromosome (Meller *et al.,* 1997). This 'painting' of the X is reminiscent of that of *Xist* RNA which coats the inactive mammalian X chromosome. *Xist* is expressed from the X-inactivation centre of the inactive X and is though to 'spread' along the one of the female X chromosomes, remodelling chromatin to form a transcriptionally inactive RNA-Barr body complex (Lee *et al.,* 1996). In situ hybridisation of *roX1* to chromosomes of which the region containing *roX1* was transposed to the Y or 2nd chromosome revealed binding of *roX1* to the transposed X-linked chromatin as well as the X chromosome (Meller *et al.,* 1997). That *roX1* RNA

can act in *trans* to recognise X-linked chromatin is in contrast to *Xist* RNA which spreads in *cis* along chromatin from the X inactivation centre.

It would be tempting to speculate that the *roX* RNAs are the same RNA component that is required for binding of MLE to the male X. However the role of the *roX* RNAs is unclear as male flies mutant for *roX1* exhibit normal MLE binding and are fully dosage compensated. Meller *et al.*, (1997) postulate that there are a family of non-homologous RNAs including *roX1* and *roX*2 that are functionally redundant. They propose that the binding of the MSL complexes to the X activates the male specific RNAs which facilitate a change in chromatin structure leading to hypertranscription of the male X. This may be by association with histone acetyl transferases or other chromatin constituents.

1.6 REGULATION OF DOSAGE COMPENSATION

The initial determinant of dosage compensation in *Drosophila* is the ratio of X chromosomes (X) to autosomes (A) (Maroni and Plaut, 1973). In females the X:A ratio is 1.0, in males it is 0.5. This ratio determines the level of X chromosome transcription by controlling the expression of the gene Sex-lethal (*Sxl*) (Reviewed by Parkhurst and Meneely, 1994). The X:A ratio itself is determined by 'counting' genes termed numerator and denominators which are members of the helix-loop-helix (HLH) family of transcription factors (Parkhurst *et al.*, 1990). The numerators are a discrete number of genes encoded on the X that are expressed at a two fold higher levels in XX embryos than XY. Autosomally encoded denominators, which are present in the same level in both sexes, compete with numerators for formation of heterodimers. In males, the elevated level of denominators relative to numerators results in the formation of inactive numerator-denominator heterodimers preventing the initiation of *Sxl* transcription. In females more numerators are present relative to denominators, allowing the formation of active numerator-numerator heterodimers which activate *Sxl* expression from its early 'establishment' promoter (Sxl_{Pe}) (Keyes *et al.*, 1992). Both sexes then begin transcription from the late 'maintenance' promoter (Sxl_{Pm}). In males a truncated inactive protein is produced. In females, *Sxl* protein

produced from the early promoter directs splicing of the *Sxl* transcript to encode an active protein. Female splicing is maintained after Sxl_{Pe} is turned off by a positive autoregulatory loop (Bell et al, 1991).

In the absence of *Sxl* protein, male differentiation and X chromosome dosage compensation occurs. In females, *Sxl* protein binds to *transformer (tra)* precursor RNA, blocking the use of a splice acceptor site, which forces the splicing apparatus to use a downstream site. (Belote *et al.*, 1989) This female-specific *tra* RNA codes for functional TRA protein, which is required for female differentiation but not viability. Since loss of function *Sxl* mutants cause female lethality (Cline, 1978), and *Sxl* products are required continuously, it was expected that *Sxl* protein would bind to the RNA of at least one of the proteins required for dosage compensation. *Sxl* protein binds to poly uridine (polyU) tracts in mRNA, consisting of eight or more Us. The presence of an adenine residue immediately 5' of the polyU tract strengthens binding several fold (Samuels *et al.*, 1994).

msl-2 transcripts from the same ORF are present in both sexes, but MSL-2 protein is detected only in males (Bashaw and Baker, 1995; Kelley *et al.*, 1995 and Zhou *et al.*, 1995). A 133 nucleotide intron which contains two *Sxl* binding sites is present in the 5' UTR of *msl-2* RNA. This intron is spliced out in males but retained in females (Bashaw and Baker, 1995; Kelley *et al.*, 1995 and Zhou *et al.*, 1995). Four additional *Sxl* binding sites are present in the 3'UTR which is retained in both sexes. The presence of *Sxl* binding sites in the 3' and 5' UTR is essential for full repression of MSL-2 in females as mutation of either the 3' or the 5' sites results in a dramatic increase of MSL-2 in females (Bashaw and Baker, 1997; Kelley *et al.*, 1997). Double mutants in which all *Sxl* binding sites have been removed display only a modest increase in female MSL-2 indicating that the sites at each end function synergistically to repress MSL-2 expression. Bashaw and Baker (1997) suggest that the binding of *Sxl* protein at both ends alters the structure of the RNA by circularisation, thus preventing access of translational machinery.

Mutation of the splice junctions of intron in the 5'UTR does not prevent rescue of *msl-2* mutant males, therefore removal of the intron is not required for *msl-2* translation (Bashaw and Baker, 1997; Kelley *et al.*, 1997). It is possible the intron has

no biological significance except to provide *Sxl* binding sites in females. In *Drosophila virilis* the three 5' sites are not present within an intron (Bashaw and Baker, 1997), yet males still display dosage compensation (Bone and Kuroda, 1996; Marin *et al.*, 1996).

1.6.1 *Sxl* May Mediate a Second Method of Dosage Compensation

A second method of dosage compensation that is independent of the MSLs has been proposed to occur in *Drosophila*. The first evidence for this was that the female lethality of *Sxl* mutant females (Cline, 1978) (which has been presumed to result from a failure to repress expression of the *msls*) is not rescued by mutation of *msl-2*, *msl-1* or *mle*. (Uenoyama et al.; 1982) (*msl-3* has not been tested but should display the same phenotype because of the co-dependence of the *msls*). This suggests that *Sxl* controls some other vital process other than repression of the *msls* in females.

A probable target of *Sxl* protein is the X-linked gene *runt*. *runt* is required during the blastoderm stage for normal segmentation of *Drosophila* embryos (Nüsslein-Volhard and Wieschaus, 1980). Males with one dose of a hypomorphic *runt* allele produce the same degree of segmentation defects as a female with two doses, demonstrating that the gene is dosage compensated (Gergen, 1987). Normal dosage compensation of *runt* is dependent on *Sxl*, but mutation of *mle*, *msl-1* or *msl-2* does not alter the wild type expression level of *runt* (Gergen, 1987), indicating that it must be dosage compensated by a mechanism independent of the *msls*.

Examination of the *runt* transcript revealed three *Sxl* binding sites in its 3'UTR (Kelley *et al.*, 1995). In addition, the timing of *Sxl* expression in the female embryo overlaps with that of *runt* (Gergen, 1987), placing *Sxl* as a prime candidate for direct regulation of *runt*, probably by down regulation of translation or RNA stability. Unlike the full repression of *msl-2* by *Sxl*, to be dosage compensated appropriately, the level of *runt* protein must only be decreased by a factor of two. *Sxl* protein binds to six sites in the 3' and 5'UTR of *msl-2* mRNA to achieve full repression (Kelley *et al.*, 1997; Bashaw and Baker, 1997). As only three sites are present in the *runt* 3'UTR, *Sxl* should only decrease *runt* expression by half. This is supported by the results of transfection experiments (Bashaw and Baker, 1997). A reporter gene

construct containing the *hsp70* promoter and the *lacZ* coding sequence placed between the *msl-2* 3' and 5' UTRs was transfected into *Drosophila* SL-2 cells. Mutation of the three sites in the 3' UTR resulted in a 1.7 fold decrease in expression and mutation of the three sites in the 5' UTR resulted in a 2 fold repression of *lacZ*.

There are two models for the relationship between MSL mediated and *Sxl* mediated dosage compensation. The first is that *Sxl* controls 'early' dosage compensation in the embryo prior to 'late' dosage compensation mediated by the MSLs. The main source of evidence for this is that a group of genes, including *runt*, are expressed before the MSLs become functional (Polito *et al.*, 1990; Gergen and Wieschaus, 1986). *runt* and *Sxl* (transcribed from Sxl_{Pe}) mRNA is detected by mid stage four of embryogenesis but MSL binding to the X chromosome does not occur until the end of stage five (Gergen, 1987). Early dosage compensation by *Sxl* may have evolved to regulate genes that are transcribed before the MSLs become functional (Franke *et al.*, 1996).

The fact that *Sxl* may act to provide a means of dosage compensation for 'early' X-linked genes does not exclude it from playing a role in dosage compensation of 'later' X-linked genes. The second model is that *Sxl* and the MSLs act in parallel throughout development on separate sets of genes. By this model, some X-linked genes are upregulated in males by the MSLs, some are down regulated in females by *Sxl*, and some are not dosage compensated (see section 1.7.1). Evidence for this model was produced by a computer search for genes with three or more polyU stretches in their 3' UTRs. 20 genes were revealed, all of which (excluding *msl-1* and *msl-2*) were X-linked (Kelley *et al.*, 1995). Furthermore, there is a *Sxl* allele, Sxl^{f2}, that is fully functional in 'early' dosage compensation, such as in dosage compensation of *runt*, but defective in 'late' functions such as development and maintenance of female differentiation. If the only role of *Sxl* in dosage compensation is at the early stage, it would be expected that the female lethality caused by the allele would be rescued by *mle* or *msl-1* mutants. This is, however, not the case, (Bernstein and Cline, 1994; Lucchesi and Skripsky, 1981; Skripsky and Lucchesi, 1982) arguing that *Sxl* plays a vital role in dosage compensation during later development.

1.7 *CIS*-ACTING REGULATORY SEQUENCES

1.7.1 Dosage Compensation Regulatory Elements (DCREs)

Little is known about the *cis*-acting sequences present on the X chromosome with which the MSLs interact. These sequences will be referred to as dosage compensation regulatory elements or DCREs. At least some DCREs are probably small and tightly linked to specific genes as X-linked genes carried as transgenes on the autosomes usually remain at least partially dosage compensated in males (Ghosh *et al.*, 1989; Hazelrigg *et al.*, 1984; Krumm *et al.*, 1995; McNabb and Beckendorf, 1986; Pirrotta *et al.*, 1985).

In complementary experiments, where cloned autosomal genes are carried on the X chromosome, a doubling of their expression occurs in males (Spradling and Rubin, 1983), demonstrating that the sequences do not have to be an intrinsic part of the gene. When a larger autosomal fragment of several hundred kb containing the *Aldox* gene was translocated to the X, the expression of *Aldox* was not increased in males (Roerdanz *et al.*, 1976), suggesting that the *cis*-acting elements can confer transcriptional upregulation only onto nearby sequences, or that autosomal genes are bounded by insulating sequences which block hyperactivation. Support for the former idea is derived from the fact that not all genes on the X chromosome are dosage compensated. Non dosage compensated genes are scattered along the length of the X chromosome and can reside next to dosage compensated genes. *LSP1α*, an X-linked gene that codes for the alpha subunit of larval serum protein 1, is not dosage compensated and may have only recently been transposed to the X chromosome (Brock and Roberts, 1982). Two similar genes are present on the autosomes whose products may mask the differences in gene dosage between males and females (Roberts and Evans-Roberts, 1979). The expression of *LSP1α* is increased in males when the gene is relocated to ectopic X-linked sites, demonstrating that it is intrinsically capable of being dosage compensated (Ghosh *et al.*, 1989). This, and the fact that *LSP1α* lies adjacent to the dosage compensated *L12* gene (Ghosh *et al.*, 1992) lends support to the hypothesis that *cis*-acting control occurs over relatively small distances.

1.7.2 DCREs Have Not Yet Been Identified

The search for *cis-acting* sequences has, to date, proved fruitless. Most investigations have involved performing progressive deletions of X-linked transgenes carried on the autosomes in order to isolate a consensus sequence required for dosage compensation.

By this method, Levis *et al.* (1985) delimited the flanking region of the X-linked *white* gene sufficient for dosage compensation to 400bp upstream and 157bp downstream of the gene. Pirrotta *et al.*, (1985) further delimited the sequence to 210 bp upstream. When *white* gene, which was inserted into ectopic sites on the X chromosome, had sequences gradually removed from its 5' end there was also a corresponding progressive decline in dosage compensation (Qian and Pirrotta, 1995). On replacement of the whole promoter with the autosomal *hsp83* promoter, partial dosage compensation still occurred. Qian and Pirrotta (1995) concluded that DCREs appear to consist of multiple elements present in the promoter region, and at least some are in the coding or intronic regions of the gene, however, no consensus DCRE sequence could be identified.

A 1.9kb fragment containing the *Sgs-4* coding region remains dosage compensated when relocated to autosomal sites (McNabb and Beckendorf, 1986), but sequence comparison of its promoter with two autosomal alleles failed to highlight any base changes specific to the non compensated alleles (Hofmann and Korge, 1987).

Similarly, Krishnan and Ganguly (1995) compared the 5' regions of the *Arrestin B* gene which is autosomal in *Drosophila melanogaster* but X-linked in *Drosophila miranda.* Five tandem repeats of a TGGGCNR heptanucleotide and a 29 bp palindromic sequence were unique to *D. miranda* but it has not been determined whether these are the sequences that target the MSLs to the X chromosome in males.

1.7.3 Mono and Dinucleotide Repeats Correlate with Dosage Compensation

Two dinucleotide repeats, CA/TG_n and CT/AG_n, and one mononucleotide repeat, C/G_n, are present on the X chromosome at twice the level they are present on the autosomes. (Huijser *et al.*, 1987; Lowenhaupt *et al.*, 1989; Pardue *et al.*, 1987). The density of these >50 base pair sequences appears to be correlated with dosage

compensation. The left arm of the autosomal chromosome three of *D. melanogaster* has been translocated to the X chromosome in *D. pseudoobscura* and *D. miranda* and has acquired the ability to dosage compensate in these species (Pardue *et al.*, 1987). This new arm of the X chromosome also gained a higher density of CA/GT_n similar to that seen on other X chromosomes and the ability to dosage compensate appears to be correlated with the acquisition of high levels of CA/GT_n (Pardue *et al.*, 1987).

These repeats are all capable of adopting a non B form of DNA when subjected to negative supercoiling *in vitro* and may be involved in the adoption of a decondensed X chromatin structure (Lowenhaupt *et al.*, 1989). It is probable, however, that even if they do play a role in dosage compensation that they are not the recognition signals that distinguish the X chromosomes from the autosomes because they are present at significant levels on all chromosome arms. Furthermore, there is no direct evidence that any of these sequences have a role in dosage compensation.

1.7.4 Why Have No DCREs Been Identified?

The main limitation of searching for DCREs by assaying for dosage compensation of X-linked genes is that X-linked transgenes carried on the autosomes are generally only partially dosage compensated.

This may in some cases be attributed to technical difficulties associated with quantitating the level of gene expression by Northern Hybridisation and RNase Protection Assays. These methods may not be accurate enough to quantitate two fold differences in expression, and are not very reproducible. Other methods such as the spectrophotometric eye pigment assay used in the study of the *white* and *mini-white* genes is non-linear with respect to the rate of transcription. Transgenes at autosomal locations exhibit full dosage compensation, as males with one dose produced at least twice as much pigment as one dose females, however females with two doses display a two to three fold higher level of pigmentation than males with one dose (Hazelrigg *et al.*, 1984; Levis *et al.*, 1985; Pirrotta *et al.*, 1985). This discrepancy makes it extremely difficult to analyse whether full dosage compensation is occurring or not. Furthermore, many genes are expressed in a stage and/or tissue specific manner and

the level of expression may vary depending on the age of the fly. This makes it extremely difficult to compare expression between males and females.

Another obvious drawback is that the X-linked gene under study must remain intact enough that its product can be assayed. This means the sequence cannot be subdivided through the coding region, preventing further delimitation of the sequence containing the DCRE(s).

Apart from the discrepancies associated with technical difficulties, there are two hypotheses to account for many X-linked transgenes being only partially dosage compensated when transduced to autosomes. One is that there are insufficient DCREs present to confer full dosage compensation. An alternative hypothesis is that the autosomal chromatin environment compromises the ability of the transgene to be dosage compensated fully (Qian and Pirrotta, 1995). The use of insulator elements to flank the transgene supports this idea (Roseman *et al.*, 1995).

1.8 INSULATOR SEQUENCES ENHANCE EXPRESSION OF X-LINKED GENES AT AUTOSOMAL SITES

1.8.1 Suppressor of Hairy-wing Protects *mini-white* from Position Effects

The suppressor of Hairy-wing [su(Hw)] gene encodes a zinc finger protein that binds to a repeated motif which has the properties of an insulator region. It can disrupt enhancer function and protect *mini-white* against position effects (Roseman *et al.*, 1993). Transgenic flies carrying autosomal insertions of the X-linked *mini-white* flanked by su(Hw) binding regions exhibited complete or almost complete dosage compensation and in a su(Hw) mutant background, only incomplete dosage compensation resulted. su(Hw) is thought to protect *mini-white* from 'repressive autosomal position effects', possibly by separating *mini-white* into a topologically distinct domain which facilitates decondensation of modified chromatin (Roseman, 1995). Partial dosage compensation, therefore, seems to result from an inhibitory effect of the autosomal environment, rather than a lack of dosage compensation elements.

1.8.2 Specialised Chromatin Structures (SCS and SCS') are Domain Boundaries

Another class of insulator sequences are the Specialised Chromatin Structures (SCS and SCS'). In their natural state SCS and SCS' flank the *D. melanogaster* 87A7 heat shock locus. SCS and SCS' were identified as constitutive DNase I hypersensitive site whose nuclease cleavage pattern alters on activation of the heat shock genes (Udvardy *et al.*, 1985). They are sites of action for topoisomerase II in vivo after heat induction (Udvardy and Schedl, 1993) which is consistent with them acting as domain boundaries. A domain is a higher order chromatin structure which allows the activity of a gene to only be affected by its resident regulatory environment. It allows protection from regulatory effects of the surrounding chromosomal DNA by a domain boundary, thus a domain boundary can establish a domain of independent gene activity.

SCS and SCS' sequences placed on either side of the *white* gene protect it from positive and negative position effects *in vivo* (Kellum and Schedl, 1991). Furthermore, SCS and SCS' prevent enhancers from activating the *hsp70-lacZ* reporter gene when inserted between an enhancer and the *hsp70* promoter (Kellum and Schedl, 1992). The ability of subfragments of SCS to repress activation by the eye and testis enhancers of the white gene was tested. Deletion of sequences from some of the hypersensitive sites within SCS decreased enhancer blocking activity, but multimerisation of subfragments, each with partial activity, from one hypersensitive site restored full enhancer blocking activity (Vazquez and Schedl 1994). This suggests that the boundary effect is additive and dependent on a certain number of cis-acting SCS subfragments, rather than specific sequences.

1.9 RESEARCH OBJECTIVES

This study has two objectives. The main objective is to develop a new system which will be used to isolate DCREs. The second objective is to determine whether *Sxl* has the ability to dosage compensate genes throughout development.

1.9.1 A New Approach to Identifying DCREs

The first objective of this project is to develop a reporter gene assay which can be used to screen X chromosomal DNA for DCREs. This involves the use of the *E. coli lacZ* gene fused to the *armadillo* promoter (this fusion will be referred to as *arm-lacZ*). The *arm* promoter was chosen because it is constitutive and active in all tissues at all stages of development (Riggleman *et al.*, 1989; Vincent et al, 1994). *arm-lacZ* will be flanked by SCS and SCS' insulator elements. Portions of DNA from the *D. melanogaster* X chromosome will be placed immediately upstream of the armadillo promoter and microinjected into *D. melanogaster* embryos. Any X-linked sequences that contain DCREs will confer dosage compensation onto *arm-lacZ* in males, producing twice the level of *lacZ* activity in males than females.

There are three main advantages of this assay system over previous methods. Firstly, insulation of the construct will ensure that any DCREs present will confer full dosage compensation onto *arm-lacZ* which will not be perturbed by negative regulatory effects of the autosomal chromatin environment at the point of insertion. Secondly, the use of a constitutive, ubiquitous, promoter ensures that male to female ratios will not be affected by slight differences in age of the flies, or by tissue specific expression. Lastly, the use of a reporter gene allows subdivision of an X-linked sequence without affecting the expression of the gene.

The specific objectives are as follows:

1. To microinject *arm-lacZ* into *D. melanogaster* embryos and assay autosomal insertions to ensure equal activity in males and females. That is, one dose in males and one dose in females gives a 1:1 male to female ratio of β-galactosidase expression.
2. To generate and assay one dose males and one dose females with X-linked insertions of *arm-lacZ* to ensure the armadillo promoter responds fully to the dosage compensation machinery (A 2:1 male to female ratio will confirm that *arm-lacZ* can be fully dosage compensated).
3. To flank *arm-lacZ* with SCS and SCS′ insulators and microinject into *D. melanogaster*.

4. To assay autosomal insertions to ensure the addition of SCS and SCS' does not affect the 1:1 male to female ratio.

5. To clone ten portions of X-linked DNA upstream of the *armadillo* promoter and microinject each into *D. melanogaster*.

6. To assay one dose male and one dose female flies with autosomal insertions. Lines which produce a 2:1 male to female ratio contain DCREs within the X-linked DNA which can dosage compensate *arm-lacZ*.

1.9.2 Evaluation of the Role of *Sxl* in Dosage Compensation

The second objective of this study makes use of the *arm-lacZ* assay system to determine whether *Sxl* can dosage compensate X-linked genes in females throughout development, purely by the presence of *Sxl* binding sites in the 3 ' UTR of a gene.

The specific objectives are as follows:

1. To insert three *Sxl* binding sites into the *lacZ* 3 ' UTR of pHF11.

2. To microinject the construct into *D. melanogaster* and obtain three autosomal transformants.

3. To assay male and female transgenic flies for *lacZ* activity. If the ratio of β-galactosidase activity alters such that males express twice as much β-galactosidase activity as females, this will prove that *Sxl* has to capability to down-regulate an X-linked gene provided it contains three or more *Sxl* binding site in its 3 ' UTR.

2. MATERIALS AND METHODS

2.1 PLASMIDS AND COSMIDS

Plasmids and cosmids used in this study are described in Table 1.

2.2 BACTERIAL STRAIN

The bacterial strain used in this study is described in Table 1.

2.3 BACTERIAL MEDIA

2.3.1 Luria Broth (LB)

Luria Broth contained (g/l): tryptone, 10; yeast extract, 5 and NaCl, 5. For LB agar 25g/L of agar (Davis) was added to LB medium before autoclaving.

2.3.2 Terrific Broth (TB)

Terrific Broth contained (g/900 ml): tryptone, 12; yeast extract, 24 and 4 ml glycerol. 100 ml 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 was added after autoclaving.

2.3.3 SOB

SOB contained (g/l): tryptone, 20; yeast extract, 5; NaCl, 0.5 and KCl, 0.2. After autoclaving the media was supplemented with (g/l): $MgCl_2$, 0.2 and $MgSO_4$, 2.5.

2.3.4 SOC

SOC contained (g/L): tryptone, 20; yeast extract, 5; NaCl, 0.5 and KCl, 0.2. After autoclaving the media was supplemented with (g/L): $MgCl_2$, 0.2; $MgSO_4$, 2.5 and sterile glucose 3.6.

Table 1. Bacterial Strain, Plasmids and Cosmids

Bacterial Strain, Plasmid or Cosmid	Relevant Characteristics	Source
Bacterial Strain		
Esherischia coli		
DH5α	ϕ80d*lacZ*ΔM15Δ(*lacZ*YA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K-,m_K+) *pho*A *sup*E44 λ⁻ *thi*-1*gyr*A96 *rel*A1	Hanahan (1983)
Plasmids		
pUChsπΔ2,3	7.3 kb, pUC18 containing 3.6 kb P element coding sequences, *hsp 70* promoter and *ry* 3 ' flanking region	Rio and Rubin (1985)
pCaSpeR-arm-βgal	13.8 kb, pCaSpeR (pUC, *mini-white*, P-element 5 ' and 3 ' ends) containing 1.7 kb *Eco*RI/*Kpn*I *armadillo* promoter and 4.4 kb *lacZ* with SV40 3 ' UTR	J.P. Vincent[a]
pElba 6	5.2 kb, pBSII SK+ containing 1.8 kb SCS in *Xho*I site and 0.5 kb SCS ' in *Xba*I site	P. Schedl[b]
pCaSpeR-KN	7.9 kb, pCaSpeR with *Kpn*I and *Not*I sites added and *Hin*dIII and *Bam*HI sites removed	P. Schedl
pHF6	10.3 kb, pCaSpeR-KN containing 2.4 kb *Kpn*I/*Not*I SCS-SCS ' fragment from Elba 6	This study
pCaSpeR4	7.9 kb, pCaSpeR with additional *Xho*I (x2), *Stu*I, *Hpa*I (x3), *Sal*I, *Spe*I, *Sfi*I, *Not*I, *Sst*II, *Kpn*I sites with *Sst*I and *Eco*RI sites removed.	V. Pirrotta[c]
pHF7	7.9 kb, pCaSpeR4 with *Eco*RI site removed	This study
pHF8	7.9 kb, pCaSpeR4 with *Eco*RI and *Spe*I sites removed	This study
pHF9	10.3 kb, pHF8 containing 2.4 kb *Kpn*I/*Not*I SCS-SCS ' fragment from Elba 6	This study
pHF10	10.3 kb, pHF9 containing *Eco*RI-*Spe*I linker	This study
pHF11	16.36 kb, pHF10 containing 6.2 kb *Eco*RI/*Hin*dIII (partial fill in) *arm-lacZ* fragment	This study

Table 1. continued

p23E12 7.7	23.9 kb, pHF11 containing 7.7 kb *Eco*RI fragment from p23E12	This study
p23E12 4.4	20.6 kb, pHF11 containing 4.4 kb *Eco*RI fragment from p23E12	This study
p185B11 6.6	22.8 kb, pHF11 containing 6.6 kb *Eco*RI fragment from p185B11	This study
p185B11 5.0	21.2 kb, pHF11 containing 5.0 kb *Eco*RI fragment from p185B11	This study
p24F3 7.4	23.6 kb, pHF11 containing 7.4 kb *Eco*RI fragment from p24F3	This study
p154H3 17.5	33.7 kb, pHF11 containing 17.5 kb *Eco*RI fragment from p154H3	This study
p11E3 15.4	31.6 kb, pHF11 containing 15.4 kb *Eco*RI fragment from p11E3	This study
p11E3 6.2	22.4 kb, pHF11 containing 6.2 kb *Eco*RI fragment from p11E3	This study
p144A7 12.8	29.0 kb, pHF11 containing 12.8 kb *Eco*RI fragment from p144A7	This study
p123B12 9.0	25.8 kb, pHF11 containing 9.0 kb *Eco*RI fragment from p123B12	This study
pHF12	9.2 kb, pBSII KS- containing 6.2 kb *Pst*I-*Eco*RI *arm-lacZ* fragment	This study
pHF13	9.4 kb, pBSII KS- containing 6.2 kb *Pst*I-*Eco*RI *arm-lacZ* fragment with 170bp *runt* PCR product (*Sxl* orientation) in *Mfe*I site of *arm-lacZ*	This study
pHF14	9.4 kb, pBSII KS- containing 6.2 kb *Pst*I-*Eco*RI *arm-lacZ* fragment with 170bp *runt* PCR product (non *Sxl* orientation) in *Mfe*I site of *arm-lacZ*	This study
pHF15	9.2 kb, pBSII KS- containing 6.2 kb *Pst*I-*Eco*RI *arm-lacZ* fragment with 40 bp linker (*Sxl* orientation) in *Mfe*I site of *arm-lacZ*	This study
pHF16	9.2 kb, pBSII KS- containing 6.2 kb PstI-*Eco*RI *arm-lacZ* fragment with 40 bp linker (non *Sxl* orientation) in *Mfe*I site of *arm-lacZ*	This study
pHF17	16.53 kb, pHF11 containing 170 bp *runt* PCR product (*Sxl* orientation) in *Mfe*I site of *lacZ* 3 ' UTR	This study

Table 1. continued

pHF18	16.53 kb, pHF11 containing 170 bp *runt* PCR product (non *Sxl* orientation) in *Mfe*I site of *lacZ* 3 ' UTR	This study
pHF19	16.4 kb, pHF11 containing 40 bp linker (*Sxl* orientation) in *Mfe*I site of *lacZ* 3 ' UTR	This study
pHF20	16.4 kb, pHF11 containing 40 bp linker (non *Sxl* orientation) in *Mfe*I site of *lacZ* 3 ' UTR	This study

Cosmids

23E12	pLorist 6 containing genomic *D. melanogaster* DNA from region 1A of the X chromosome	*Drosophila* Genome Project[d]
185B11	pLorist 6 containing genomic *D. melanogaster* DNA from region 1EF of the X chromosome	*Drosophila* Genome Project
24F3	pLorist 6 containing genomic *D. melanogaster* DNA from region 1EF of the X chromosome	*Drosophila* Genome Project
154H3	pLorist 6 containing genomic *D. melanogaster* DNA from region 2A of the X chromosome	*Drosophila* Genome Project
11E3	pLorist 6 containing genomic *D. melanogaster* DNA from region 1B of the X chromosome	*Drosophila* Genome Project
144A7	pLorist 6 containing genomic *D. melanogaster* DNA from region 1F of the X chromosome	*Drosophila* Genome Project
123B12	pLorist 6 containing genomic *D. melanogaster* DNA from region 2A of the X chromosome	*Drosophila* Genome Project

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[c] V. Pirrotta, Department of Zoology, University of Geneva, Geneva, Switzerland

[d]Drosophila Genome Project, Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology - Hellas, Vassilika Vouton, 71110 Heraklion Crete, Greece

2.3.5 2 x YT

2 x YT contained (g/L): tryptone, 16; yeast extract, 10 and NaCl, 5.

2.3.6 Antibiotics and Media Additives

170 μg/ml of ampicillin or kanamycin was added to LB, LB agar (section 2.3.1), TB (section 2.3.2) and 2 x YT (section 2.3.5) when required. 25 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (Xgal) in dimethylformamide and 25 μg/ml of Isopropylthio-β-galactosidase (IPTG) were added to LB agar when required. 170 μg/ml of chloroamphenicol in ethanol was added to LB agar when required.

2.4 MAINTENANCE OF BACTERIAL CULTURES

For long term storage, 3 ml of LB (section 2.3.1) was inoculated with a loop of culture and grown overnight at 37°C. 700 μl of culture and 30 μl of sterile glycerol were combined in a sterile cryotube and stored at -70°C.

When required, a loopful of the culture was streaked out on an LB plate containing the appropriate antibiotic, incubated at 37°C overnight then kept two weeks at 4°C.

2.5 FLY STOCKS AND TRANSGENIC FLY STOCKS

Fly stocks and transgenic fly stocks used in this study are described in Table 2.

2.6 FLY MEDIA

2.6.1 Cornmeal Agar

6.4 g agar (Davis), 56 g cornmeal, 19 g yeast and 600 ml H_20 were combined and stirred over medium heat until boiling. 77 g sugar and 2 g methyl paraben dissolved in 20 ml 95% ethanol were added. The porridge was stirred and poured into 20 ml vials (Labserve), 100 ml Schott bottles or 60 x 15 mm tissue culture dishes (Corning or Falcon).

Table 2. *Drosophila melanogaster* Fly Stocks

Fly Strain	Use	Source
y w	Microinjection recipient strain	M. Ashburner[a]
w; In(3LR)TM3, Sb ry^{rk}e/In(3LR)TM6, Tb e	Determination of chromosomal linkage	M. Ashburner
y w; L^{2}/CyO,Cy pr cn^{2} y+	Determination of chromosomal linkage	M. Ashburner
w; Sp/CyO,Cy pr cn^{2}; ry^{506} Dr{P[ry+] Δ2-3}/In (3LR)TM6	Mobilisation of construct to new genomic sites	Drosophila Stock Centre[b]

Transgenic Fly Stocks

Fly Stock	Chromosomal Linkage	Source
arm-lacZ #1: {*w+*} *y w*	2nd	This study
arm-lacZ #2: {*w+*} *y w*	2nd	J.P. Vincent[c]
arm-lacZ #3: {*w+*} *y w*	2nd	J.P. Vincent
arm-lacZ X #1: {*w+*} *y w*	X	J.P. Vincent
arm-lacZ X #2: {*w+*} *y w*	X	This study
arm-lacZ X #3: {*w+*} *y w*	X	This study
HF11 #1 : {*w+*} *y w*	2nd	This study
HF11 #2: {*w+*} *y w*	3rd	This study
HF11 #3: {*w+*} *y w*	3rd	This study
HF11 X #1: {*w+*} *y w*	X	This study
123B12 9.0 #1: {*w+*} *y w*	3rd	This study
123B12 9.0 #1: {*w+*} *y w*	2nd	This study
144A7 12.8 #1: {*w+*} *y w*	3rd	This study
144A7 12.8 #2: {*w+*} *y w*	3rd	This study
185B11 6.6 #1: {*w+*} *y w*	2nd	This study
185B11 6.6 #2: {*w+*} *y w*	3rd	This study
185B11 6.6 #3: {*w+*} *y w*	4th	This study
11E3 6.2 #1: {w+} *yw*	2nd	This study
11E3 6.2 #2: {w+} *yw*	2nd	This study
185B11 5.0 #1 {w+} *yw*	2nd	This study
185B11 5.0 #3 {*w+*} *yw*	2nd	This study
11E3 15.4 #1 {*w+*} *yw*	3rd	This study

Table 2. Continued

11E3 15.4 #2 {*w+*} *yw*	3rd	This study
24F3 7.4 #1 {*w+*} *yw*	2nd	This study
23E12 7.7 #1 {*w+*} *yw*	2nd	This study
HF17 #1 {*w+*} *yw*	3rd	This study
HF17 #2 {*w+*} *yw*	2nd	This study
HF17 #3 {*w+*} *yw*	2nd	This study
HF19 #1 {*w+*} *yw*	3rd	This study
HF19 #2 {*w+*} *yw*	3rd	This study
HF19 #3 {*w+*} *yw*	2nd	This study

[a]M. Ashburner, Department of Genetics, Cambridge University, Cambridge, U.K.

[b]Drosophila Stock Centre, Bloomington, Indiana, USA

[c]J.P. Vincent, Laboratory of Molecular Biology, Cambridge University, Cambridge, U.K.

2.6.2 Formula 4-24 (Carolina Biological Supply Company)

7 ml of H_20 was added to 1.5g of Formula 4-24 in a vial and mixed.

2.7 MAINTENANCE OF FLY STOCKS

All stocks were kept in vials at 18°C and or 22°C. Every four weeks (for 22°C stocks) or six weeks (for 18°C stocks) flies were turned into new cornmeal agar vials (section 2.6.1). When in use, flies were bred in 100 ml bottles to expand the stock and kept at 25°C.

2.7.1 Setting Fly Crosses

Five virgin females (section 2.7.2) and five males were mated in a cornmeal agar vial (section 2.6.1). To expand the stock, approximately 20 virgin females and 20 males were mated in bottles. Adult flies were removed after one week. Progeny emerged three to five days later.

2.7.2 Collection of Virgin Females

Virgins were collected from bottles in which flies were beginning to eclose. The bottles were cleared of emerged flies and incubated at 25°C for six to eight hours. Female flies were then collected and held in vials until required. Eight hour old flies have not yet reached sexual maturity ensuring that the female flies collected will be virgins.

2.8 SYNTHETIC OLIGONUCLEOTIDES

Synthetic oligonucleotides used in this study are described in Table 3.

2.9 BUFFERS AND SOLUTIONS

All solutions were made up to the appropriate volume with milliQ H_20, autoclaved and stored at room temperature unless otherwise stated.

2.9.1 Solutions used for Gel Electrophoresis

2.9.1.1 10 x Gel Loading Dye

10 x gel loading dye contained Ficoll 400 20 % (w/v); EDTA (pH 8.0) 0.1 M and bromophenol blue 0.24 % (w/v). Gel loading dye was not autoclaved.

2.9.1.2 1 x TAE Buffer (Tris Acetate EDTA Buffer)

1 x TAE contained 40 mM Tris acetate 1.4 % (v/v) glacial acetic acid and 1 mM EDTA (pH 8.5).

2.9.1.3 1 x TBE Buffer (Tris Borate EDTA Buffer)

1 x TBE contained 89 mM Tris HCl; 89 mM boric acid and 2.5 mM Na_2EDTA.

2.9.2 Solutions used for Preparation of Plasmid or Cosmid DNA

2.9.2.1 GTE (Glucose/Tris/EDTA)

GTE contained 50 mM glucose; 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA (pH 8.0).

2.9.2.2 TE Buffer 10/1 (Tris EDTA Buffer)

TE Buffer 10/1 contained 10 mM Tris HCl and 1 mM Na_2EDTA.

2.9.2.3 TE Buffer 10/0.1

TE Buffer 10/0.1 contained 10 mM Tris HCl and 0.1 mM Na_2EDTA.

2.9.2.4 Phenol (Tris Equilibrated)

Phenol crystals were heated at 65 °C until melted. The bottle was filled with deionised H_20, shaken to form a fine emulsion and placed at 4 °C until the phases separated. The upper phase was removed and hydroxyquinoline was added to a final concentration of 0.1%. An equal volume of 0.5M Tris-HCl (pH 8.0) was added and mixed. After phase separation, the upper aqueous phase was removed. An equal volume of 0.1 M Tris-HCl was added, the solution mixed and the upper aqueous phase removed. This step was repeated until the aqueous Tris-HCl phase had a pH of 8. The phenol was overlayed with an equal volume of 0.1 M Tris-HCl (pH 8) and stored at 4 °C in a foil covered bottle. Phenol was not autoclaved.

Table 3. Synthetic Oligonucleotides

Oligonucleotide[a]	Sequence 5' to 3'	Use
Sequencing primers		
SCS1	ATCACAATCAGTTCCTGCGCAG	Sequencing of SCS-SCS' polylinker
Cspr4XbBm	TTCGAGGTCGACTCTAGAGGAT	Sequencing of SCS-SCS' polylinker
T7	GTAATAGGACTCACTATAGGGC	Sequencing of pHF13, pHF14, pHF15 and pHF16
Oligonucleotide Linkers		
ESp1	AATTCGGCAGCGCACTAGTC	Forms *Eco*RI-*Spe*I linker with ESp2
ESp2	AATTGACTAGTGCGCTGCCG	Forms *Eco*RI-*Spe*I linker with ESp1
SxlA	AATTCAAAAAAAATGCTAGCCAAA AAAAATACTAGTCAAAAAAAATC	Forms linker with SxlTop Top containing three *Sxl* binding sites
SxlTop	AATTGATTTTTTTTGACTAGTATTTT TTTTGGCTAGCATTTTTTTTG	Forms linker with SxlA containing three *Sxl* binding sites
PCR primers		
*Runt*1	GTCGAATTCCATAGCTAACTAGTTG TAACACTC	PCR amplification of 170 bp of *runt* 3' UTR
*Runt*2	GTTCCAATTGGCGCTAAAACTTATG GCTAGAC	PCR amplification of 170 bp of *runt* 3' UTR

[a] All oligonucleotides except T7 (Stratagene) were custom made from Life Technologies.

2.9.2.5 Potassium acetate (pH 4.8)

Potassium acetate contained 3 M KAc and 11.5 % glacial acetic acid. Potassium acetate was not autoclaved.

2.9.3 Solutions used for Transformation of DNA

2.9.3.1 DnD

DnD contained 1 M DTT; 90 % (v/v) DMSO and 10 mM KAc. DnD was filter sterilised and stored in 1 ml aliquots at -20° C.

2.9.3.2 K-MES

K-MES contained 0.5 M 2[N-morpholino]ethone sulphonic acid (MES). MES was adjusted to pH 6.2 with concentrated KOH. K-MES was not autoclaved and was stored at -20° C

2.9.3.3 Transformation Buffer (TFB)

TFB contained 10 mM K-MES; 45 mM $MNCl.4H_20$; 10 mM $CaCl_2.2H_20$; 3 mM $HCoCl_3$ and 100 mM KCl. TFB was filter sterilised and stored at 4° C.

2.9.4 Solutions used for Quantitation of DNA

2.9.4.1 1 x TNE Buffer

1 x TNE contained 0.1 M NaCl; 10 mM Tris-HCl and 1 mM EDTA (pH 7.4).

2.9.4.2 Working Dye Solution B

Working dye solution B contained 1 x TNE and 1.0 μg/ml Hoechst 33258 solution. Working dye solution B was not autoclaved.

2.9.5 Solutions for Colony Lifts and Hybridisation

2.9.5.1 100 x Denhardt's Solution

100 x Denhardt's solution contained (g/l): Ficoll 400, 10; Polyvinylpyrrolidone, 10 and BSA, 20. Denhardt's solution was stored at -20° C.

2.9.5.2 20 x SSC (Standard Saline Citrate)

20 x SSC contained 3 M NaCl and 0.2 M Na_3citrate.$2H_20$.

2.9.5.3 Salmon sperm DNA

Salmon sperm DNA was dissolved in H_20 at a concentration of 10 mg/ml. NaCl was added to 0.1 M. The solution was extracted with phenol and chloroform and the aqueous phase recovered. The DNA was sheared by passing it rapidly 12 times through an 18 gauge needle. After ethanol precipitation it was resuspended at a concentration of 10 mg/ml in H_20. The solution was boiled for 10 minutes and stored at -20°C in 1 ml aliquots. Immediately before use, the DNA was boiled for 5 minutes and chilled on ice.

2.9.5.4 APH (Aqueous Prehybridisation/Hybridisation Solution)

APH contained 5 x SSC; 5 x Denhardt's Solution; 1 % SDS (w/v) and 100 µg/ml denatured salmon sperm DNA. APH was not autoclaved.

2.9.6 Solutions for Detection with DIG

2.9.6.1 Neutralisation Solution (pH 7.4)

Neutralisation Solution contained 1.5 M NaCl and 0.5 M Tris-HCl.

2.9.6.2 Denaturation Solution

Denaturation Solution contained 0.5 N NaOH and 1.5 M NaCl.

2.9.6.3 Maleic Acid Buffer (pH 7.5)

Maleic Acid Buffer contained 100 mM maleic acid and 150 mM NaCl.

2.9.6.4 Washing Buffer

Washing Buffer contained maleic acid buffer plus 0.3% Tween. Washing buffer was not autoclaved.

2.9.6.5 Detection Buffer (pH 9.5)

Detection Buffer contained 100 mM Tris HCl and 100 mM NaCl.

2.9.7 Solutions for Microinjection

2.9.7.1 Injection Buffer (pH 6.8)

Injection buffer contained 0.1 mM NaH_2P0_4 ; 0.1 mM Na_2HP0_4 and 5 M KCl. Injection buffer was stored in 1 ml aliquots at -20°C.

2.9.8 Solutions for β-Galactosidase Assays

2.9.8.1 β-Galactosidase Assay Buffer

β-Galactosidase assay buffer contained 50 mM KH_2P0_4 and 50 mM K_2HP0_4. 1 mM $MgCl_2$ was added after autoclaving. β-galactosidase assay buffer was stored in 1 ml aliquots at -20°C.

2.10 PREPARATION OF PLASMID DNA

2.10.1 Large Scale Preparation of Plasmid or Cosmid DNA

1 ml of LB (section 2.3.1) was inoculated with a loopful of bacteria and grown for five hours with moderate shaking at 37°C. This was used to inoculate a 500 ml flask of LB, TB (section 2.3.2) or 2 x YT (section 2.3.5) supplemented with ampicillin (for plasmids) or kanamycin (for cosmids) (section 2.3.6) which was shaken for 16 to 24 hours at 37°C. Plasmid or cosmid DNA was prepared by following Unit 1.7.1 of Current Protocols in Molecular Biology (1989) Cells were harvested in GSA bottles by centrifugation at 6000 rpm in a Sorvall RC5C centrifuge and resuspended in 9 ml GTE (section 2.9.2.1). 1 ml of 25 mg/ml hen egg white lysozyme (Sigma) in GTE was added, the solution mixed by inversion and allowed to stand 10 minutes at room temperature to aid lysis. 20 ml of 0.2 N NaOH/1% SDS solution was added and mixed in gently by inversion until the solution became homogeneous and cleared. After standing 10 minutes, 10 ml of 5 M KAc (section 2.9.2.5) was added and the solution mixed gently by inversion until the viscosity was reduced and a thick white precipitation formed. The solution was incubated 10 minutes on ice and centrifuged at 13 000 rpm for 10 minutes at 4°C. The supernatant was poured through sterile cheese cloth or a 80 μm cell strainer (Falcon) into a sterile SS34 tube. 0.6 ml of isopropanol was added per 1 ml of filtrate collected, the solution mixed and left 10 minutes at

room temperature. The pellet was recovered by centrifugation at 11 500 rpm for 10 minutes at room temp in a Heraeus Labofuge. The supernatant was poured off and the pellet dried under vacuum in a SpeedVac Concentrator (Savant).

Purification of the closed circular plasmid and cosmid DNA was based on Unit 1.4.2 of Sambrook et al., (1989). The pellet was resuspended in 4 ml TE (section 2.9.2.2) and transferred to a 50 ml polypropylene tube (Falcon). 4.4 g caesium chloride and 0.4 ml of ethidium bromide were added and the solution mixed by inversion. After centrifugation at 4100 rpm for 5 minutes at room temperature, the clear red solution under the 'furry' precipitate was transferred to a 6 ml ultracentrifuge tube (Sorvall). The tube was filled with additional TE/CsCl/EtBr solution and crimped. Centrifugation was carried out at 55 000 rpm for 18 hours in a Sorvall Combi Ultracentrifuge. The closed circular plasmid band was removed from the ultracentrifuge tube with an 18 gauge needle, if necessary under UV light. The ethidium bromide was extracted with isoamyl alcohol (IAA). An equal volume of TE saturated IAA was added to the solution and shaken for two minutes. After the phases separated completely, the upper organic phase was removed. This was repeated until all the pink colour was removed from the aqueous phase. The DNA solution was diluted three fold in TE to prevent precipitation of the caesium chloride, and ethanol precipitated (section 2.11.3).

Alternatively, plasmid and cosmid DNA were prepared using the Qiagen Plasmid Maxi Kit with the Qiagen-tip-500 according to the manufacturers instructions. The following modification was employed: For all cosmids and plasmids over 16 kb, the volume of buffers P1, P2 and P3 was doubled to 20 ml to increase yields. The DNA yield still proved to be variable and was usually very low, therefore cosmids and large plasmids were purified using a caesium chloride/ethidium bromide gradient.

2.10.2 Small Scale Preparation of Plasmid DNA

Plasmid DNA preparations were carried out according to the method of Birnboim and Doly (1979) and Birnboim (1983). 3 ml sterile LB (section 2.3.1) was inoculated with a single bacterial colony and grown to saturation with moderate shaking at 37°C overnight. 1.5 ml of cells were centrifuged 1 minute at 13 000 rpm in a Heraeus

Biofuge 13. The supernatant was poured off and the pellet resuspended in 100 µl GTE (section 2.9.2.1). 200 µl 0.2 N NaOH /1% SDS was added and mixed by inversion and the solution was placed on ice for 5 minutes. 150 µl of 5 M KAc (section 2.9.2.5) was added then the solution was vortexed at maximum speed for 2 seconds. After standing on ice 5 minutes, the solution was centrifuged for 3 minutes at 13000 rpm to pellet cellular debris and chromosomal DNA. The supernatant was carefully pipetted into a new microcentrifuge tube and mixed with 800 µl absolute ethanol. Plasmid DNA was pelleted by centrifugation at 13000 rpm, washed with 1 ml 70 % ethanol then dried under vacuum for 5 minutes in a SpeedVac concentrator (Savant). The pellet was resuspended in 30 µl TE (section 2.9.2.2) and stored at -20°C.

High quality plasmid DNA for direct automatic sequencing and cloning was prepared using the Biorad Quantum Prep® Plasmid Miniprep Kit following the manufacturer's instructions.

2.11 PURIFICATION OF DNA

2.11.1 Purification of DNA after PCR

Following amplification by the Polymerase Chain Reaction, DNA was purified using the Qiagen QIAquick PCR Purification Kit according to the manufacturer's instructions.

2.11.2 Phenol/Chloroform Extraction of DNA

An equal volume of Tris equilibrated phenol (section 2.9.2.4) and chloroform were added to the DNA solution and shaken for two minutes. The solution was centrifuged at 13000 rpm for 5 minutes. The upper aqueous phase was transferred to a fresh microcentrifuge tube, and an equal volume chloroform was added. The solution was shaken and centrifuged as before. The upper aqueous phase was transferred to a fresh microcentrifuge tube and ethanol precipitated (section 2.11.3).

2.11.3 Ethanol Precipitation of DNA

1/10th volume of 3M NaAc and 2 volumes of absolute ethanol were added to the DNA solution and mixed. After 2 - 3 hours at -20°C or 30 minutes at -70°C the DNA

was pelleted at 13000 rpm for 15 minutes. The pellet was washed with 70 % ethanol, dried under vacuum for 5 minutes and resuspended in TE (section 2.9.2.2) or sterile H_20.

2.12 DETERMINATION OF DNA CONCENTRATION

2.12.1 Determination by Comparison to DNA Ladder

The Lambda *Hin*dIII/*Sst*II ladder was run alongside DNA of unknown concentration. The concentration was estimated by comparison to the intensity of bands of the ladder.

2.12.2 Spectrophotometric Determination of DNA Concentration

The DNA of unknown concentration was diluted in TE (section 2.9.2.2) and the absorbance measured at 260 nm after adjusting the spectrophotometer to zero with TE. An OD of 1 corresponds to 50 μg/ml of double stranded DNA, therefore the absorbance reading was multiplied by 50 and the dilution factor to give the DNA concentration in μg/ml.

2.12.3 Fluorometric Determination of DNA Concentration

The Hoefer Scientific TKO 100 Fluorometer was adjusted to zero against 2 ml of Working Dye Solution B (section 2.9.4.2). 2 μl of 100 μg/ml calf thymus DNA was added to 2 ml Working Dye Solution B to give a reading of 100. If the reading was not exactly 100 the scale was altered. 2 ml of sample DNA was added to 2 ml Working Dye Solution B and measured. The reading was the concentration of the DNA in ng/μl, i.e. DNA giving a reading of 100 has a concentration of 100 ng/μl.

2.13 AGAROSE GEL ELECTROPHORESIS

DNA was size fractionated by electrophoresis on agarose gels in 1 x TBE buffer (section 2.9.1.3). The concentration of agarose ranged from 0.5 %, to separate high molecular weight (> 10 kb) fragments, to 2 % which separated small (< 300 bp) fragments. The appropriate amount of agarose was added to 1 x TBE buffer and

microwaved until melted. When cooled to 50°C it was poured into a casting tray and allowed 15 - 45 minutes to set. DNA fragments containing 1 x loading dye were loaded into the appropriate wells alongside 10 µl of λ DNA digested with *Hin*dIII and *Sst*II or 10 µl of 1 kb ladder (BRL). Gel electrophoresis was carried out in a Horizon or Biorad minigel apparatus for 1 - 2 hours at 70 - 90 Volts. The gel was then stained for 15 - 30 minutes in 2 µg/ml ethidium bromide and destained in H_20. The gel was visualised under short wave UV light and photographed using Polaroid 667 film or the Gel Documentation System (Alpha Innotech).

2.13.1 DNA Extraction from Seaplaque Agarose

After the appropriate restriction digest, DNA was size fractionated on a 0.8 - 1% TAE (section 2.9.1.2) seaplaque agarose gel. The gel was stained in ethidium bromide and the desired fragment was excised with a scalpel under UV light. The DNA was initially purified from the agarose using the Promega Wizard™ PCR DNA Purification Kit (agarose method) according to the manufacturer's instructions. The DNA yields from this kit were extremely variable and often too low to be useful. The Qiagen QIAquick Gel Extraction Kit was then used with the following modification: An extra one minute centrifugation after the final wash was performed to remove residual wash buffer. This method yielded higher concentrations of DNA.

2.14 RESTRICTION DIGESTION OF DNA

2.14.1 Restriction Enzymes

All enzymes were from New England Biolabs (NEB) except *Asp*718 and *Sst*II from Boehringer Mannheim.

20 Units/µl: *Eco*RI, *Xba*I, *Hin*dIII, *Sac*II, *Pst*I

10 Units/µl: *Asp*718, *Sst*II, *Not*I, *Spe*I, *Cla*I, *Stu*I, *Mfe*I

5 Units/µl: *Nhe*I

2.14.2 Analytical Restriction Enzyme Digestion

Restriction enzyme digestions for analysis of plasmids following transformation (section 2.18) were performed in commercially prepared buffer using 2 µl of DNA and 10 units of the appropriate restriction enzyme(s) which constituted less than 1/10 of the final volume of the digestion. 0.1 µl of RNase was added to each digest, or 1 µl was added to a mastermix of buffer and enzyme when multiple digests were performed. When required by a specific enzyme, BSA was added to a final concentration of 0.1 mg/ml. Digestions were incubated at 37°C for 2-3 hours or overnight, in which case only 5 units of enzyme per reaction was used. 1 x gel loading dye (section 2.9.1.1) was added to each digest which was then size fractionated on an agarose gel (section 2.13).

2.14.3 Preparative Restriction Digestion

5 to 40 µg DNA was digested using 3 - 10 Units of the appropriate enzyme(s) per µg of DNA. 200 - 500ng of digested DNA was run on a gel to determine whether complete digestion has occurred. If not, more enzyme was added and the DNA was further digested overnight. When complete digestion had occurred, the enzyme was heat inactivated at 65°C for 20 minutes. If the enzyme could not be heat inactivated, a Phenol/Chloroform Extraction (section 2.11.2) was carried out.

2.15 AMPLIFICATION OF DNA BY THE POLYMERASE CHAIN REACTION

A mix was prepared on ice containing the following: 1 µl of 10 µM *runt*1 primer, 1 µl of 10 µM *runt*2 primer, 2 µl of 2 µM dNTPs, 2 µl of genomic DNA and 11.5 µl of sterile H_20. A second mix was prepared on ice containing the following: 2 µl of 10 x buffer (Mg^{2+}) and 0.5 µl of *Pwo* Polymerase (Boehringer Mannheim). The two mixes were combined on ice. A negative control containing no genomic DNA was also prepared. The reaction tubes were placed in a thermal cycler preheated to 95°C. The following cycles were used to amplify the DNA:

Cycle 1, denaturation at 94°C for 2 minutes; cycles 2-4, denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 45 seconds; cycles 5-34, denaturation at 94°C for 30 seconds, annealing/extension at 72°C for 1 minute; cycle 35, extension at 72°C for 5 minutes; hold at 4°C.

2.16 SUBCLONING

2.16.1 Filling in 5' Overhangs with Klenow

Following restriction enzyme digestion (section 2.14) and heat inactivation, 1 U/μg Klenow (NEB) and 33 μM of dNTPs were added to 50 μg/ml DNA. The solution was incubated at 25°C for 15 minutes then heat inactivated at 75°C for 10 minutes. Alternatively, if the restriction enzyme could not be heat inactivated, a phenol/chloroform extraction (section 2.11.2) was performed and the DNA resuspended at 50 μg/ml in 1 x EcoPol buffer (NEB) before addition of Klenow and dNTPs.

2.16.2 Removal of 3' or 5' Overhangs with Mung Bean Nuclease

Following restriction enzyme digestion (section 2.14) and heat inactivation, 1U/μg Mung Bean Nuclease (NEB) and 1 mM $ZnS0_4$ were added to 100 μg/ml DNA. The solution was incubated at 30°C for 30 minutes and heat inactivated at 75°C for 10 minutes. Alternatively, if the restriction enzyme could not be heat inactivated, a phenol/chloroform extraction (section 2.11.2) was performed and the DNA resuspended at 100μg/ml in 1 x Mung Bean Nuclease buffer (NEB) before addition of Mung Bean Nuclease and $MgS0_4$.

2.16.3 Removal of 3' Overhangs with T4 DNA Polymerase

Following restriction enzyme digestion (section 2.14) and phenol/chloroform extraction (section 2.11.2), DNA was resuspended in 1 x T4 DNA Polymerase buffer (Life Technologies). 1 Unit of T4 DNA polymerase (Life Technologies) and 0.1 mM dNTPs were added per μg DNA and incubated at 11°C at 20 minutes. The enzyme was heat inactivated at 75°C for 10 minutes.

2.16.4 Removal of 5' Phosphate Groups from Vector DNA

5' Phosphate groups were removed from vector DNA prior to ligation to prevent vector self religation. 1 Unit Calf Intestinal Alkaline Phosphatase (NEB) was added per pmol DNA ends and incubated for 30 minutes at 37°C then the enzyme was heat inactivated at 75°C for 10 minutes. Calf Intestinal Alkaline Phosphatase is active in all NEB buffers.

2.16.5 Addition of 5' Phosphates to Oligonucleotides by T4 Kinase

Synthetic oligonucleotides were ethanol precipitated (section 2.11.3) to remove ammonium ions which inhibit T4 kinase (Life Technologies). 0.5 µg of oligonucleotide was added to 1 x T4 kinase buffer, 1 mM ATP and 30 Units T4 Kinase. The solution was incubated at 37°C for 60 minutes then heated to 75°C for 10 minutes.

2.16.6 Annealing Oligonucleotides

Oligonucleotides were resuspended at 100 µmol/L in sterile TE (section 2.9.2.3). 25 µg of the two appropriate oligonucleotides was combined with NaCl to a concentration of 300 µM and the volume was made up to 500 µl with sterile H_20. The solution was heated to 80°C then removed from the heat and left until cooled to room temperature. If this method was not successful, oligonucleotides were ethanol precipitated (2.11.3) before being annealed. To assist reannealing, the solution was held for one hour at a temperature slightly below the Tm's of the oligonucleotides and then cooled.

2.17 DNA LIGATION

Ligations of DNA fragments were carried out in 1 x ligase buffer with 1 µl of T4 DNA ligase (New England Biolabs) in a final volume of 10 - 25 µl. The ligation mixture contained 200 ng vector DNA and insert DNA at a 2 - 3 fold molar excess of insert to vector. Vector DNA was treated with calf intestinal alkaline phosphatase (section 2.16.4) to prevent self religation unless being ligated with annealed oligonucleotides which did not contain 5'phosphate groups. 100 ng of annealed

oligonucleotides was added to the ligation which was a 200 to 400 molar excess of insert DNA. Ligations were incubated overnight at 18°C or at 14°C when ligating *Eco*RI cohesive ends.

2.18 TRANSFORMATION OF PLASMID DNA

Plasmids were transformed into *Esherischia coli* DH5α cells by the method of Hanahan (1983). DH5α cells were streaked out for single colonies from a glycerol stock onto an LB agar plate (section 2.3.1) and incubated at 37°C overnight. Several 2 mm colonies were picked off the plate and dispersed into 1 ml SOB (section 2.3.3) medium by vortexing. The cells were inoculated into a 1 litre Erlenmeyer flask containing 30 ml of SOB and incubated at 37°C until the cell density reached 4 - 7 x 10^7 viable cells/ml (OD_{550} of 0.45 - 0.55). The culture was collected into an SS34 tube and chilled on ice for 10 minutes. The cells were pelleted by centrifugation in a Sorvall RC5C centrifuge at 3000 rpm for 15 minutes at 4°C. The supernatant then was poured off and the cells resuspended in TFB (2.9.3.2) to 1/3 of the original culture volume. Cells were pelleted as before and resuspended in TFB to 1/12.5 of the original culture volume. 7 µl of DnD (section 2.9.3.1) was added per 200 µl of cell suspension and mixed by swirling the tube for several seconds. The cells were incubated on ice for 20 minutes then a second equal aliquot of DnD was added and the cells were incubated a further 10 minutes on ice. 210 µl aliquots of cells were pipetted into chilled 15 ml polypropylene tubes (Falcon). 1 µl of ligation solution was added and swirled to mix. 1 µl of pUC monomer DNA was added to 210 µl of cells as a positive control. The cells were incubated 30 minutes on ice and heat shocked by placing in a 42°C waterbath for 90 seconds. After chilling on ice, 800 µl of SOC was added to each tube then the cells were incubated at 37°C with moderate shaking for 60 minutes. 100 µl of each culture was spread onto the appropriate LBamp (section 2.3.6) plate. The remaining culture was transferred to a microcentrifuge tube and centrifuged for 1 minute at 13000 rpm to pellet the cells. Most of the supernatant was poured off and the cells were resuspended in the remaining drops and spread onto the

appropriate plate. The plates were left to dry before incubating at 37°C overnight in an inverted position.

A transformation frequency of approximately 2 x 10^8 amp^R colonies/μg pUC was usually achieved. When a high frequency of transformation (> 1 x 10^9 amp^R colonies/μg pUC) was required, MAX efficiency DH5α™ Competent cells (Life Technologies) were used according to the manufacturer's instructions.

2.19 COLONY HYBRIDISATION

2.19.1 Colony Lifts

Hybond N 80 mm nylon filters (Amersham Life Science) were placed onto LBamp plates (sections 2.3.1 and 2.3.6). 50 to 100 colonies were picked and patched onto each nylon filter and then onto a master plate in a grid pattern. The plates were incubated at 37°C until the colonies reached a width of 0.5 to 1 mm. The filters were then transferred to LBamp plates containing 170 μg/ml chloramphenicol and incubated overnight. The master plates and filters were marked in the same places in an asymmetric pattern with an 18 gauge needle. The master plate was then stored at 4°C. Four pieces of 3MM paper (Whatman) were soaked with either 10 % SDS, denaturation solution (section 2.9.6.2), neutralisation solution (section 2.9.6.1) or 2 x SSC (section 2.9.5.2). The filters were removed from the plates and placed colony side up in the four solutions for 5 minutes each then placed on a dry sheet of 3MM paper for 30 minutes. The filters were sandwiched between two sheets of 3MM paper and baked at 80°C for 2 hours.

2.19.2 Preparation of DIG labelled probe

1 μg of probe was prepared using the DIG-High Prime labelling kit (Boehringer Mannheim) according to the manufacturer's instructions.

2.19.3 Hybridisation and Post Hybridisation Washes

The filters were wet with 6 x SSC (section 2.9.5.2) and placed in a round plastic container 10 cm in diameter. 1 ml APH (section 2.9.5.4) solution was added per 10

cm^2 of filter. Up to six filters were hybridised in the same container. The filters were prehybridised at 68°C for 3 hours, then the denatured DIG labelled probe was added to a concentration of 5 μg/ml to 20 ml APH. Hybridisation was carried out overnight at 68°C. The filters were then washed on a shaker as follows: 2 x 5 mins in 2 x SSC/0.1 % SDS at room temp, 2 x 5 mins in 1 x SSC/0.1 % SDS at room temp, 2 x 15 mins in 1 x SSC/0.1 % SDS (preheated to 68°C) at 68°C then rinsed in 2 x SSC.

2.19.4 Colorimetric Detection with NBT/BCIP

After post hybridisation washes, the filters were equilibrated for 2 minutes in washing buffer (section 2.9.6.4) then incubated in 1% blocking solution (Boehringer Mannheim) in maleic acid buffer (section 2.9.6.3) for 60 minutes. Antibody solution was prepared by the addition of 6 μl Anti-Digoxygenin-AP (Boehringer Mannheim) to 30 ml blocking solution. After a 30 minute incubation in the antibody solution, the filters were washed twice for 15 minutes each in 100 ml washing buffer. To detect the colour precipitate, the filters were firstly equilibrated in 10 ml detection buffer (section 2.9.6.5) then incubated in the dark in colour substrate solution (45 μl NBT and 35 μl BCIP in 10 ml detection buffer). The colour precipitate took 15 minutes to form.

2.20 MICROINJECTION OF *D. MELANOGASTER* EMBRYOS

2.20.1 Co-Precipitation of Plasmid DNA

40 μg of plasmid DNA and 12 μg of pUChsπΔ2,3 (section 2.1) were added together and the volume was made up to 100 μl with TE (section 2.9.2.2). For plasmids of 16 to 34 kb, 50 to 80 μg of plasmid DNA was used proportionally per 12 μg of pUChsΔ2,3. The DNA was ethanol precipitated (section 2.11.3) and resuspended in 100 μl of injection buffer (section 2.9.7.1). Prior to use, the DNA solution was centrifuged at 13000 rpm for 20 minutes to remove particles that may block the needle. 3 μl was loaded into the microinjection needle or Femtotip using a microloader (Eppendorf).

2.20.2 Collection of Embryos

Approximately 200 flies were placed in a plastic tripour beaker which was inverted onto a 60 x 15 mm plate of cornmeal agar (section 2.6.1) with a blob of yeast paste on it. These chambers were placed in the dark at 15 - 20°C for three days to allow the flies to acclimatise. When embryos were required, the plates were changed approximately every 30 minutes and the fresh embryos collected off the plates with a fine wet paintbrush. The embryos were transferred to double sided tape (Scotch 3M or Sellotape) placed in two 2 cm rows on a microscope slide.

2.20.3 Dechorionation of Embryos

Using fine forceps, the chorions of the embryos were teased away by rolling the embryos along the double sided tape. The width of the tape was scored 2 mm from the right edge. The dechorionated embryos were lined up along the scored edge of the tape. Five minutes was allowed for as many embryos as possible (usually approximately 30) to be dechorionated. All the tape was removed except for the 2 mm strip containing the embryos which were then dehydrated.

2.20.4 Dehydration of Embryos

The embryos were dehydrated by placing the microscope slide into a glass petri dish filled with silica gel and covering with the glass lid. Dehydration was carried out for 0 to 5 minutes depending on temperature, humidity and other unknown factors that affected the softness or firmness of the embryos. Various dehydration times were tested until the embryos were flaccid enough not too burst upon microinjection, but firm enough not to buckle. After dehydration the embryos were covered with halocarbon oil (Series 700, Halocarbon Products Corporation) or paraffin oil (Whiterex 334, Pauling Industries Ltd) to halt dehydration.

2.20.5 Microinjection of Embryos

DNA was microinjected according to Spradling and Rubin (1982). A transjector (Eppendorf) and micromanipulator (Leitz) were used in conjunction with a 0.5 μm Femtotip (Eppendorf) or a glass needle. The glass needles were pulled from 1.0 mm (outer diameter) x 0.75 mm (inner diameter) 10 cm glass filaments (A-M Systems, Inc) using a Micropipette Puller-MC2 (Ensor Scientific) with the following settings: heater: 124°C; slow pull: 8; fast pull: 6; tip delay: 70. An injection pressure of 50 to

600 kPa was used to microinject the DNA into the posterior pole of the embryo where the germ cells eventually would form. After microinjection the slides carrying the embryos were placed onto a rack in a plastic container lined with moist paper towels to increase the humidity. The container was incubated at 18°C for 24 hours and then 22°C for 24 hours. The surviving embryos were transferred to a vial of Formula 4-24 food (section 2.6.2) (40 embryos per vial), and incubated at 25°C until the flies hatched. These flies are termed the G0 generation.

2.20.6 Crossing Adult Survivors

After hatching, G0 flies were mated to the *y w* recipient stock in separate vials. Each female was mated with two males and each male was mated with five virgin females (section 2.7.2). The adults were removed after one week and the vials incubated at 25°C until the progeny (G1) hatched.

2.20.7 Identification of Transformants and Establishment of a Transformant Stock

G1 flies were examined twice daily for transformants. Transformants (w^+) had eye colours ranging from pale yellow to wild type. If the gametes of a G0 fly were transformed, the number of transformant flies in the corresponding G1 vial ranged from 1 in 4 to 1 in 100. Transformant males were crossed with five *y w* virgin females (section 2.7.2). If there were no transformant males, a transformant female was mated to two *y w* males. If a vial contained transformant flies with different eye colours, this indicated multiple insertions of the construct, therefore males with each eye colour were crossed to obtain stock of flies with each insertion. Heterozygous G2 males and virgin females were crossed. G3 progeny emerged at a 1:2:1 ratio of homozygous w^+: heterozygotes: homozygous *w*. A single homozygous male and virgin female were mated to ensure the establishment of a homozygous line.

2.21 DETERMINATION OF CHROMOSOMAL LINKAGE OF INSERT

To determine if the insertion was on the 2nd or 3rd chromosome, the following crosses were carried out. Five w^+ flies from each transformant line were mated separately with five *w; In(3LR)TM3,Sb* ry^{rk}*e/In(3LR)TM6, Tb e* and five *y w;* L^2 */CyO,*

Cy pr cn^2 y^+ virgin females (section 2.7.2). *Sb* w^+ and *Cy* w^+ progeny from these crosses were mated with virgin *y w* females. If the insertion was on the 2nd chromosome, then all *Cy* progeny would be *w*. If the insertion was on the 3rd chromosome then all *Sb* progeny would be *w*.

To determine if the insert was on the X chromosome, five w^+ males from each transformant line were mated with five *y w* virgin females. If the insertion was on the X chromosome, only female progeny could receive an X chromosome from their father, therefore all male progeny would be *w*.

2.22 MOBILISATION OF CONSTRUCT TO NEW GENOMIC SITES

To generate different chromosomal insertions of a construct, transformant flies containing a 2nd chromosome insertion of the construct were crossed to a stock containing an active transposase (Δ2-3 element with *Dr* marker) on the 3rd chromosome. This could mobilise the P elements and the intervening construct to new sites. Firstly, males from the stock containing the 2nd chromosome insertion were crossed to *w; In(3LR)TM3, Sb* ry^{rk}*e/In(3LR)TM6, Tb e* virgin females (section 2.7.2) to provide a 3rd chromosome balancer (*Sb* marker) to prevent recombination of the transposase into the transformant stock. *w+ Sb* virgin female progeny from this cross were selected and mated with *w; Sp/CyO, Cy pr* cn^2*;* ry^{506} *Dr {P[ry+]Δ2-3}/In(3LR)TM6* males. w^+, *Cy, Sb, Dr* virgin female progeny were mated with *y w* males. Progeny which were w^+ and *Cy* would contain a transposition of w^+ to a different chromosome. Linkage crosses (section 2.21) were then performed.

2.23 β-GALACTOSIDASE ASSAYS

β-Galactosidase assays were based on the method of Simon and Lis (1987). 12 male flies or 9 female flies were hemisected by cutting between the abdomen and thorax. The heads and thoraxes were retained and ground in a 1 ml homogeniser (Kontes) in 500 µl of assay buffer (section 2.9.8.1). The homogenate was transferred to a

microcentrifuge tube and an additional 500 µl of assay buffer was added. The homogenate was centrifuged at 13 000 rpm for 1 minute to remove cellular debris. 25 µl of the extract from homozygotes, or 50 µl from heterozygotes was added to assay buffer containing 1 mM Chlorophenol red-β-galactopyranoside monosodium salt (CPRG) in a microcentrifuge tube to a final volume of 1 ml. Assays were performed in triplicate for each sample. The samples were incubated at 37°C. After 30 minutes, each sample was transferred to a quartz cuvette and the absorbance read at 574 nm using a Shimadsu spectrophotometer. The samples were incubated a further 30 minutes and the absorbance read again. A graph of absorbance versus time (minutes) was constructed and a straight line through zero plotted for each sample. The differences in the amount of protein in each sample was standardised by measuring the wet weight of the flies and by performing total protein assays (section 2.23.1). β-galactosidase activity was measured as Δabsorbance/min/mg fly weight, and Δabsorbance/min/µg protein. *y w* flies (the microinjection recipient strain) were assayed to measure the activity of endogenous β-galactosidase in the gut (Appendix 2). When the average endogenous β-galactosidase activity was subtracted from the male and female activities of transgenic fly lines, the male to female ratio was altered by ≤ 2%, therefore the endogenous β-galactosidase activity was ignored.

2.23.1 Total Protein Assays

Total protein assays were carried out using the Bio-Rad Protein Microassay procedure according to the manufacturer's instructions. 0 to 18 µg/ml of bovine gamma immunoglobulin (Biorad) was used as the protein standard. A standard curve was constructed by plotting absorbance at 595 nm versus µg/15 µl protein. 15 µl of each fly homogenate was assayed in triplicate, the average absorbance calculated and the amount of protein determined using the standard curve.

3. RESULTS

3.1 EVALUATION OF THE *arm-lacZ* ASSAY SYSTEM

The isolation of DCREs using the *arm-lacZ* system relies on the premise that if DCRE(s) are present in the X-linked DNA inserted upstream of *arm-lacZ*, they will promote dosage compensation of *lacZ* in males, producing a 2:1 male to female ratio of β-galactosidase activity. In developing this system, two assumptions have been made:

1. That *lacZ* will be expressed equally in males and females in the absence of DCREs. This assumption was addressed by measuring the β-galactosidase activity of transgenic flies carrying autosomal insertions of *arm-lacZ.*
2. That the *armadillo* promoter can respond to the DCREs to facilitate dosage compensation in males. X-linked insertions of *arm-lacZ* were assayed for β-galactosidase activity to determine whether the *armadillo* promoter could respond to the dosage compensation machinery,

3.1.1 β-Galactosidase Assays of Transgenic Flies Carrying Autosomal Insertions of *arm-lacZ*

The plasmid pCaSpeR-arm-βgal consists of the *armadillo* promoter fused to the *Escherischia coli lacZ* gene (*arm-lacZ*) (Vincent *et al.,* 1994). pCaSpeR-arm-βgal DNA obtained from J.P. Vincent (Table 2) was microinjected into *y w D. melanogaster* embryos (section 2.20). One transgenic line was produced and linkage crosses showed the insertion to be on the 2nd chromosome (section 2.21). Two more transgenic lines containing insertions of *arm-lacZ* on the second chromosome were obtained from J.P. Vincent (Table 2).

β-Galactosidase assays (section 2.23) were performed on heterozygous (one dose) and homozygous (two dose) males and females from each transgenic line and the male to female ratios of β-galactosidase activity were calculated (Table 4). In all experiments,

males expressed the same amount of β-galactosidase as females (within error), confirming that *lacZ* is not under any sex specific control.

3.1.2 β-Galactosidase Assays of Transgenic Flies Carrying X-linked Insertions of *arm-lacZ*

If the *armadillo* promoter is responsive to the dosage compensation machinery, transgenic flies carrying X-linked insertions of *arm-lacZ* will express twice the level of β-galactosidase as females. An X-linked transgenic fly stock was obtained from J-P Vincent (Table 2), and two more were generated by mobilising *arm-lacZ* to new genomic sites with a P-element transposase (section 2.22). The β-galactosidase activity of one and two dose females was compared with that of one dose males (Table 5). Males with one dose of *arm-lacZ* expressed the same level of β-galactosidase as two dose females and twice that of one dose females, indicating that *arm-lacZ* responds fully to the dosage compensation machinery in males.

3.2 INSULATION OF *arm-lacZ* WITH SCS AND SCS' ELEMENTS

To protect against the possible repressive effects of the autosomal chromatin environment, the SCS and SCS' insulating elements were subcloned on either side of *arm-lacZ*. SCS and SCS' were supplied on pElba 6 from P. Schedl. The simplest cloning method was to subclone SCS and SCS' into a new CaSpeR vector and then subclone *arm-lacZ* into the polylinker between them.

3.2.1 Insertion of the SCS and SCS' Elements into a pCaSpeR Vector

CaSpeR-KN, a CaSpeR transformation vector with convenient *Kpn*I and *Not*I restriction sites (Appendix 1) was also obtained from P. Schedl. The cloning strategy is described in Figure 1.

Table 4. β-Galactosidase Assays of Transgenic Flies Carrying Autosomal Insertions of *arm-lacZ*.

Three different transgenic lines were assayed. For each line, the expression of one dose males and one dose females was compared, as was two dose males and two dose females. Each assay was performed four times and standardised using total protein measurement or total fly wet weight. The standard error represents the variability between the four experiments.

Line	Chromo some	Dose Male	Dose Female	M/F Ratio		Standard Error
arm-lacZ #1	2	1	1	Protein	1.00	0.06
				Fly weight	1.12	0.06
		2	2	Protein	1.16	0.06
				Fly weight	1.20	0.03
arm-lacZ #2	2	1	1	Protein	1.07	0.04
				Fly weight	1.14	0.05
		2	2	Protein	1.14	0.03
				Fly weight	1.15	0.01
arm-lacZ #3	2	1	1	Protein	1.03	0.02
				Flyweight	1.11	0.06
		2	2	Protein	0.98	0.06
				Fly weight	0.91	0.05

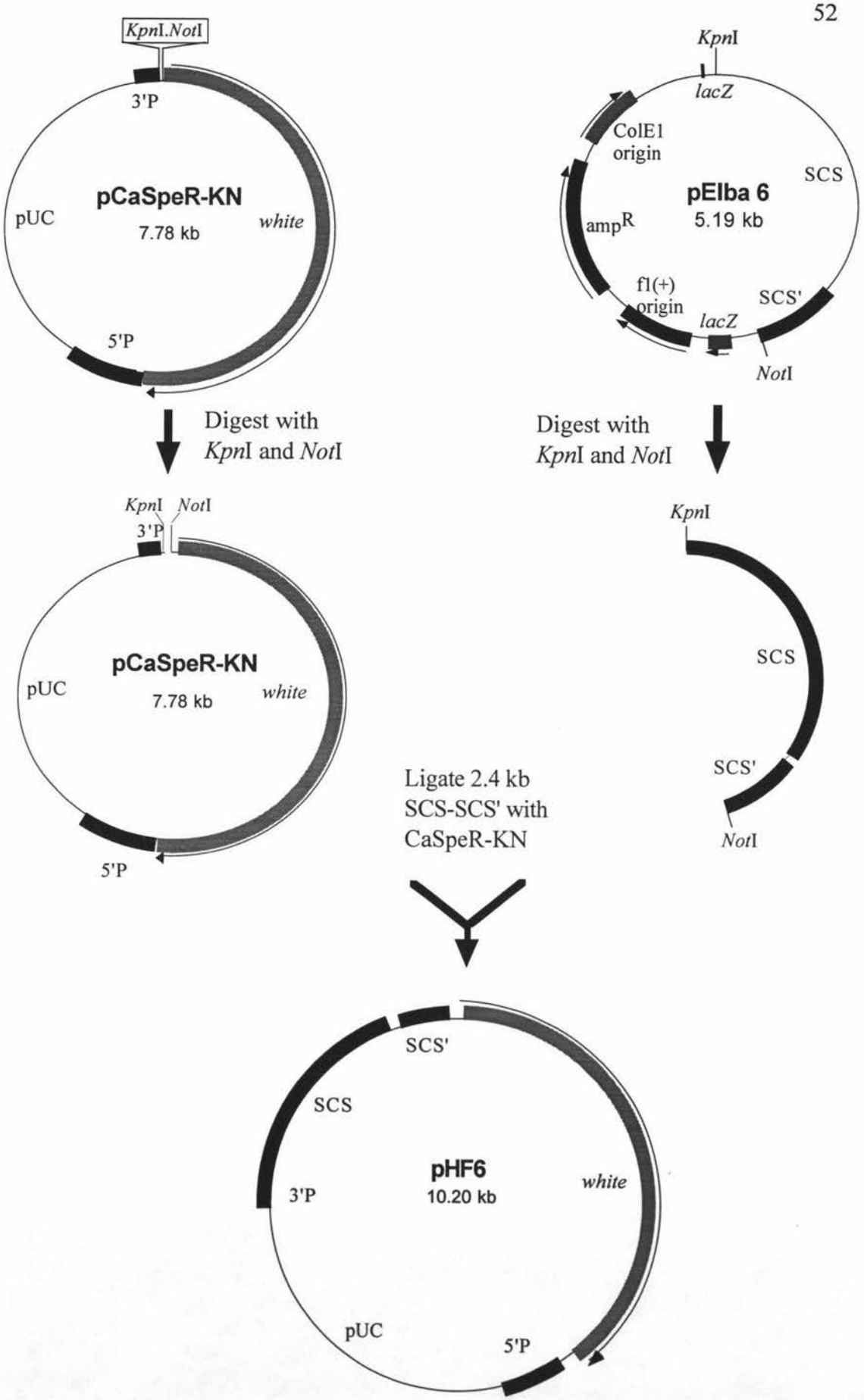
Table 5. β-Galactosidase Assays of Transgenic Flies Carrying X-linked Insertions of *arm-lacZ*.

Three different transgenic lines were assayed. For each line, the expression of one dose males and one dose females was compared, as was one dose males and two dose females. Each assay was performed four times and standardised using total protein measurement or total fly wet weight. The standard error represents the variability between the four experiments.

Line	Chrom osome	Dose Male	Dose Female	M/F Ratio		Standard Error
arm-lacZ X#1	X	1	1	Protein	1.96	0.19
				Fly weight	2.04	0.14
		1	2	Protein	0.95	0.06
				Fly weight	0.94	0.02
arm-lacZ X#2	X	1	1	Protein	1.84	0.02
				Fly weight	1.81	0.03
		1	2	Protein	1.03	0.02
				Fly weight	1.05	0.04
arm-lacZ X#3	X	1	1	Protein	1.74	0.05
				Flyweight	1.93	0.05
		1	2	Protein	0.85	0.02
				Fly weight	0.95	0.04

Figure 1. Insertion of SCS and SCS ' Elements into pCaSpeR-KN.

1. Digest pElba6 with *Not*I and *Kpn*I and isolate 2.4 kb SCS-SCS ' fragment.
2. Digest pCaSpeR-KN with *Not*I and *Kpn*I and ligate with 2.4 kb SCS-SCS ' fragment to form pHF6.


KpnI.NotI
3'P
pCaSpeR-KN
7.78 kb
pUC
white
5'P
KpnI
lacZ
ColE1
origin
SCS
pElba 6
5.19 kb
ampR
f1(+)
origin
SCS'
lacZ
NotI
Digest with
KpnI and NotI
Digest with
KpnI and NotI
KpnI NotI
3'P
pCaSpeR-KN
7.78 kb
pUC
white
5'P
KpnI
SCS
SCS'
NotI
Ligate 2.4 kb
SCS-SCS' with
CaSpeR-KN
SCS'
SCS
pHF6
10.20 kb
3'P
white
pUC
5'P

The 2.4 kb *Not*I/*Kpn*I SCS-SCS' fragment was ligated (section 2.17) with *Not*I/*Kpn*I digested pCaSpeR-KN and transformed into *E. coli* (section 2.18). Analytical restriction digestions (section 2.14.2) of the recombinant plasmid (called pHF6) revealed that there was an extra *Eco*RI site in the pCaSpeR-KN polylinker that was not specified on the restriction map. As a unique *Eco*RI site was required for insertion of the X-linked DNA, the 'extra' *Eco*RI site had to be removed. After digestion of pCaSpeR-KN with *Eco*RI, the 5' overhangs were filled in with Klenow (section 2.16.1) and the plasmid was religated (section 2.17) and transformed (section 2.18). Subsequent restriction digestions (section 2.14.2) showed that although the *Eco*RI site had been removed, the crucial *Not*I site had also been removed. As CaSpeR-KN was constructed from pCaSpeR by replacement of an *Eco*RI site with a *Not*I site, it is possible that on addition of the *Not*I site, an *Eco*RI site was unknowingly recreated on each side of the *Not*I site.

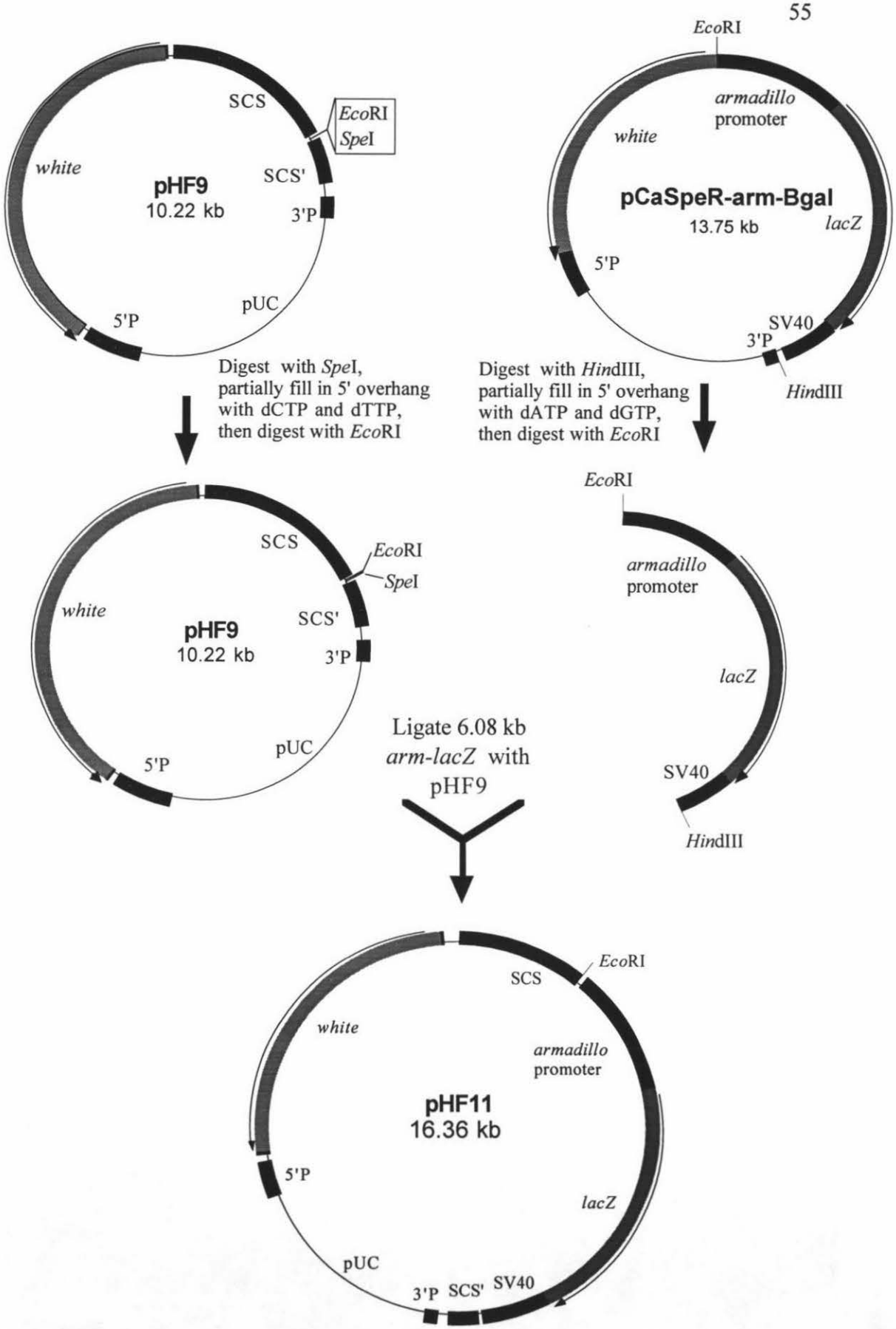
A new cloning strategy was devised. The only other pCaSpeR transformation vector to contain *Not*I and *Kpn*I sites was pCaSpeR4 (Appendix 1). *Eco*RI and *Spe*I sites were also present in the polylinker which had to be removed because after addition of SCS-SCS', unique *Eco*RI and *Spe*I site were required in the polylinker between SCS and SCS' for insertion of *arm-lacZ*. pCaSpeR4 was digested with the *Eco*RI, the 5' overhangs were filled in with Klenow, and the vector was religated to form plasmid pHF7. This plasmid was digested with *Spe*I then the same procedure was performed to form pHF8 containing one *Not*I and one *Kpn*I site and no *Eco*RI or *Spe*I sites. The *Not*I/*Kpn*I SCS-SCS' fragment was cloned into pHF8 digested with *Not*I and *Kpn*I to form pHF9.

3.2.2 Insertion of *arm-lacZ* Between the SCS and SCS' Elements

To insert *arm-lacZ* between SCS and SCS', the cloning strategy according to Figure 2 was followed. *arm-lacZ* and the polylinker between SCS and SCS' both contained *Eco*RI sites that could be used for cloning, however no other common restriction sites were present. The two 3' outermost bases of the *Spe*I and *Hin*dIII restriction sites are complementary therefore if the two innermost 5' bases of the sticky end are filled in with Klenow and the appropriate dinucleotide triphosphates (dNTPs) (section 2.16.1), *Spe*I and

Figure 2. Insertion of *arm-lacZ* between SCS and SCS ' of pHF9

1. Digest pCaSpeR-arm-βgal with *Spe*I, fill in the two innermost bases of the *Spe*I sticky end with dCTP and dTTP then further digest with *Eco*RI.
2. Isolate 6.2 kb *arm-lacZ* fragment.
3. Digest pHF9 with *Hin*dIII, fill in the two innermost bases of the *Hin*dIII sticky end with dATP and dGTP then further digest with *Eco*RI.
4. Ligate 6.2 kb *arm-lacZ* fragment with pHF9.


SCS
EcoRI
SpeI
white
pHF9
10.22 kb
SCS'
3'P
pUC
5'P
EcoRI
armadillo
promoter
white
pCaSpeR-arm-Bgal
13.75 kb
lacZ
5'P
SV40
3'P
HindIII
Digest with SpeI,
partially fill in 5' overhang
with dCTP and dTTP,
then digest with EcoRI
Digest with HindIII,
partially fill in 5' overhang
with dATP and dGTP,
then digest with EcoRI
SCS
EcoRI
SpeI
white
pHF9
10.22 kb
SCS'
3'P
pUC
5'P
EcoRI
armadillo
promoter
lacZ
SV40
HindIII
Ligate 6.08 kb
arm-lacZ with
pHF9
SCS
EcoRI
white
armadillo
promoter
pHF11
16.36 kb
5'P
lacZ
pUC
3'P
SCS'
SV40

*Hin*dIII can be joined by a two base pair ligation. pCaSpeR-arm-βgal was digested as described and the 6.2 kb *arm-lacZ* fragment gel purified (section 2.13.1). The fragment was ligated (section 2.17) with pHF9 (also digested as described above) and transformed (section 2.18) but no transformants were obtained. To increase the chances of producing a transformant, the following changes to the cloning strategy were made:

1. The vector was gel purified (section 2.13.1) to reduce uncut vector background
2. *Hin*dIII and *Spe*I 5' overhangs were made fully blunt with Klenow (section 2.16.1) before ligation
3. Instead of using the *Hin*dIII site of pCaSpeR-arm-βgal, the *Pst*I site was blunt ended with Mung Bean Nuclease (section 2.16.2) and ligated to the blunt *Spe*I site. This alternative strategy was tried as a 3 kb *Hin*dIII fragment from pUC is also produced from the *Hin*dIII/*Eco*RI digestion of pCaSpeR-arm-βgal. When present in a ligation mixture, this fragment can self ligate and be transformed at a relatively high efficiency because it contains ampicillin resistance and an origin of replication. Some of the recombinant plasmid produced from the unsuccessful ligations were approximately 3 kb, suggesting that the 3 kb fragment was contaminating the 6.2 kb *arm-lacZ* fragment.

After multiple transformations, no transformants were obtained. To determine if there was an error in the SCS-SCS' polylinker, a sequencing primer (SCS1, Table 3) was designed to 3' end of SCS. The automated sequencing reaction was unsuccessful, therefore a primer to the CaSpeR4 polylinker (Cspr4XbBm, Table 3) was designed to sequence through the 0.5 kb SCS element and SCS-SCS' polylinker. The sequence revealed an 'extra' unspecified *Eco*RI site at the 5' end of the SCS' element. Digestion at both *Eco*RI sites had removed the intervening *Spe*I site producing a vector with two *Eco*RI ends, hence preventing ligation with the 6.2 kb *arm-lacZ* fragment.

Oligonucleotides were designed which, when annealed, formed a linker containing a *Spe*I site flanked by *Eco*RI cohesive ends, only one of which reconstituted an *Eco*RI site when ligated to *Eco*RI digested pHF9. The resultant plasmid, pHF10, was

sequenced using the Cspr4XbBm primer to ensure only one *Eco*RI site and one *Spe*I site were present.

arm-lacZ was subcloned into pHF10 by a *SpeI/Hin*dIII partial fill-in and *Eco*RI ligation as described previously. Initially no transformants were obtained from 18 minipreparations of plasmid DNA (section 2.10.2). Colony hybridisation (section 2.19) using a 1 kb fragment of the *arm* promoter as a probe (section 2.19) was successful in identifying a transformant which was named pHF11 (Appendix 1). Restriction digestions confirmed that a unique *Eco*RI site was present between SCS' and *arm* for subcloning the X-linked DNA (Figure 3).

Prior to insertion of X-linked DNA, pHF11 was microinjected into *D. melanogaster* to confirm that *arm-lacZ* was still expressed equally in males and females and to determine whether the SCS and SCS' element can block dosage compensation.

3.2.3 β-Galactosidase Assays of Transgenic Flies Carrying Autosomal Insertions of pHF11

Three lines carrying autosomal insertions of pHF11 were obtained from transformation of pHF11. β-Galactosidase assays (section 2.23) were performed on one dose males and females and two dose males and females from the three lines. The results are presented in Table 6. In all cases, males and female expression was essentially identical, confirming that the SCS and SCS' elements do not alter the 1:1 ratio of expression.

3.2.4 β-Galactosidase Assays of Transgenic Flies Carrying X-linked Insertions of pHF11

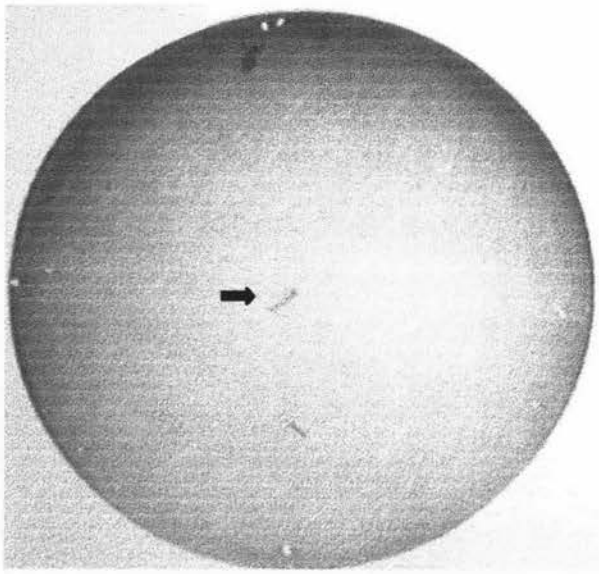
One transgenic line containing pHF11 inserted onto the X chromosome was produced. β-Galactosidase assays (section 2.23) were performed on one dose males compared with one dose and two dose females. The results are summarised in Table 7. *arm-lacZ* remained dosage compensated, therefore the SCS and SCS' elements cannot block dosage compensation.

Figure 3. Colony Hybridisation and Analytical Restriction Enzyme Digestion of Positive Clones

A digoxygenin labelled 1 kb fragment of the *armadillo* promoter was used to probe filters carrying lysed and baked colonies transformed with 6.2 kb *arm-lacZ* fragment + pHF10 ligation mixture. A filter containing a positive clone is shown. The positive clone is marked by an arrow. The topmost colony is a positive control containing pCaSpeR-arm-βgal DNA.

Restriction Digestion of pHF11 DNA

Lane 1. λ *Hin*dIII/*Sst*II ladder; Lane 2. *Pst*I digestion of pHF11 produced the expected fragments of 8.4, 6.7 and 1.3 kb; Lane 3. *Pst*I/*Eco*RI digestion of pHF11 allows cleavage of the 6.7 kb *Pst*I fragment into a 6.1 and a 0.6 kb fragment, confirming the presence of a single *Eco*RI site between SCS ' and the *arm* promoter.



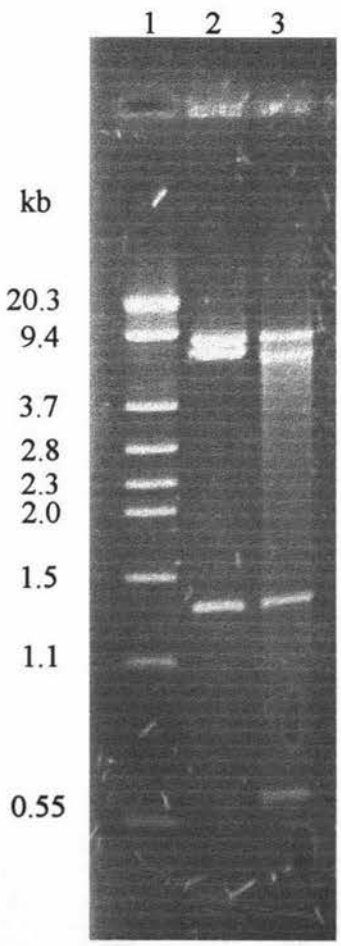

1
2
3
kb
20.3
9.4
3.7
2.8
2.3
2.0
1.5
1.1
0.55

Table 6. β-Galactosidase Assays of Transgenic Flies Carrying Autosomal Insertions of pHF11.

Three different transgenic lines were assayed. For each line, the expression of one dose males and one dose females was compared, as was two dose males and two dose females. Each assay was performed four times and standardised using total protein measurements and total fly wet weight. The standard error represents the variability between the four experiments.

Line	Chromo some	Dose Male	Dose Female	M/F Ratio		Standard Error
HF11 #1	2	1	1	Protein	1.07	0.05
				Fly weight	1.12	0.06
		2	2	Protein	1.10	0.06
				Fly weight	0.92	0.22
HF11 #2	2	1	1	Protein	0.99	0.02
				Fly weight	0.96	0.02
		2	2	Protein	1.06	0.02
				Fly weight	1.14	0.07
HF11 #3	2	1	1	Protein	1.02	0.05
				Flyweight	1.13	0.02
		2	2	Protein	1.04	0.02
				Fly weight	1.01	0.02

Table 7. β-Galactosidase Assays of Transgenic Flies Carrying an X-linked Insertion of pHF11.

The expression of one dose males was compare with that of one dose and two dose females. Each assay was performed four times and the standard error represents the variability between the four experiments.

Line	Chromo some	Dose Male	Dose Female	M/F Ratio		Standard Error
HF11 X#1	X	1	1	Protein	1.66	0.02
				Fly weight	1.73	0.04
		1	2	Protein	1.02	0.07
				Fly weight	1.07	0.05

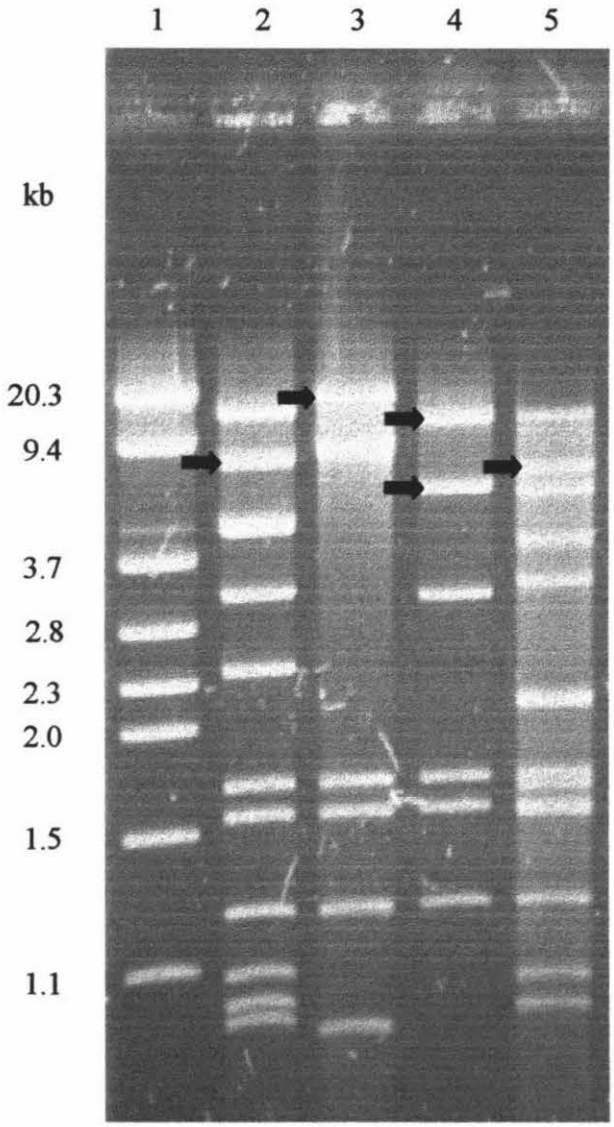
3.3 INSERTION OF X-LINKED DNA INTO pHF11

X-linked DNA sequences were selected by examination of polytene chromosome spreads stained with anti-MSL and H4Ac16 antibodies. The assumption was made that the areas that the antibodies bound to were MSL target sites, and were, therefore, more likely to contain DCREs. Regions 1A, 1B, 1E, 1F and 2A were selected from the bright staining tip of the X chromosome. A cosmid contig spanning ~84% of the X chromosome has been constructed as part of the European Drosophila Genome project (Madueño *et al.*, 1995). Seven cosmids from the five regions selected from the X were obtained. Cosmid DNA was digested with *Eco*RI which cleaves genomic DNA on average once every 4.1 kb. A range of 8 up to 19 fragments were produced from the cosmid digests (Figure 4). Four fragments of 1.7, 1.6, 1.2 and 0.05 kb were derived from internal *Eco*RI sites within the vector. Because there were no *Eco*RI sites in the polylinker, two vector fragments of 0.29 and 0.39 kb which flanked either side of the polylinker were present on the ends of two of the X-linked fragments. As the smallest fragment selected for screening would be over 4 kb, this small amount of vector DNA was considered to be insignificant.

Each cosmid digest was fractionated on a seaplaque agarose gel (section 2.13) and the largest fragments excised and gel purified (section 2.13). Ten fragments of 4.4 to 17.5 kb (as estimated from the Molecular Weight determination function of the Gel Documentation System) were ligated (section 2.17) with *Eco*RI digested (section 2.14.3), phosphatase treated pHF11 (section 2.16.4) and transformed into *E. coli* (section 2.18) Transformants of the ten plasmids were obtained and the presence of the correct insert was confirmed by performing an *Eco*RI/*Pst*I digestion on the plasmid DNA (section 2.14.2). Fragments produced that corresponded to the vector DNA were 8.4, 6.2, 1.3 and 0.5 kb. The remaining bands were derived from the insert which matched the bands produced from the *Pst*I digestion of the corresponding gel purified fragment. Plasmids were named as the cosmid the fragment was derived from and the size of the fragment, i.e. p24F3 7.4 is a fragment of 7.4 kb derived from the cosmid 24F3 (Table 1).

Figure 4. *Eco*RI Digestion of Cosmid DNA

Sample *Eco*RI digestions of four of the seven cosmaid as shown. Lane 1. *Hin*dIII/*Sst*II ladder. Lane 2. *Eco*RI digestion of cosmid 23E12. Lane 3. *Eco*RI digestion of cosmid 154H3. Lane 4. *Eco*RI digestion of cosmid 11E3. Lane 5. *Eco*RI digestion of cosmid 24F3. Arrows denote the *Eco*RI fragments that were cloned into pHF11.


1
2
3
4
5
kb
20.3
9.4
3.7
2.8
2.3
2.0
1.5
1.1

The plasmids were grown up in LBamp and plasmid DNA extracted (section 2.10.1). Plasmid DNA was microinjected (section 2.20) at a concentration of 0.4 μg/μl to 0.8 μg/μl, the concentration increasing with larger plasmids to keep the molar ratio of plasmid DNA: helper DNA (pUChsπΔ2,3) constant. Initially four plasmids were microinjected using Eppendorf 0.5 μm femtotips. None of the 40 G0 adult flies resulting from injection of 11E3 15.4 or 46 adults from injection of 24F3 7.4 were transformant. This transformation frequency was extremely low, considering that 1 transformant per 12 adult flies was obtained from microinjection of pHF11. Two possibilities were considered for the lack of transformants:

1. That the new brand of CsCl (Boehringer Mannheim, Molecular Biology Grade 99.5% purity) that was used for injection of these plasmids (but not pHF11) was not pure enough compared with the original CsCl used (Ajax Chemicals, 99.9% purity).
2. That the 0.5 μm (± 0.3 μm) femtotips were too fine to be used for the injection of large plasmids and the DNA was sheared as it passed through the tip. 1μm tips are thought not to shear plasmid DNA (Ashburner, 1989) and Haenlin (1985) suggests using larger tips of 3-8 μm for injection of larger plasmids or cosmids.

Two transformants each of the third and fourth constructs were subsequently obtained. If the DNA was low quality due to the lower purity of the CsCl, it would be expected that no transformants would be produced, suggesting that it may have been the size of the needle that was causing the low transformation frequency. The four transformants that were produced could have resulted from microinjection with a slightly broken needle (needles often break during the course of microinjection). Furthermore, the variation in the size of the femtotip is stated as ± 0.3 μm, therefore the largest tip could be up to four times wider than the smallest.

The remaining plasmids were injected with larger 'self pulled' tips which were pulled from glass capillary tubes (A-M Systems) with a needle puller (Ensor Scientific) (section 2.20.5). The exact size of the 'self pulled' tips is unknown, however they are visually estimated to be at least twice the diameter of a femtotip. Due to time constraints and mechanical failure of the transjector while the ninth construct was being injected, the tenth construct was not injected. The results of the transformations are summarised in Table 8.

It is unclear why no transformants were obtained from microinjection of p154H3 17.5 or the second round of microinjection of p24F3 7.4. As there were only 25 adult survivors from the p24F3 7.4 injections, the lack of transformants could be attributed to chance. This is not the case with p154H3 17.5, as there were 90 adult survivors. The plasmid is extremely large (approximately 34 kb) therefore it could be unstable although it is not significantly larger than p11E3 15.4 of which two transformants were produced. The injection buffer (section 2.9.7.1) cannot be blamed, as the same batch was used throughout, but it is possible that the impurities in the CsCl affected this plasmid more negatively than others.

Linkage crosses (section 2.21) were performed on all transgenic lines to determine chromosomal linkage. For each construct, β-galactosidase assays (section 2.23) were performed on one dose males and females with autosomal insertions. The results are summarised in Table 9. For all constructs, the male to female ratio of β-galactosidase activity was approximately 1:1, therefore no DCREs that can confer dosage compensation onto *arm-lacZ* are present within the X-linked DNA.

Table 8. Frequency of P-element Mediated Transformation of Constructs Containing X-linked DNA into *D. melanogaster* Embryos

Nine constructs were injected into *D. melanogaster* embryos using either femtotips (Eppendorf) or self-pulled needles. The relative frequencies of transformation are shown by comparison of the number of adult injection survivors (G0) compared with the number of transformants for each line.

Construct	Type of needle used	No. of G0 adult injection survivors	No. of transformants
24F3 7.4	femtotip	45	0
11E3 15.4	femtotip	39	0
123B12 9.0	femtotip	53	2[a]
144A7 12.8	femtotip	45	2
185B11 6.6	self-pulled	34	4[a,b]
11E3 6.2	self-pulled	40	2
24F3 7.4	self-pulled	25	0
185B11 5.0	self-pulled	31	4[a,c,d]
11E3 15.4	self-pulled	54	2
154H3 17.5	self-pulled	90	0
24F3 7.4	self-pulled	60	3[e]
23E12 7.7	self-pulled	20	1

[a]The construct inserted into two different polar nuclei or two insertions occurred in the same polar nuclei, resulting in two segregating transgenic lines from the same G0 fly

[b] One line had activity too low to assay

[c] one line was X-linked

[d] one line was poorly viable and died out before it could be assayed

[e] Two lines were X-linked

Table 9. β-Galactosidase Assays of Transgenic Flies Carrying Autosomal Insertions of pHF11 Containing Portions of X-linked DNA.

Assays were performed on one dose males and one dose females. For each line, each assay was performed four times and standardised using total protein measurement or total fly wet weight. The standard error represents the variability between the four experiments.

Line	Chromosome	M/F Ratio		Standard Error
123B12 9.0 #1	3	Protein	1.06	0.02
		Fly weight	1.08	0.07
123B12 9.0 #2	2	Protein	1.03	0.04
		Fly weight	1.06	0.05
144A7 12.8 #1	3	Protein	1.07	0.04
		Fly weight	1.18	0.07
144A7 12.8 #2	3	Protein	0.95	0.05
		Fly weight	1.09	0.07
185B11 6.6 #1	2	Protein	0.97	0.02
		Fly weight	1.05	0.03
185B11 6.6 #2	3	Protein	1.01	0.04
		Fly weight	1.07	0.04
185B11 6.6 #3	4	Protein	1.15	0.01
		Fly weight	1.10	0.04
11E3 6.2 #1	2	Protein	1.04	0.04
		Fly weight	1.12	0.03
11E3 6.2 #2	2	Protein	1.07	0.11
		Fly weight	1.09	0.09
185B11 5.0 #1	2	Protein	1.05	0.04
		Fly weight	0.99	0.03
185B11 5.0 #3	2	Protein	1.02	0.02
		Fly weight	1.02	0.02

Table 9. continued

11E3 15.4 #1	3	Protein	1.01	0.05
		Fly weight	1.07	0.05
11E3 15.4 #2	3	Protein	1.01	0.02
		Fly weight	1.07	0.06
23E12 7.7#1	2	Protein	1.00	0.03
		Flyweight	1.04	0.02

3.4 EVALUATION OF THE ROLE OF *SXL* IN DOSAGE COMPENSATION

3.4.1 Insertion of *Sxl* Binding Sites into pHF11

The 3'UTR of *lacZ* in pHF11 is derived from the mammalian SV40 virus and contains the polyA signals of the early gene. Examination of this sequence did not reveal any unique restriction sites suitable for the insertion of *Sxl* binding sites. One *Mfe*I site was present, however there were additional *Mfe*I sites in the *white* gene and SCS element. To allow cloning into the *Mfe*I site in the 3'UTR without interference by the other sites, the following strategy was employed: After restriction digestion (section 2.14.3) of pHF11 with *Pst*I and *Eco*RI, the *arm-lacZ* fragment was gel purified (section 2.13.1) and ligated (section 2.17) into *Pst*I and *Eco*RI digested pBluescript KSII- (pBS KSII-). As pBS KSII- does not contain any *Mfe*I sites, this plasmid (named pHF12) contained a unique *Mfe*I site in the *lacZ* 3'UTR.

3.4.2 Selection of *Sxl* Binding Sites

Sxl binding sites could be obtained in two ways. The first was to design oligonucleotides containing three *Sxl* binding sites which could be annealed to form a linker. The second was to amplify them by PCR from the 3'UTR of one of the 20 genes known to contain three or more *Sxl* binding sites (Kelley *et al.*, 1995). Although the sequence of the *Sxl* binding site has been determined (Samuels *et al.*, 1994) it is not known whether the length or nature of the intervening sequences between the *Sxl* binding sites has any effect on regulation by *Sxl*. For this reason it was decided that both methods of selection would be applied.

The *Sxl*Top oligonucleotide (Table 3) contains three *Sxl* binding sites separated by an *Nhe*I and a *Spe*I site. These restriction sites both do not cut pHF11 so can be used to insert additional *Sxl* binding sites at a later date if desired. The *Sxl*A oligonucleotide is complementary to *Sxl*Top and together, when annealed, they form a linker with *Mfe*I cohesive ends. The oligos were designed such that, when annealed, the 3' end of the linker contains the sequence 5' G/CTTAA 3'. This cohesive end is complementary to that of *Mfe*I, but when ligated forms the sequence 5' GAATTG 3', which does not reconstitute the *Mfe*I restriction site (5' CAATTG 3'). This serves as a directional

tool to determine the orientation of the linker once inserted by digestion with *Mfe*I and a nearby restriction site such as *Xba*I.

*Sxl*Top and *Sxl*A were annealed (section 2.16.6) and ligated (section 2.17) into pHF12. As no transformants were initially produced, two new strategies were tried. The first was to increase the annealing efficiency of the oligos by heating to 100°C then cooling to 45°C (9°C below Tm) and holding for one hour before ligation. The second approach was to kinase the ends of the oligos (section 2.16.5) which allowed phosphatase treatment of the vector (section 2.16.4) and therefore prevented vector religation. Analytical restriction digestion (section 2.14.2) of minipreparations of plasmid DNA (section 2.10.2) revealed that the kinasing method was successful. The orientation of the linker in each recombinant was determined by *Mfe*I/*Xba*I digestion. The orientation producing *Sxl* binding sites produced 4.25, 3.9, 0.7 and 0.22 kb sized fragments and the opposite orientation produced fragments of 4.2, 3.9, 0.75 and 0.17 kb. It was desirable to clone the linker in both directions as the direction in which polyA stretches instead of polyT *Sxl* binding sites were present would serve as a negative control. Plasmids containing the oligo in each direction were obtained and named pHF15 (containing *Sxl* binding sites) and pHF16 (containing polyA sites). They were both sequenced using the T7 primer (Stratagene, Table 3) which confirmed that only one copy of the insert was present in both plasmids.

To obtain *Sxl* binding sites that are present *in vivo*, the 3' UTR sequences of several of the X-linked genes containing three or more *Sxl* binding sites were examined. 156 bp of the *runt* 3'UTR was selected for PCR amplification as it contained three well spaced *Sxl* binding sites and was large enough to be easily visualised on a gel.

Two 33 bp PCR primers, *runt*1 and *runt*2 (Table 3) were designed to amplify the 156 bp product. An *Eco*RI site was placed at the 5' end of *runt*1 and an *Mfe*I site was placed at the 5' end of *runt*2. *Eco*RI and *Mfe*I share the same 5' TTAA 3' cohesive ends therefore after digestion with these two enzymes, the PCR product can be directly inserted into the *lacZ Mfe*I site. The *Eco*RI site will not be reconstituted, allowing the orientation of the PCR product to be determined by double digestion with *Mfe*I and *Xba*I.

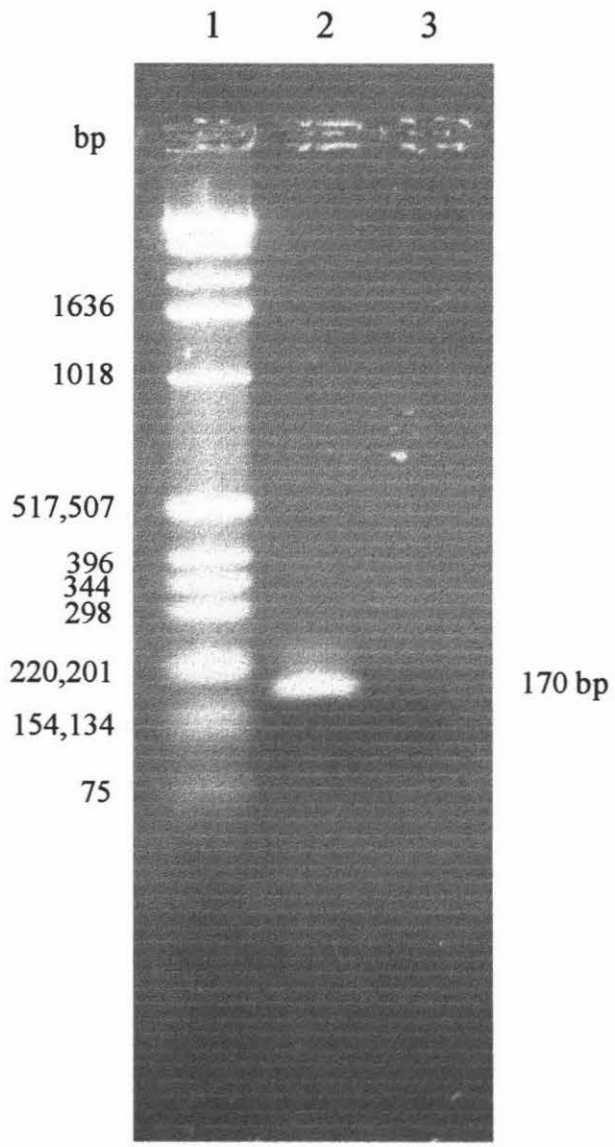
The polymerase chain reaction (section 2.15) was performed on *D. melanogaster* genomic DNA (prepared by K.J. Frith) using the *runt*1 and *runt*2 primers. A 170 bp product was amplified (Figure 5) and purified with the Qiagen PCR purification kit (section 2.11.1). Following an *Eco*RI/*Mfe*I double digest, the PCR product was cloned into *Mfe*I digested pHF12. *Mfe*I/*Xba*I double digests were performed on minipreparations of plasmid DNA (section 2.10.2) to determine the orientation of the inserts. Fragments of 4.23, 3.9, 0.86 and 0.17 kb were produced when the insertion was in the orientation giving the *Sxl* binding sites, and fragments of 4.23, 3.9, 0.7 and 0.33 kb were produced when the insertion was in the opposite orientation. Plasmids containing the PCR product in each orientation were obtained and named pHF13 (containing *Sxl* binding sites) and pHF14 (containing polyA stretches). They were sequenced using the T7 primer (Stratagene) which confirmed that there were no errors in either of the sequences.

3.4.3 Reinsertion of *arm-lacZ* into pHF10

The two 6.2 kb *arm-lacZ* fragments containing the linker and the two 6.4 kb *arm-lacZ* fragments containing the PCR product in both orientations were cloned into pHF10 in a similar method that *arm-lacZ* was cloned initially into pHF10. pHF13, pHF14, pHF15 and pHF16 were digested with *Pst*I which was blunt ended with Mung Bean Nuclease (section 2.16.2) then digested with *Eco*RI. Similarly, the *Spe*I site of pHF10 was also made blunt by Mung Bean Nuclease then digested with *Eco*RI. Following size fractionation on a seaplaque gel (section 2.13.1), excision and gel purification (section 2.13.1), the four *arm-lacZ* fragments were ligated (section 2.17) with pHF10 and transformed (section 2.18). *Xba*I digestions (section 2.14.2) of minipreparations of plasmid DNA (section 2.10.2) produced fragments of 10.6, 4.2 and 1.5 kb, which confirmed the presence of *arm-lacZ*, however fragments of the sizes expected from *Mfe*I/*Xba*I digestions were not produced. Three of the four plasmids were missing the *Mfe*I site and one of the plasmids containing

Figure 5. Amplification of 170 bp of *runt* 3 ' UTR by Polymerase Chain Reaction

Lane 1, 1kb ladder (BRL); lane 2, 170 bp *runt* PCR product; lane 3; negative control in which no genomic DNA was added.


1
2
3
bp
1636
1018
517,507
396
344
298
220,201
154,134
75
170 bp

the linker was missing the *Spe*I site, indicating that the linker (or part thereof) was no longer present. This result was unusual, considering that digestion with *Xba*I produced fragments of the approximate sizes expected for *arm-lacZ*. As the *Mfe*I site is only 150 bp from the 3' end of *arm-lacZ* it was concluded that the Mung Bean Nuclease may have 'nibbled' into the 3' end of *arm-lacZ*, in three cases as far as the *Mfe*I site (and therefore into the linker or PCR product). The small differences in size of the shortened fragments could not be discerned on a gel. The cloning was repeated using T4 DNA polymerase (section 2.16.3) to blunt the ends in place of Mung Bean Nuclease and restriction digestion of four recombinant plasmids yielded fragments of the expected sizes. The plasmids containing the PCR products were named pHF17 (containing *Sxl* binding sites) and pHF18 (containing polyA stretches). The plasmids containing the linkers were named pHF19 (containing Sxl binding sites) and pHF20 (containing polyA stretches).

Large scale preparations of plasmid DNA were performed on the four plasmids and pHF17 and pHF19 were microinjected into *D. melanogaster* embryos (section 2.20). Due to time constraints and mechanical failure of the transjector, pHF18 and pHF20 were not microinjected. Three transformants each of pHF17 and pHF19 were generated.

3.4.4 β-Galactosidase Assays of Transgenic Flies Carrying Autosomal Insertions of pHF17

One dose males and one dose females carrying autosomal insertions of pHF17 were assayed for β-galactosidase activity. The results are presented in Table 10. One dose males produced 1.33 ± 0.03, 1.38 ± 0.03, 1.46 ± 0.03 (standardised with total fly weight) and 1.33 ± 0.04, 1.31 ± 0.03 and 1.43 ± 0.04 (standardised with total protein) times the β-galactosidase activity than one dose females.

Table 10. β-Galactosidase Assays of Transgenic Flies Carrying Autosomal Insertions of pHF17

Assays were performed on one dose males and one dose females from three different transgenic lines. Each assay was performed four times and standardised using total protein measurement or total fly wet weight.

Line	Chromo some	Dose Male	Dose Female	M/F Ratio		Standard Error
HF17 #1	3	1	1	Protein	1.33	0.03
				Fly weight	1.33	0.04
HF17 #2	2	1	1	Protein	1.31	0.03
				Fly weight	1.38	0.03
HF17 #3	2	1	1	Protein	1.43	0.04
				Flyweight	1.46	0.03

3.4.5 β-Galactosidase Assays of Transgenic Flies Carrying Autosomal Insertions of pHF19

One dose males and one dose females carrying autosomal insertions of pHF19 were assayed for β-galactosidase activity. The results are presented in Table 11. One dose males produced the same level (within error) of β-galactosidase activity as one dose females.

Table 11. β-Galactosidase Assays of Transgenic Flies Carrying Autosomal Insertions of pHF19

Assays were performed on one dose males and one dose females from three different transgenic lines. Each assay was performed four times and standardised using total protein measurement or total fly wet weight.

Line	Chromo some	Dose Male	Dose Female	M/F Ratio		Standard Error
HF19 #1	3	1	1	Protein	1.00	0.02
				Fly weight	1.01	0.04
HF19 #2	3	1	1	Protein	1.08	0.03
				Fly weight	1.09	0.04
HF19 #3	2	1	1	Protein	1.02	0.02
				Flyweight	1.07	0.01

4. DISCUSSION

4.1 DEVELOPMENT OF THE *arm-lacZ* SYSTEM

The *arm-lacZ* assay system has been developed primarily as a new method to isolate DCREs which will eliminate previous problems encountered in the search for DCREs. Genes studied have often been expressed in a stage and/or tissue specific manner and the level of expression has varied depending on the age of the fly which has made it difficult to compare male and female expression.

β-galactosidase assays of flies carrying *arm-lacZ* showed that *lacZ* was expressed at a sufficient level to be assayed spectrophotometrically. The 1:1 male to female ratio of β-galactosidase activity produced from flies carrying autosomal insertions of *arm-lacZ* showed that *lacZ* was not expressed preferentially in either sex in the head or thorax (section 3.1.1).

Previous attempts at isolation of DCREs have been unsuccessful partly because the genes studied displayed only partial dosage compensation when present as transgenes on autosomes. It is not known whether the incomplete dosage compensation is due to insufficient DCREs being present on the transgene or repression by a negative autosomal chromatin environment. This study also raises the possibility that partial dosage compensation could be due to the presence of *Sxl* binding sites in the 3'UTR. Both *white* and *Pgd* show partial dosage compensation (Qian and Pirrotta, 1995; Scott and Lucchesi, 1991) and each contains *Sxl* binding sites in the 3'UTR. However, *Zw* also contains a *Sxl* binding site in the 3'UTR but does not show partial dosage compensation (Fouts *et al.*, 1988).

arm-lacZ was flanked by the SCS and SCS' insulator elements in an attempt to prevent repression of full dosage compensation if any DCREs should be isolated. β-Galactosidase assays of autosomal insertions of pHF11 ('insulated' *arm-lacZ*) proved that the insertion of SCS and SCS' on either side of *arm-lacZ* did not alter the 1:1 ratio of *lacZ* expression (section 3.2.3).

4.1.1 SCS and SCS' Cannot Block Dosage Compensation.

β-Galactosidase assays of one dose males and one dose females carrying an X-linked insertion of pHF11 revealed that the SCS and SCS' elements cannot block dosage compensation of *arm-lacZ* when in the presence of the X chromosome environment (section 3.2.4). This is in agreement with the results of Kellum and Schedl (1991) and Vazquez and Schedl (1994) who flanked *mini-white* with SCS and SCS' and found that one dose males retained the same eye colour as two dose females. In similar assay, Roseman *et al.* (1995) found that that The *Su(Hw)* binding region also cannot block dosage compensation of *mini-white*. These results suggest that the nature of DCREs in different to that of conventional transcriptional enhancers whose actions can be blocked by both the SCS and SCS' insulators and the *Su(Hw)* binding site (in the presence of *Su(Hw)* protein). The SCS and SCS' elements are thought to organise the 30 nm chromatin fibre into a distinct higher order chromatin domain of independent gene activity. DCREs, when present on the X chromosome, are capable of perturbing this structure. Kellum and Schedl (1991) suggest that the X chromosomal dosage compensation system has a global effect that is epistatic to the effects of insulators such as SCS and SCS'.

4.1.2 Are SCS and SCS' Good Insulators Against Position Effects?

Position effect is the term used to describe the effects that positive and negative regulatory elements that surround the site of insertion have on the expression of a transgene. SCS and SCS' have been reported to protect *mini-white* and *white* from repressive and enhancing position effects respectively (Kellum and Schedl, 1991). When placed between an *hsp70:lacZ* fusion and the upstream *yp-1* enhancer, SCS and SCS' can both block expression of *lacZ* in the fat body and in the same manner they block enhancement of *white* expression by the *white* enhancer (Kellum and Schedl, 1992; Vazquez and Schedl, 1994)

It would be expected that if SCS and SCS' are effective at insulating *arm-lacZ* from position effects that 'insulated' *arm-lacZ* should express approximately the same level of *lacZ* in all four lines (three autosomal and one X-linked). Comparison of the level of β-galactosidase activity (expressed as ΔOD/min/μg protein and ΔOD/min mg fly weight revealed a difference of approximately 3.5 fold between the highest and lowest mean activity (Appendix 2). Given that SCS and SCS are reported to be very effective

insulators against position effects, the variability in expression between the lines may be higher than expected. The fact that the rates vary only approximately three fold in the absence of insulators (Appendix 2) suggests, however, that the *armadillo* promoter may be relatively resistant to position effect (although more lines would have to be generated to show this convincingly). The eye pigment assays used in the study of *mini-white* (Kellum and Schedl, 1991) and the *lacZ* staining used in the experiments with *lacZ* (Kellum and Schedl, 1992; Vasquez and Schedl, 1994) are assessed visually and are not quantitative. The deposition of eye pigments is non linear with respect to gene expression and can reach saturation, therefore the levels of expression after addition of the insulators could still vary widely, but would not be detected. Furthermore, as the *white* promoter is known to be sensitive to position effect (Pirrotta *et al.,* 1985) it is unclear whether the differences in expression they observed were in the region of 1 - 2 fold or 50 fold. In conclusion, because the levels of *lacZ* expression did not vary much before addition of the insulators, it cannot be concluded whether SCS and SCS' were effective at insulating *arm-lacZ* from position effects.

4.1.3 Can SCS and SCS' Protect X-linked Transgenes from the Autosomal Chromatin Environment?

Kellum and Schedl (1991) observed that the eye colour of male transgenic flies carrying the *white* gene flanked by SCS and SCS' was less than that of females carrying two copies, indicating that the gene was not fully dosage compensated. This implies either that the SCS insulators are ineffective at protecting against the negative effects of the chromatin environment, or that *white* contains insufficient DCREs to promote full dosage compensation. The same result was obtained from insulation of *white* with the *Su(Hw)* binding region (Qian and Pirrotta, 1995). In contrast, Roseman *et al.* (1995) reported full dosage compensation of *white* when flanked with *Su(Hw)* binding region. In the absence of the *Su(Hw)* binding protein, only partial dosage compensation was achieved. The only difference between the two experiments was that 8 kb of X-linked *yellow* gene was present upstream of *Su(Hw)* binding sites and *white* in Roseman *et al.*'s study. It can be argued that the extra X-linked sequence promoted dosage compensation of *white* and that incomplete dosage compensation occurred in the absence of *Su(Hw)* due to the negative autosomal chromatin environment. It is also conceivable that the additional X-linked sequence assisted

insulation of *white* by providing an X- chromatin environment between autosomal chromatin and *mini-white*, which lessen the repressive effect that the autosomal chromatin environment previously had on *mini-white*. The SCS and SCS' insulators were used exclusively in this study as the *Su(Hw)* insulators could not be obtained.

4.2 ISOLATION OF DCRES

Transformants of eight constructs containing fragments of sizes 5 - 15.4 kb cloned into pHF11 were obtained by P-element mediated germline transformation (section 3.3). Results from β-galactosidase assays performed on one dose males and one dose females from each transgenic line revealed a 1:1 male to female ratio (section 3.3), therefore no DCREs were isolated. Homozygous (two dose) males and females carrying an autosomal insertion of 144A7 12.8 also expressed the same level of β-galactosidase (Appendix 2). There are three alternative hypotheses to explain why no DCREs were isolated:

1. The X-linked sequences were chosen from areas of the X that are devoid of genes
2. There were genes were present in the X-linked fragments, but there were no DCREs present
3. DCREs were present within the X-linked fragments but could not confer dosage compensation onto *arm-lacZ*. These three possibilities will be discussed in turn.

4.2.1 Are Genes Present Within the X-linked Sequences?

The seven cosmids from which the X-linked fragments were taken have not yet been sequenced therefore the nature of the sequences is unknown. The *Appl* gene which codes for a beta amyloid protein precursor does, however, map to region 1B3-1B10 which is contained within the X-linked DNA from cosmid 11E3.

One third of the *D. melanogaster* genome is heterochromatic, the majority being constitutive heterochromatin which surrounds the centromeres and is present as large blocks over half the X the whole Y chromosome. The region 1A - 1B of the X where the X-linked fragments map to is not considered to contain constitutive

heterochromatin (Hartl and Lozovskaya, 1995) however this does not exclude the possibility that there are small regions of β-heterochromatin within the regions of X-linked DNA chosen for this study. Of the remaining euchromatic regions (which contain the vast majority of the coding regions) 80% contains single copy DNA and 20% consists of moderately repeated sequences (Hartl and Lovoskaya, 1995). It has been concluded from reassociation kinetics that there are approximately 16 000 different transcripts produced from the *D. melanogaster* genome during the life of a fly, or 1 per 7 kb of euchromatin (Levy *et al.,* 1981). If each transcript was from a different coding unit this would correspond to 1 gene per 7 kb of genomic DNA. The same estimate was obtained from analysis of the 315 kb region containing the *rosy* and *Ace* genes which gives rise to 43 transcripts - or 1 per 7 kb (Hall *et al.,* 1984; Gausz *et al.* 1986; Bossy *et al.,* 1984). Two independent insertions of autosomal genes into region 1F have become partially dosage compensated (Spradling and Rubin, 1983) suggesting this area is euchromatic (P elements insert preferentially into euchromatin) and that there may be DCREs present in the surrounding regions. By putting these data together and considering that, in total, 70 kb of X-linked DNA from the seven cosmids was screened, it is very unlikely that all the X-linked sequences were derived from non coding heterochromatic DNA.

An alternative hypothesis is that there are no DCREs associated with any genes present on the X-linked fragments. Most X-linked genes that have been studied have been found to be dosage compensated, however a small number of structural genes lack dosage compensation (Lucchesi and Manning, 1987). It is admissible that the X-linked sequences could be derived from a region containing non dosage compensated genes, however there is no evidence of non dosage compensated genes being clustered in a region. Moreover, non dosage compensated genes can reside next to dosage compensated genes (Ghosh *et al.,* 1992). It seems remote that the lack of identification of DCREs is due to all the X-linked fragments containing non dosage compensated genes. Assuming, therefore, that dosage compensated genes are present but do not contain DCREs, then the DCREs would be much rarer than previously thought. This is in direct contrast to many studies in which X-linked transgenes transposed to autosomes remain at least partially dosage compensated, which indicates that they contain DCREs in their coding and/or regulatory regions.

The third hypothesis is that DCREs are present within the X-linked fragments, but cannot confer dosage compensation onto *arm-lacZ*. One explanation that would account for this phenomenon is that one or more of the *roX* RNAs is required in *cis* for full dosage compensation to occur. *roX1* and *roX2* are X-linked non-coding RNAs whose expression is dependent on the *msls* (Amrein and Axel, 1997; Meller *et al.*, 1997) *roX1* localises to the X chromosome in a similar pattern to that of MSL-2. Both *roX1* and *roX2* are not essential for dosage compensation, but Meller *et al.* (1997) postulate that there is a family of non-homologous RNAs including *roX1* and *roX2* that are functionally redundant. *roX1* does have the ability to bind to the X chromosome in *trans* (Meller *et al.*, 1997), however whether *roX2* can also bind in *trans* has not yet been examined. Early studies of the *cis*-acting regulatory elements required for dosage compensation focused on large translocations of hundreds of kilobases of X-linked DNA to the autosomes. Tobler *et al.* (1971) reported full dosage compensation of the X-linked v^+ gene which was carried on the T(1;3)ras translocation (regions 9F to 10C 1-2 of the X translocated to chromosome three). *roX2* is situated at region 10B within the translocated fragment. It is an attractive hypothesis that the presence of *roX2* may have contributed to the complete dosage compensation of v^+. Further study of the roles of *roX1* and *roX2* and identification of additional non coding RNAs that are dependent on the MSLs will shed more light on the contribution of non coding RNAs to dosage compensation.

Another possibility is that there are two types of DCRE: the first type are sequences that are tightly linked to the dosage compensated gene such as those present within the *white* gene (Levis *et al.*, 1985; Pirrotta *et al.*, 1985; Qian and Pirrotta, 1995). These sequences may not be sufficient to confer full dosage compensation and in the case of the *arm-lacZ* assay system, are not 'strong' enough to confer dosage compensation onto *arm-lacZ*. When X-linked transgenes are relocated to ectopic sites on the X they remain fully or even hyper dosage compensated due to the X-chromatin environment. Full dosage compensation may be assisted by the more decondensed state of the X chromosome and possibly the presence of the *roX* RNAs in *cis*. It may also be possible that there are DCREs present along the X chromosome that act over longer distances, perhaps similarly to a transcriptional enhancer although these elements can in some manner perturb higher order chromatin structures to bring about dosage

compensation of X-linked genes. Autosomal transgenes of >8 kb have been reported to become dosage compensated when transduced to the X chromosome (Spradling and Rubin, 1983), suggesting that these sequences can act over at least 8 kb, although their range is probably not over hundreds of kb, as an autosomal gene present on a translocation to the X did not become dosage compensated (Roehrdanz *et al.*, 1976). As these sequences would be rarer than other DCREs which are tightly linked to genes, it would be more difficult to isolate them, simply because they occur less often and may be some distance from the gene(s) they act upon. The full dosage compensation of the v^+ gene observed by Tobler *et al.* (1971) may also be attributed to the presence of sequences that act over longer distances(s) as part of the large translocation. Partial dosage compensation of X-linked genes transduced to the autosomes may be attributed to the repressive autosomal chromatin environment, however, another consideration is that there no are sequences present that can act over long distances to confer full dosage compensation.

4.2.2 What Could be the Nature of DCREs?

The nature of the DCREs is still a mystery. They may act as multiple of different elements specifically positioned around each X-linked gene to confer optimal dosage compensation to that gene, possibly by increasing transcriptional initiation and/or elongation. These may be short oligonucleotide sequences that are not easily identifiable on their own. Ghosh *et al.* (1989) reason that the promoters of all the genes on the X represent a 'spectrum of different strengths'. As dosage compensation always leads to a two fold increase in male X-linked gene expression, this may require a differential set of interactions between promoters and DCREs for different genes (Ghosh *et al.*, 1989).

There may be other DCRE sequences that can act over longer distances to achieve full dosage compensation. These may be short sequences such as mono- and/or di-nucleotide repeats which have been correlated with dosage compensation (Pardue *et al.*, 1987). Binding of specific proteins to these could open the chromatin structure allowing access of the MSLs to DCREs around the target gene. Binding of the MSLs would then facilitate further alteration in chromatin structure by histone acetylation

via MOF which would then assist transcription factors to gain access to their respective promoters.

Lyman *et al.* (1997) suggest that there is a subset of DCREs with high affinity for the MSLs. The MSL complexes would be assembled at these high affinity sites and only fully assembled complexes would then have the ability to associate with the rest of the MSL bindings sites which possess a lower affinity for the MSL complex. Perhaps these high affinity DCREs must be present in within the X-linked fragments to achieve dosage compensation of *arm-lacZ* on an autosome.

4.2.3 Conclusion and Future Work

Ten constructs containing X-linked DNA plus *arm-lacZ* flanked by SCS and SCS' insulators have been generated and nine were microinjected into *D. melanogaster* embryos. β-galactosidase assays of the transgenic lines showed that no DCREs were identified that could confer dosage compensation onto *arm-lacZ*. Although no DCREs were isolated, the *arm-lacZ* system has proved useful in providing further insight into the nature of DCREs. If DCREs are present though the coding, intronic and flanking regulatory regions of genes as some researchers have reported (Levis *et al.*, 1984; Qian and Pirrotta, 1995) then it would be expected that some would be present within the ~63 kb of X-linked DNA that has been screened in this study. If indeed present, these DCREs must not be capable of conferring dosage compensation onto *arm-lacZ*. This raises the possibility that there could be a second type of DCRE that is rarer and acts over longer distances to confer dosage compensation, such as that conferred onto autosomal genes when transduced to autosomes. It may be a requirement that this type of DCRE must be present to achieve full dosage compensation of an X-linked transgene when relocated to an autosome.

Another possibility is that *roX* RNA(s) are required in *cis* for full dosage compensation to occur. The next step of this study will be determine whether any of the X-linked sequences can confer dosage compensation onto *arm-lacZ* when the *roX* RNAs are present in *cis*. DNA encoding the *roX* RNAs can be obtained. Transgenic flies carrying autosomal insertions of *roX1* and *roX2* will be generated by P-element mediated germline transformation and the insertion will be mapped to a chromosome

arm by *in situ* hybridisation. The chromosome arms to which the various pHF11 constructs containing the X-linked fragments will also be mapped. Transgenic flies carrying a *roX* RNA plus a construct containing X-linked DNA on the same arms of sister chromatids will be generated by genetic crosses. Because the sister chromatids associate very tightly together in *D. melanogaster,* the *roX* RNAs may be able to facilitate dosage compensation of *arm-lacZ* even though not in *cis* on the same chromatid. If β-galactosidase assays of one dose males and females do not show dosage compensation, each *roX* RNA can be placed, by recombination, onto the same chromatid arm as the construct containing the X-linked DNA. As the *roX* RNA and the construct containing the X-linked DNA both carry the w^+ marker which produces orange eyed flies, a recombination event can be detected by the selection of flies with a darker red eye colour. β-Galactosidase assays of one dose males and one dose females will determine whether the *roX* RNAs are required in *cis* for dosage compensation to occur.

If rare DCREs exist that act over longer distances, more X-linked fragments will need to be tested. Indeed, the 63 kb of X-linked sequences that have been tested so far constitute only ~0.3% of the X chromosome (Heino *et al.*, 1994). Additional portions of X-linked DNA from euchromatic areas that the MSLs antibodies bind could also be obtained and tested. Of particular interest are a subset of sites to which MSL-1 and MSL-2 bind in the absence of MSL-3 and MLE, as these are considered to be high affinity binding sites for the MSL-1:MSL-2 complex (Lyman *et al.,* 1997).

4.3 EXAMINATION OF DOSAGE COMPENSATION INVOLVING *Sxl*

A second method of dosage compensation has been proposed to occur in *D. melanogaster* in which *Sxl* protein down-regulates the expression of a subset of X-linked genes in females, to half the level of male expression. *Sxl* protein is thought to somehow prevent translation through binding to polyU stretches in the 3'UTR of female mRNA. The aim of this study was to determine if *Sxl* could down-regulate a gene in females at the adult stage, purely by the addition of three *Sxl* binding sites to the 3'UTR.

4.3.1 *Sxl* Binding Sites Derived from the *runt* 3'UTR can Partially Down-Regulate *lacZ* in Females

β-Galactosidase assays of one dose flies carrying autosomal insertions of pHF17 revealed that the β-galactosidase activity in males was an average of 1.31 to 1.46 times that of female flies for the three lines (section 3.4.4). This corresponds to 15.5 to 23 % down-regulation of *lacZ* in females which shows that the presence of *Sxl* binding sites in the 3'UTR can promote down-regulation by *Sxl* protein. This is, however, less than half of the repression required to equalise the male and female dosage of *lacZ*.

4.3.2 Why did Only Partial Repression Occur?

The *runt* PCR product containing the *Sxl* binding sites was inserted into a convenient restriction site in the *lacZ* 3'UTR (section 3.4.1). Topologically, this may not have been optimal for the mechanism of repression by *Sxl*. If *Sxl* acts to prevent translation by altering the conformation of the mRNA, e.g. to prevent the mRNA from loading onto the ribosome, then the position of the sites within the 3'UTR may be crucial to the formation of the correct secondary structures. The distance between the polyA site and the *Sxl* binding sites may also alter the topology. The first *Sxl* binding site is 71 bp from the SV40 polyA site, however in its natural state, the first *Sxl* binding site is a similar distance of 64 bp from the *runt* polyA site. The topology may also be affected by the distance between the *Sxl* binding sites and the stop codon which is 495 bp in *runt* mRNA and 1007 bp in pHF17.

Another possibility that may account for the lack of repression is that there may be repressers within the SV40 3'UTR that inhibit the action of *Sxl* protein. Alternatively, in the natural *runt* 3'UTR there may be binding sites outside the region of the 170 bp fragment for specific proteins that interact with *Sxl* protein to increase its efficiency at preventing translation.

It could be argued that there are sufficient *Sxl* binding sites present that are in the correct part of the 3'UTR to down-regulate *lacZ* two-fold, but the amount of *Sxl* protein is limiting, which causes males to express less than twice the level of β-galactosidase than females. If this is the case, then *Sxl* would be even more limiting in

female flies homozygous for pHF17 thus the male to female ratio would be even lower than that produced by flies carrying pHF17. β-galactosidase assays were performed on two dose males and females. Males produced 1.28 to 1.49 times the level of β-galactosidase (Appendix 2), therefore it is not a lack of *Sxl* protein that is causing the partial down-regulation.

4.3.3 The Distance Between the *Sxl* Binding Sites May Affect the Ability of *Sxl* to Down-regulate

β-Galactosidase assays of one dose male and female flies carrying autosomal insertions of pHF19 showed, surprisingly, that males and females expressed the same amount of β-galactosidase (section 3.4.5). The $A(U)_7$ motif is though to have a strong affinity for *Sxl* protein (Samuels *et al.*, 1994) and two such sites in the 3 ' UTR of *msl-2* RNA act in concert with longer polyU stretches to fully repress MSL-2 expression in females. The $A(U)_8$ motif present in three copies as part of the synthetic oligonucleotide should, therefore, be at least adequate, if not optimal for binding of *Sxl*. The possibility exists that because the binding sites are only seven bp apart, they may be too close together for efficient binding of the *Sxl* protein molecules. In the *runt* 3 ' UTR, two of the *Sxl* binding sites are only eight bases apart, which suggests that the close proximity doesn't matter. The third site is 69 bases away from the next closest *Sxl* binding site. It is possible that two but not three *Sxl* binding sites can be in such close proximity. A similar situation exists with some other mRNAs which contain three or more *Sxl* binding sites (Appendix 3). *cut* mRNA (database name DMCUT) has an $A(U)_7$ and a $(U)_9$ site separated by only one base with 339 bases to the next closest site. *fsh* mRNA (database name DROFSHB) has two bases separating a $(U)_{11}$ and an $A(U)_{11}$ site with 437 bases to the next site and *gs2* mRNA (database name DMGS2) has one base separating a $(U)_8$ and a $(U)_9$ with 30 bases to the third site. It is difficult to speculate whether the two sites that are close together are as efficient in binding *Sxl* protein as the distant site. The closest that the three sites are found together is in *otd* mRNA (database name DMOTD) which has a long $(U)_{20}$ stretch separated from the other $(U)_9$ site by 30 bases.

4.3.4 The Length, Position and Number of *Sxl* Binding Sites Varies Widely Between Genes

In the initial computer search by Kelley *et al.* (1995) for mRNAs with three or more *Sxl* binding sites in their 3 ' UTRs, a *Sxl* binding site of $(U)_{15}$ or longer (such as that found in *otd* mRNA) was considered to be two sites. It is unknown whether two *Sxl* proteins can bind this polyU stretch. Further *in vitro* binding assays similar to those of Samuels *et al.* (1994) would increase understanding of this and may also shed light on whether longer polyU stretches have a higher affinity for *Sxl* protein and are, therefore, more effective at repression of translation. Mutation of the $A(U)_{12}$ site in the *msl-2* 5 ' UTR results in a higher level of MSL-2 protein in females than mutation of the $A(U)_{10}$ site. This could be interpreted to mean that a longer polyU stretch is more efficient in repression of translation, however the difference in repression could be attributed to the different positions of the *Sxl* binding sites within the 5 ' UTR.

Among nineteen of the X-linked genes reported to contain three or more *Sxl* binding sites (Kelley at al., 1995), there is considerable variability in the number of sites, the length of the polyU stretches, the distances between the sites, the distance to the polyA site and the distance to the stop codon. Comparison of the nineteen sequences (Appendix 3) did not reveal any apparent correlation between any of these variables. This suggests that if, *in vivo*, these genes are down regulated two-fold by *Sxl* as proposed by Kelley *et al.*, (1995) then each gene must have its own intrinsic system of achieving the two-fold decrease in translation. This would undoubtedly be influenced by all the variables mentioned as well as the effects of other general activators and repressers and the length of the transcript.

4.3.5 How does *Sxl* Protein Promote a Two-Fold Decrease in Translation?

Translational repression of *msl-2* has been suggested to occur via the binding of *Sxl* to the two ends of the *msl-2* RNA which causes circularisation of the RNA and blocks access of the translational machinery (Bashaw and Baker, 1997). Seven of the nineteen X-linked transcripts with three or more *Sxl* binding sites in their 3 ' UTR also have one site in their 5 ' UTR and *fog* mRNA has two, however the majority of the transcripts do not. It is possible though, that although most of the genes do not have *Sxl* binding sites in their 5 ' UTRs, there may be binding sites for other factors that *Sxl*

protein can interact with to assist in alteration of the secondary structure of the mRNAs.

The two *Sxl* binding sites in the 5'UTR of *msl-2* are more efficient in repression of translation than the four sites in the 3'UTR (Bashaw and Baker, 1997; Kelley *et al.*, 1997). As *msl-2* translation is completely repressed by *Sxl*, it seems feasible that when *Sxl* binding sites are only present at the 3'UTR they may not be able to form the mRNA into such a structure that is able to block the translational machinery as efficiently as circularised mRNA, thus some of the transcripts can be translated.

4.3.6 Conclusion and Future Work

It has been suggested that dosage compensation via *Sxl* only occurs in the embryo before the MSLs become active at early gastrulation (stage six of embryogenesis) (Franke *et al.*, 1995). The results presented in this study show that *Sxl* can partially dosage compensate *arm-lacZ* in adult females when *Sxl* binding sites are present in the 3'UTR. Provided the adult mRNA contains *Sxl* binding sites in the 3'UTR, it is a strong possibility that a subset of X-linked genes may be dosage compensated by this alternative method. Of the nineteen genes that have three or more *Sxl* binding sites in their 3'UTRs, most are expressed after early gastrulation when the MSLs become active (Bogaert *et al.*, 1987; Ciazzi *et al.*, 1990; Costa *et al.*, 1994; Finkelstein *et al.*, 1990; Higashijima *et al.*, 1992; Ishimaru *et al.*, 1993; Ramos *et al.*, 1993; Yang *et al.*, 1991; Yee *et al.*, 1993). It would be of interest to find whether these genes are also dosage compensated in an *msl* mutant background.

Although dosage compensation of *arm-lacZ* was observed in females, only 31 - 46% of the expected down-regulation occurred when a 170 bp fragment containing *Sxl* binding sites from the *runt* 3'UTR were placed in the *lacZ* 3'UTR (section 3.4.4). This result suggests that either the *Sxl* binding sites in the 3'UTR alone are not sufficient for a two-fold decrease in female protein expression, or that topologically the sites were in an inopportune position in the 3'UTR. In addition, no dosage compensation occurred when synthetic *Sxl* binding sites were introduced on a linker which implies that either additional 3'UTR sequence is required to achieve the same

level of down-regulation as that achieved by the 170 bp *runt* sequence, or that, topologically, the sites were too close together to allow optimal activity of *Sxl* protein.

The curious finding that the *Sxl* binding sites from *runt* mRNA can partially down-regulate *lacZ* in females, but the closely spaced synthetic *Sxl* binding sites cannot, allows a whole range of different experiments to be performed in an attempt to understand this phenomenon.

The first experiment that must be undertaken is to inject the 'negative control' pHF18 construct which contains 170 bp of *runt* 3'UTR in the orientation that produces polyA stretches instead of the three *Sxl* binding sites. If β-galactosidase assays of males and females carrying one dose of this construct show a 1:1 male to female ratio, then it can be confirmed that the down-regulation in females was caused by the presence of the three *Sxl* binding sites.

To prove convincingly that the three *Sxl* binding sites act together to down-regulate *lacZ* in females, one of the *Sxl* binding sites could be deleted. A fragment of the *runt* 3'UTR could be PCR amplified with the *runt*1 primer and new primer which would be designed to bind somewhere in the 69 bases between the second and third *Sxl* binding sites. By this method, only the first and second *Sxl* binding sites would be amplified by PCR. The PCR product would be cloned into pHF12 as described previously (section 3.4.2).

The partial down-regulation of *lacZ* by the *Sxl* binding sites from the *runt* 3'UTR could have been due to a lack of other binding sites present elsewhere in the *runt* 3'UTR that are required for a two-fold down regulation of *lacZ* in females. This could be tested by replacement of the SV40 3'UTR with the entire *runt* 3'UTR.

It is also a possibility that elements within the 5'UTR are required for two-fold down regulation of *lacZ* in females. Seven of the nineteen mRNAs containing three or more *Sxl* binding sites in their 3'UTRs also contain a *Sxl* binding site in their 5'UTR and one contains two (Appendix 3). A *Sxl* binding site could be inserted into the *arm-lacZ* 5'UTR to determine whether this aids down-regulation by *Sxl*. Furthermore, the

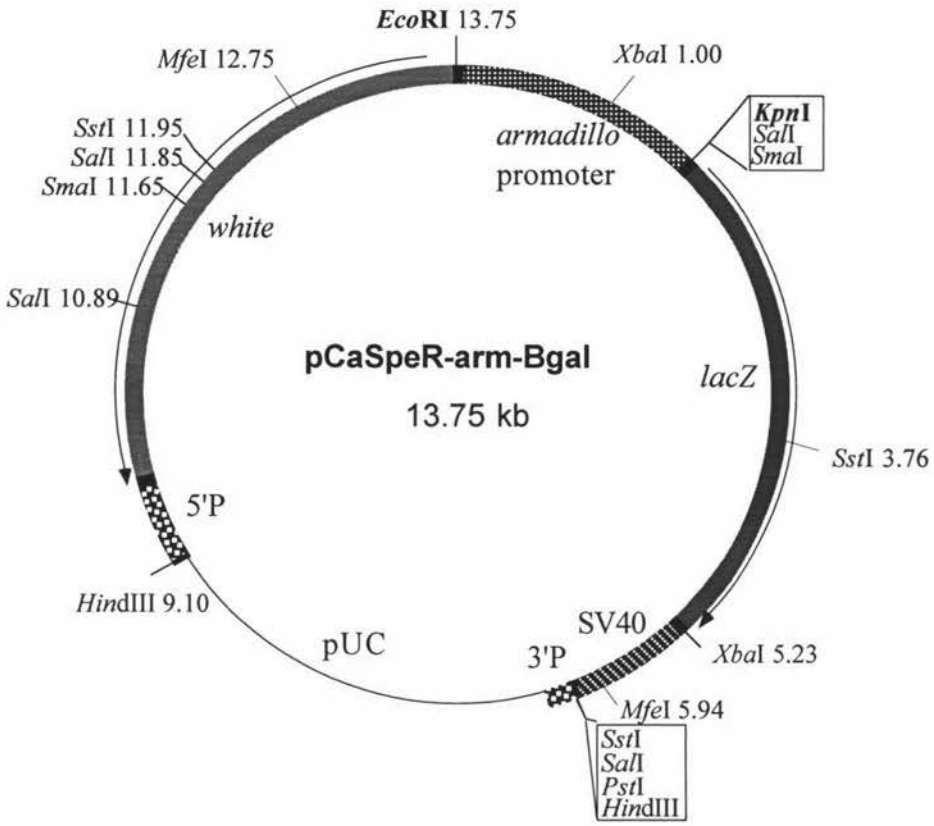
whole *runt* 5 ' UTR could be inserted into the *arm-lacZ* 5 ' UTR to determine whether there are other factors that bind the 5 ' UTR to assist *Sxl* in down-regulating *lacZ*.

To investigate whether the lack of down-regulation by the synthetic *Sxl* binding sites was due to incorrect spacing between the sites, spacer DNA could be inserted into either the *Spe*I or the *Nhe*I site, both of which are positioned between the *Sxl* binding sites (neither of these restriction enzymes cut elsewhere in pHF19) to increase the distance between two of the sites.

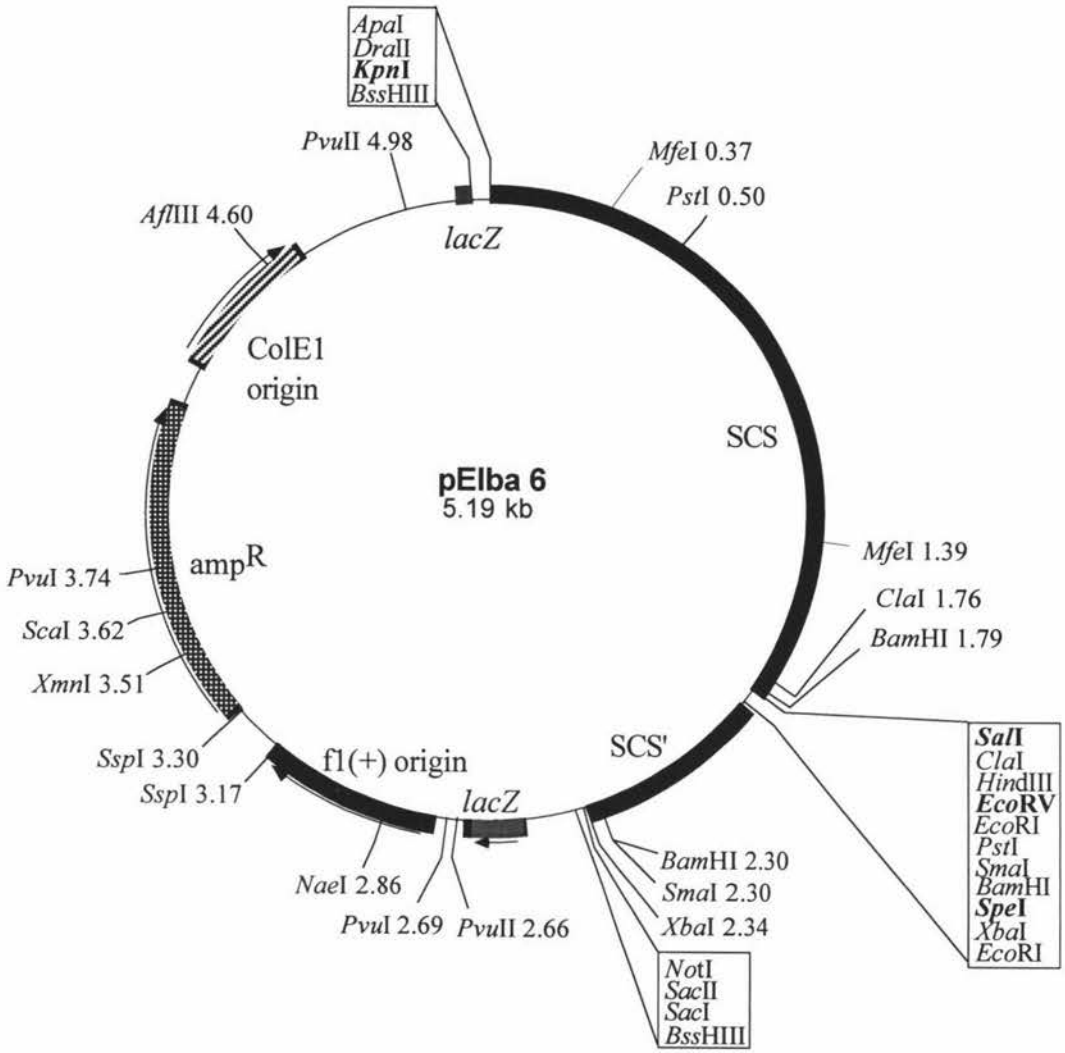
Lastly, to determine whether longer *Sxl* binding sites are more effective at repressing *lacZ* in females, pHF19 could be digested with *Nhe*I and *Spe*I which would remove the middle *Sxl* binding site. This would allow the insertion of an *Nhe*I/*Spe*I linker containing a longer *Sxl* binding site.

5. APPENDICES

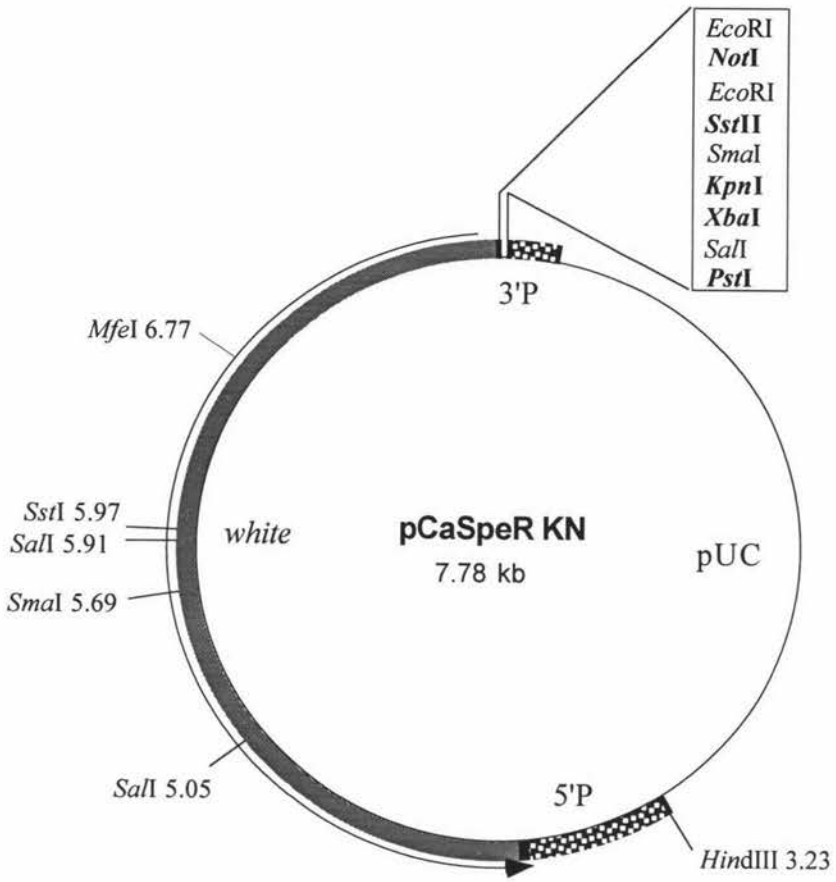
APPENDIX 1. Physical Maps of Plasmids used in this Study
Unique restriction sites are indicated in bold



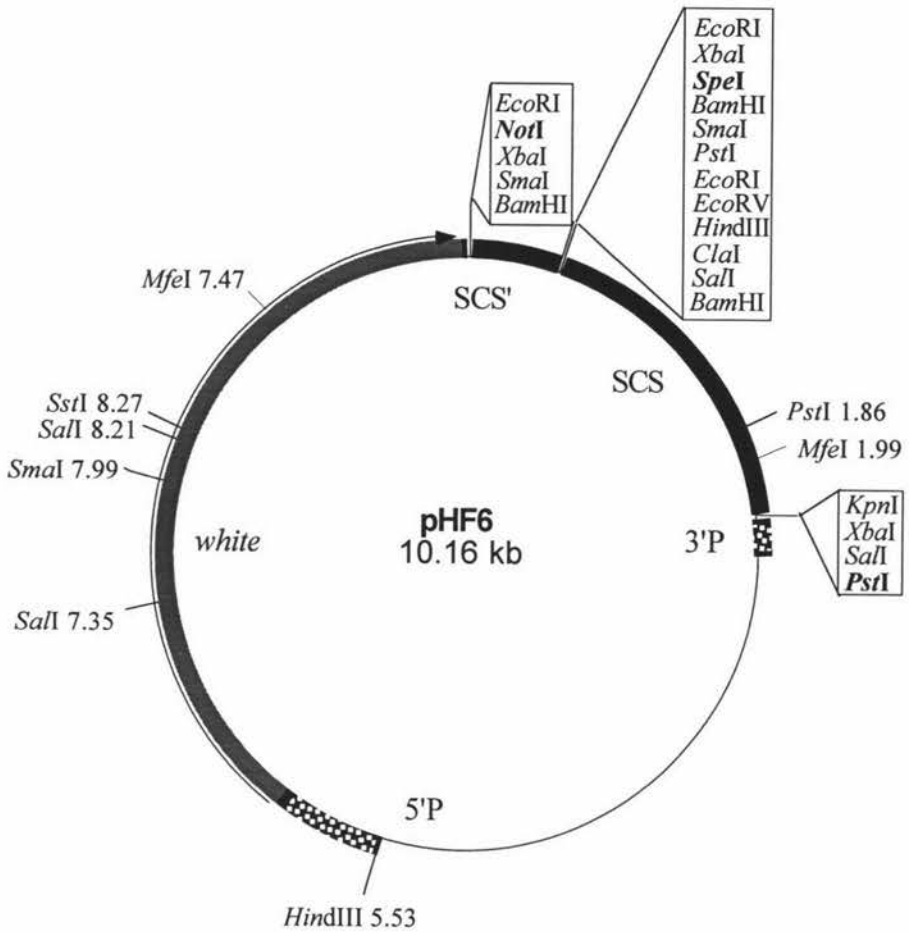
Physical Map of pCaSpeR-arm-Bgal
13.8 kb, pCaSpeR (pUC, *mini-white*, P-element 5' and 3' ends) containing 1.7 kb *Eco*RI/*Kpn*I *armadillo* promoter and 4.4 kb *lacZ* with SV40 3'UTR



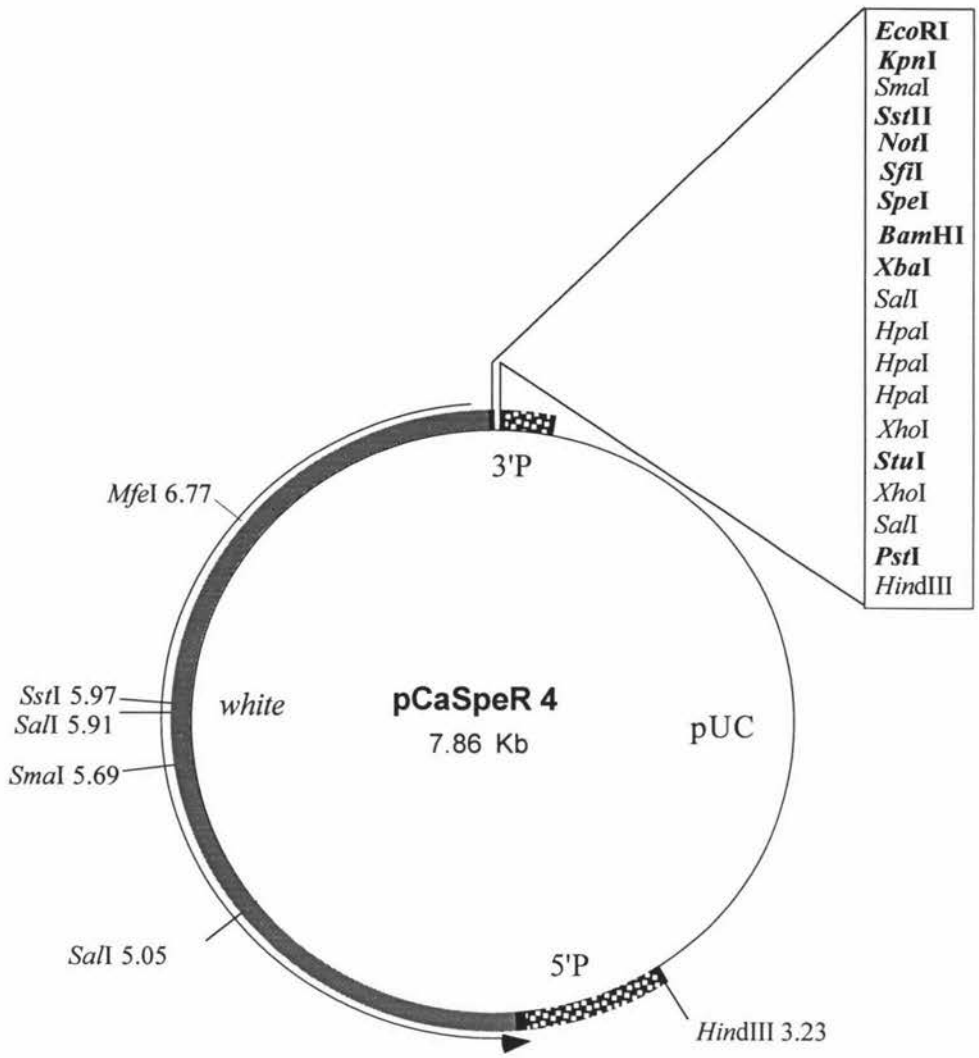
Physical Map of pElba 6
5.2 kb, pBluescript II KS+ containing 1.8 kb SCS in *Xho*I site and 0.5 kb SCS' in *Xba*I site



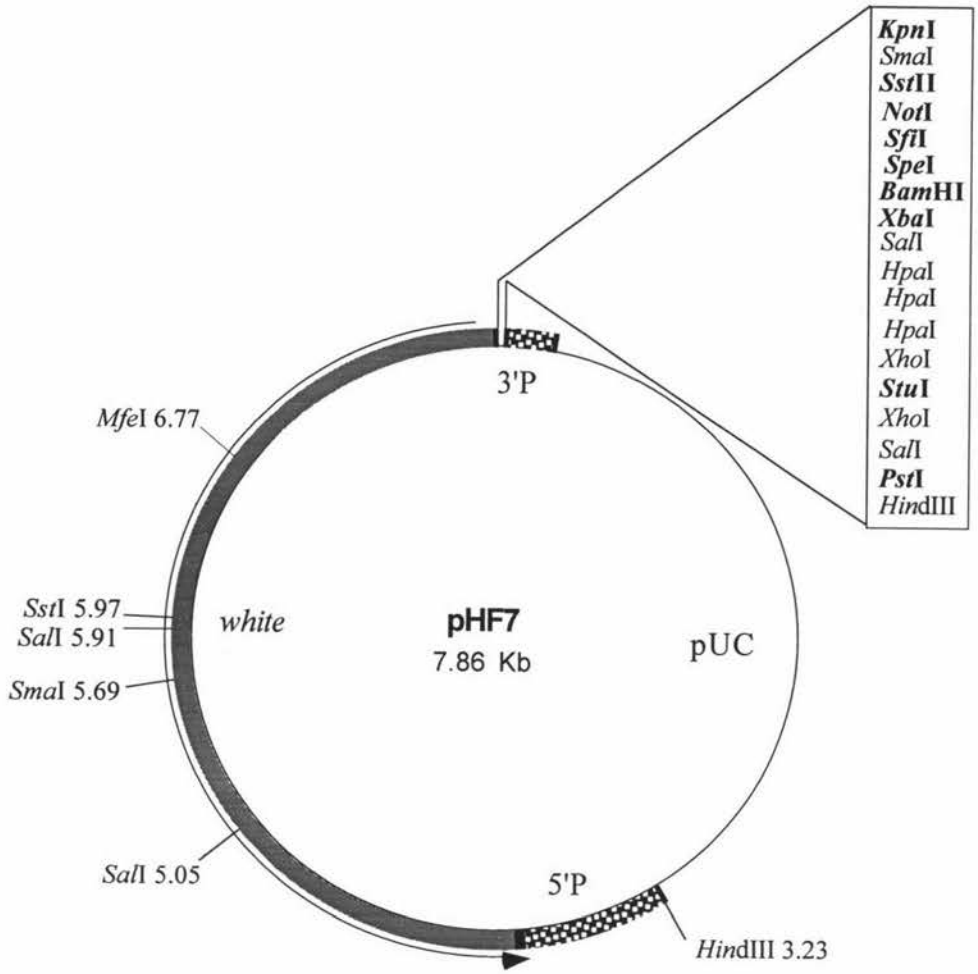
Physical Map of pCaSpeR KN
7.9 kb, pCaSpeR with *Kpn*I and *Not*I sites added and *Hin*dIII and *Bam*HI sites removed



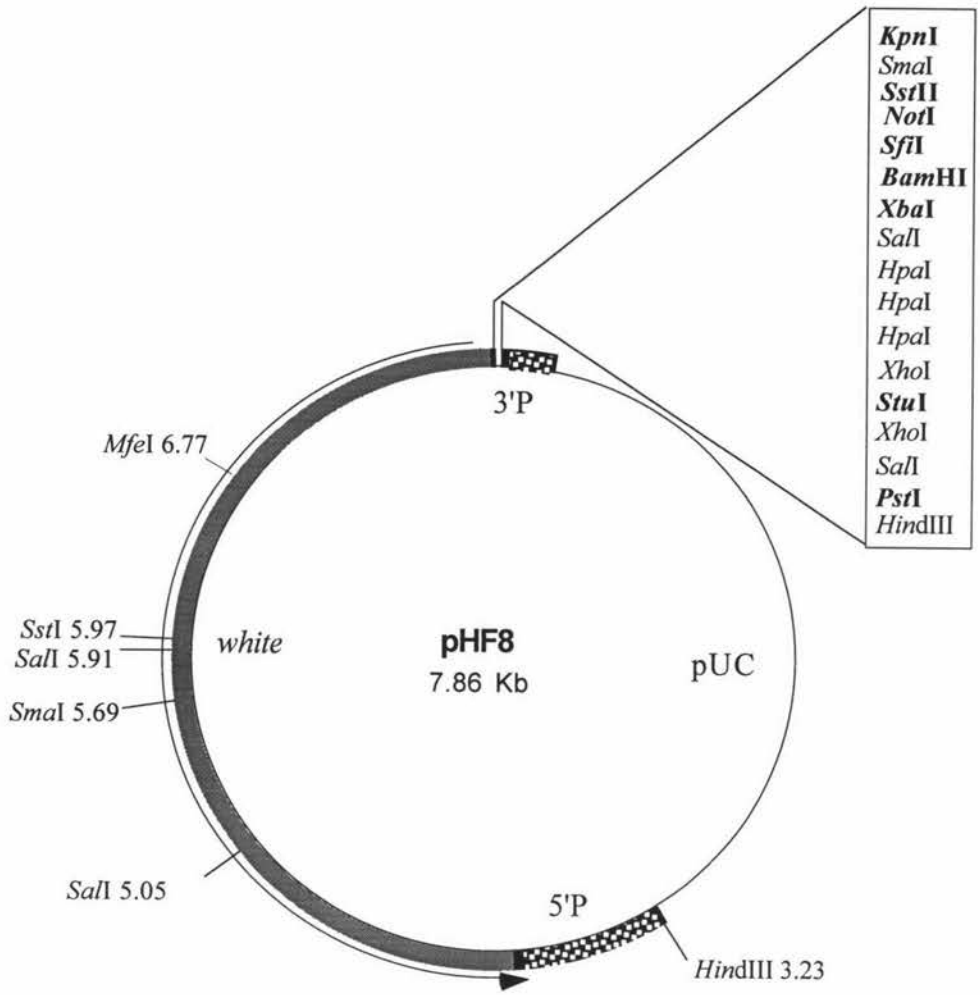
Physical map of pHF6
10.3 kb, pCaSpeR KN containing 2.4 kb *Kpn*I/*Not*I SCS-SCS' fragment from pElba 6



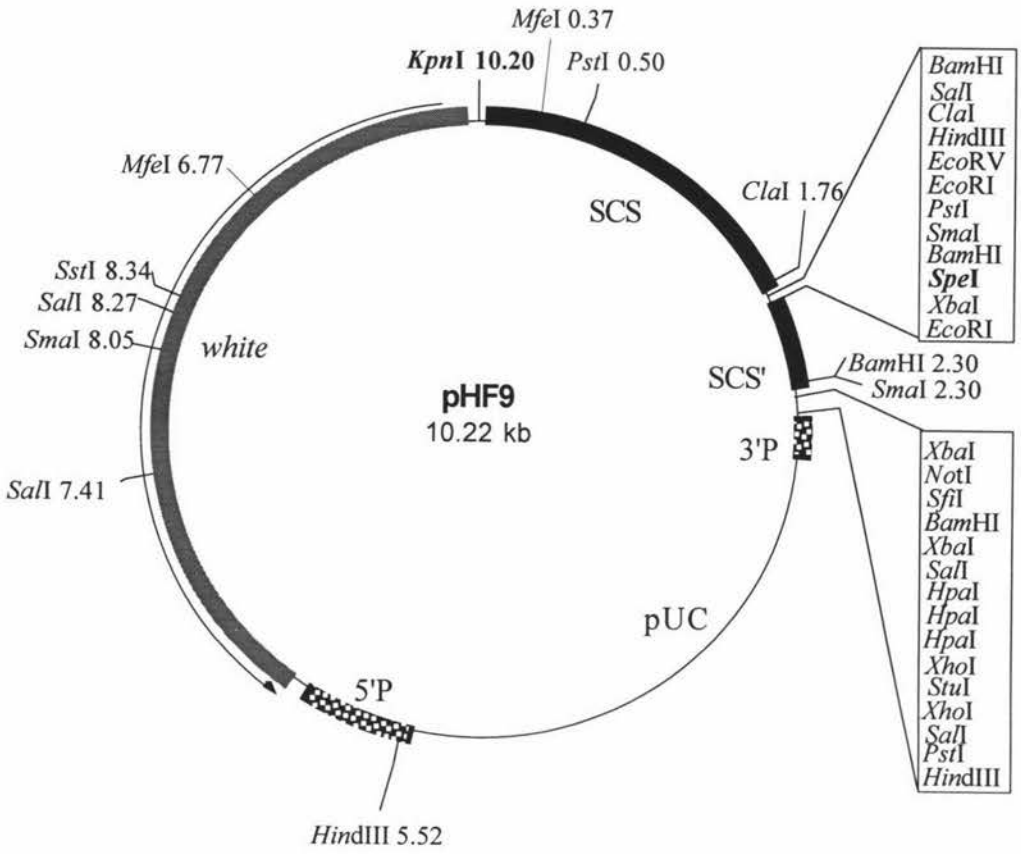
Physical Map of pCaSpeR4
7.9 kb, pCaSpeR with additional *Xho*I (x2), *Stu*I, *Hpa*I (x3), *Sal*I, *Spe*I, *Sfi*I, *Not*I, *Sst*II and *Kpn*I sites with *Sst*I site removed



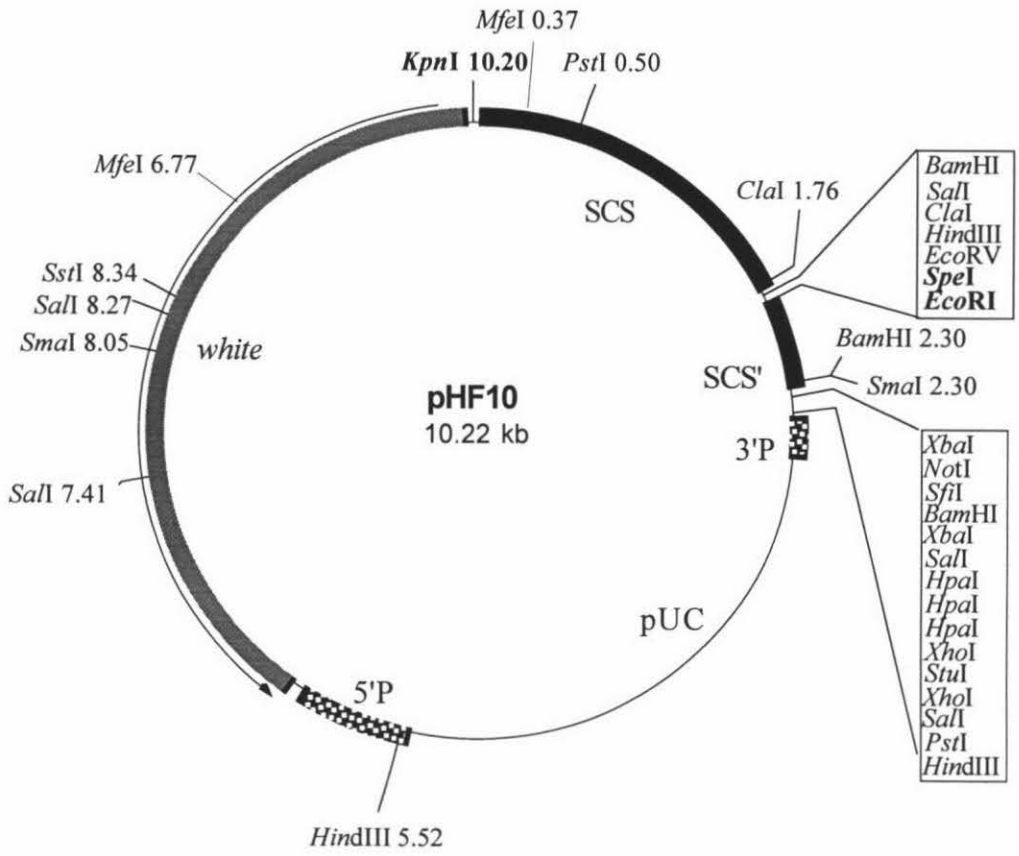
Physical Map of pHF7
7.9 kb, pCaSpeR4 with *Eco*RI site removed



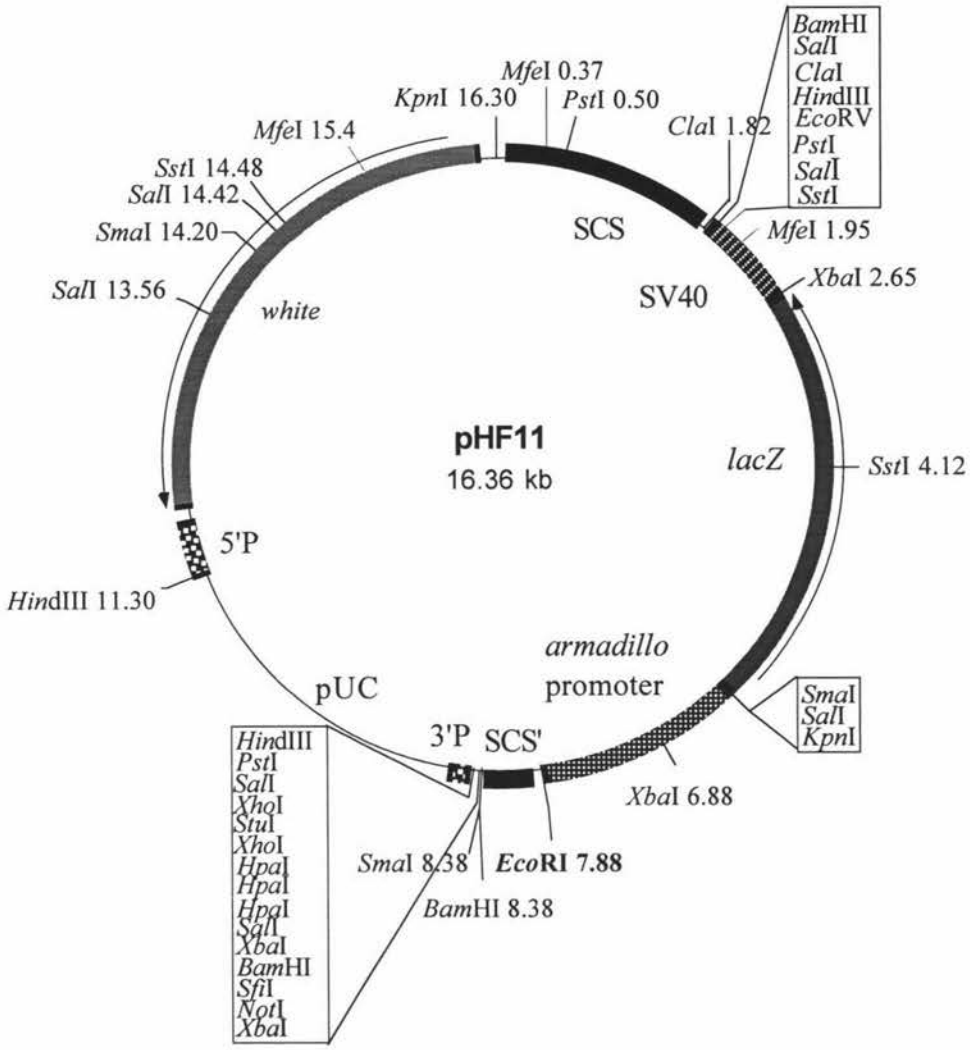
Physical Map of pHF8
7.9 kb, pCaSpeR4 with *Eco*RI and *Spe*I sites removed



Physical Map of pHF9
10.2 kb, pHF8 containing 2.4 kb *Kpn*I/*Not*I SCS-SCS' fragment from pElba6

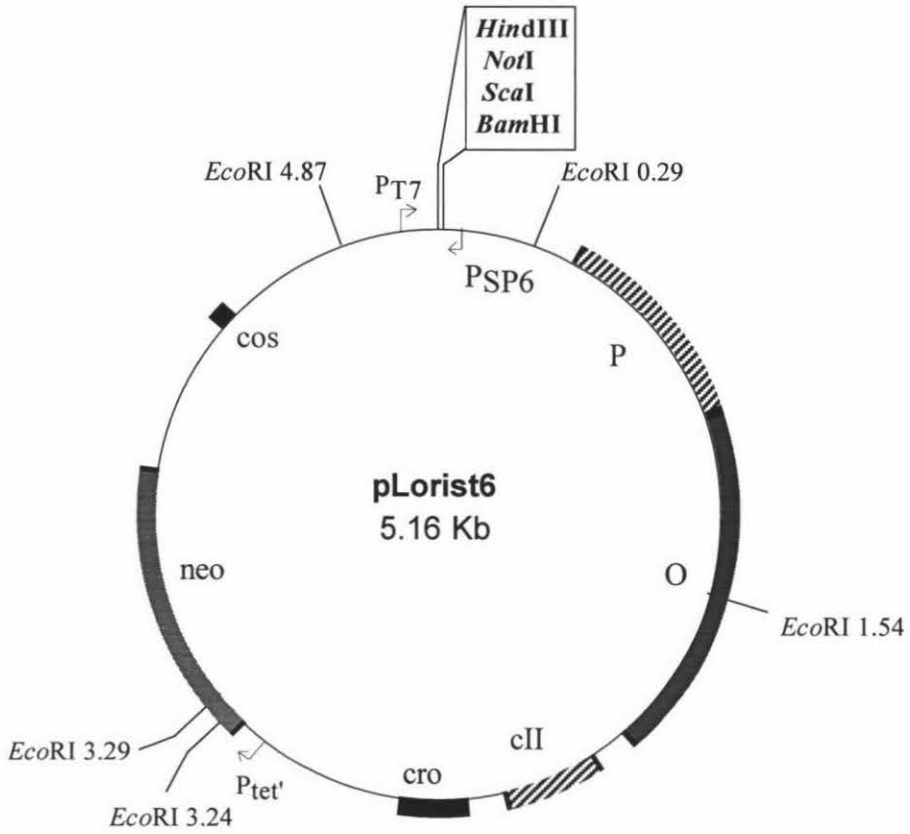


Physical Map of pHF10
10.2 kb, pHF9 containing *Eco*RI-*Spe*I linker



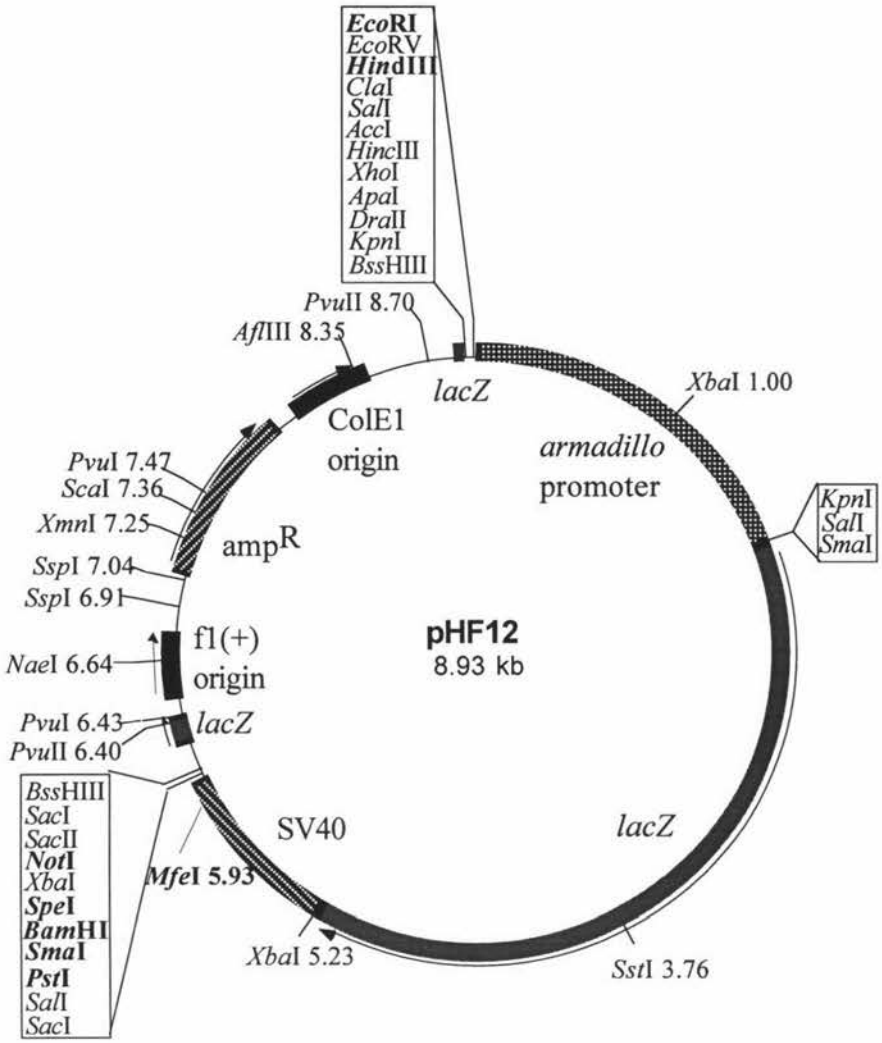
Physical Map of pHF11

16.2 kb, pHF10 containing 6.2 kb *Eco*RI/*Hin*dIII *arm-lacZ* in *Eco*RI/*Spe*I sites. 4.4 - 17.5 kb *Eco*RI fragments of *D.melanogaster* X chromosomal DNA were inserted into the *Eco*RI site of pHF11 to form the plasmids p23E12 7.7, p23E13 4.4, p185B11 6.6, p185B11 5.0, p24F3 7.4, p154H3 17.5, p11E3 6.2, p144A7 12.8 and p123B12 9.0. The X chromosomal DNA was derived from pLorist 6 based cosmids (see physical map of pLorist 6)



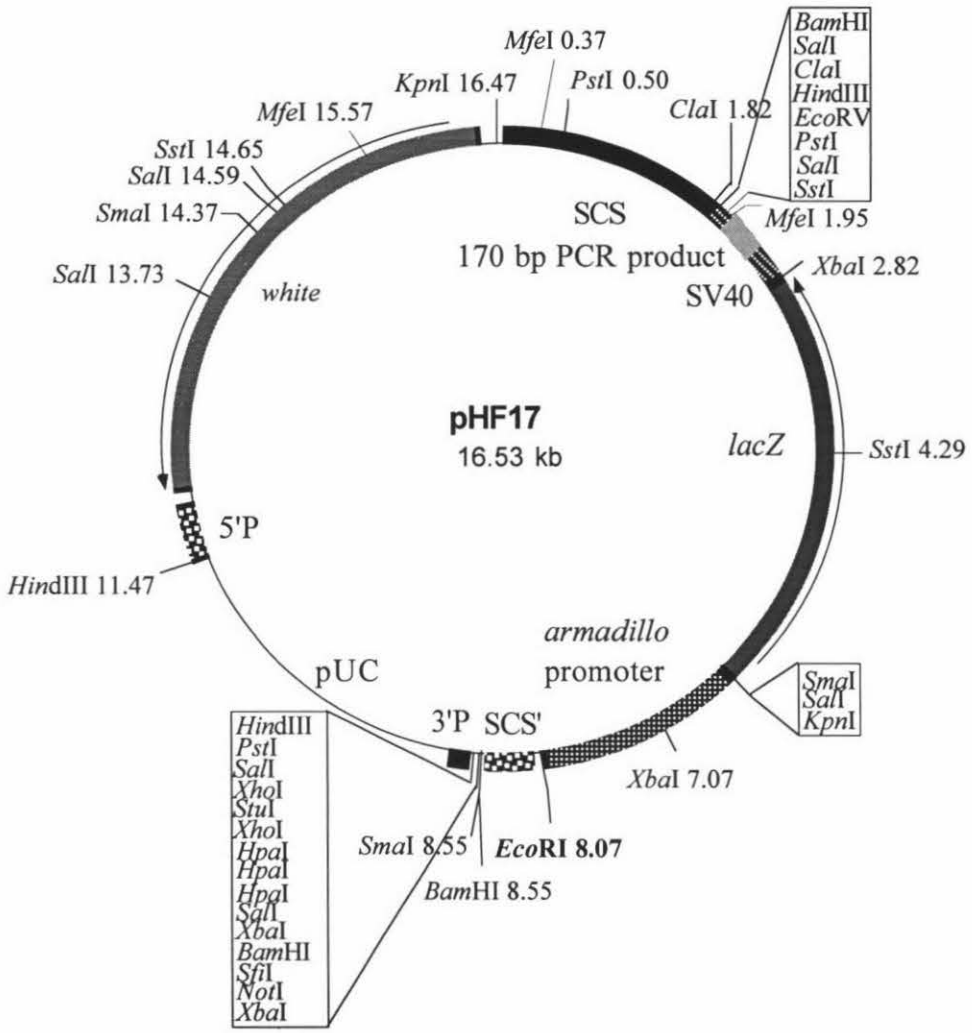
Physical Map of pLorist 6

5.2 kb. 35 - 50 kb fragments of X-linked DNA from a *Sau*3A partial digest of the *D.melanogaster* X chromosome were inserted into the *Bam*HI site to produce a cosmid library of the X chromosome (Siden-Kiamos *et al*., 1990). Cosmids used in this study were 23E12 (from region 1A of the X chromosome), 185B11 (region 1EF), 24F3 (region 1EF), 154H3 (region 2A), 11E3 (region 1B), 144A7 (region 1F) and 123B12 (region 2A)



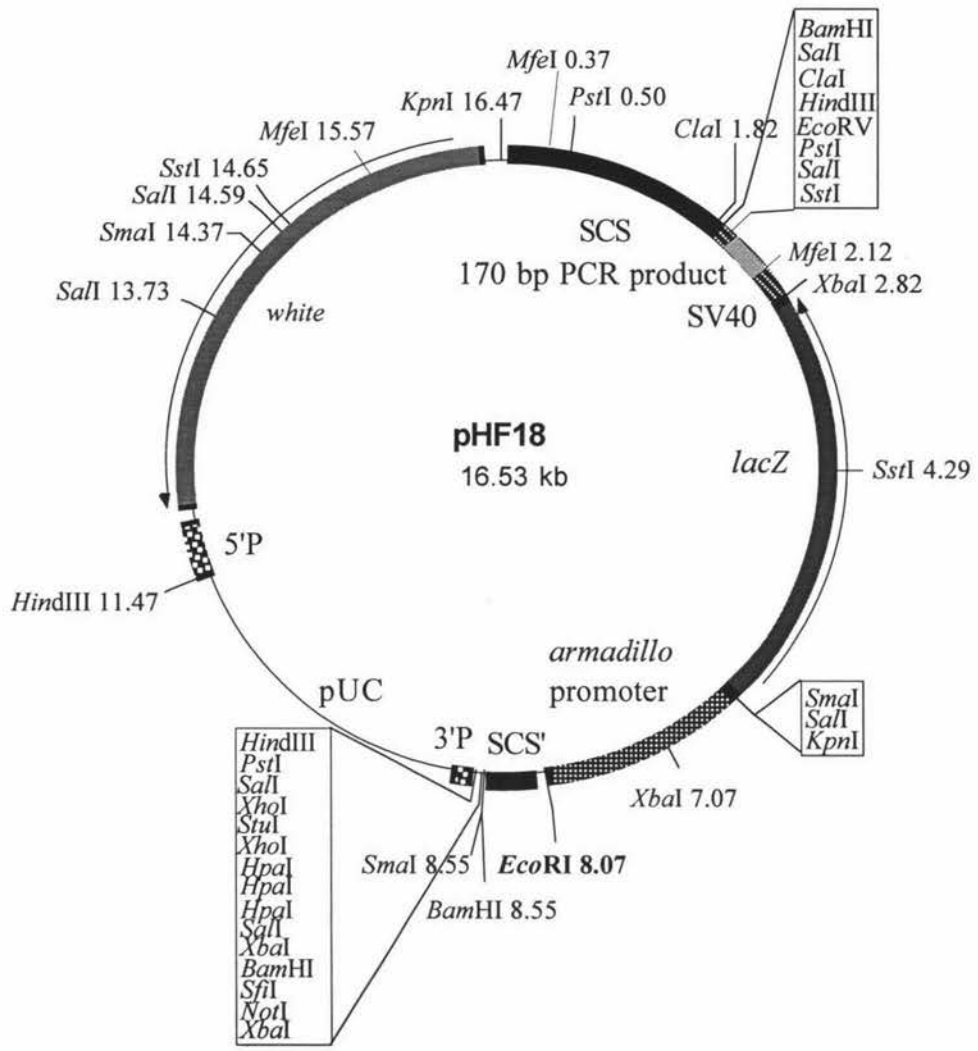
Physical Map of pHF12

8.9 kb, pBS II KS- containing 6.2 kb *Pst*I/*Eco*RI *arm-lacZ* fragment. The 40 bp linker containing *Sxl* binding sites and the 170 bp PCR product form the *runt* 3'UTR were inserted in both orientations into the *Mfe*I site in the SV40 3'UTR to produce pHF13 (170 bp *runt* PCR product in orientation producing *Sxl* binding sites), pHF14 (170 bp *runt* PCR product opposite orientation), pHF15 (40 bp linker in orientation producing *Sxl* binding sites and pHF16 (40 bp linker in opposite orientation)



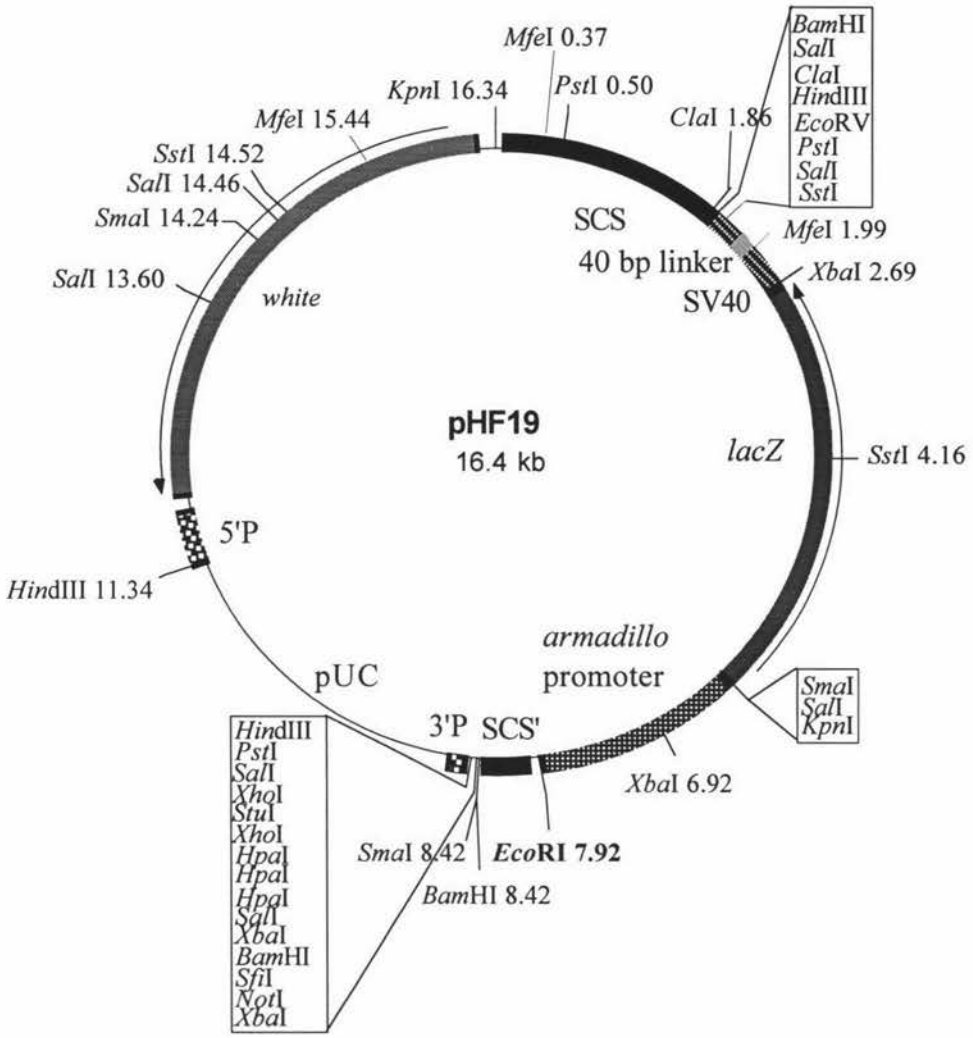
Physical Map of pHF17

16.53 kb, pHF17 was created by isolation of 6.4 kb *Eco*RI/*Pst*I (blunt) *arm-lacZ* fragment from pHF13 containing a 170 bp *runt* PCR product in the *Mfe*I site. This fragment was inserted into *Eco*RI and *Spe*I (blunt) sites of pHF10. pHF17 contains the *runt* PCR product in the orientation that produces *Sxl* binding sites



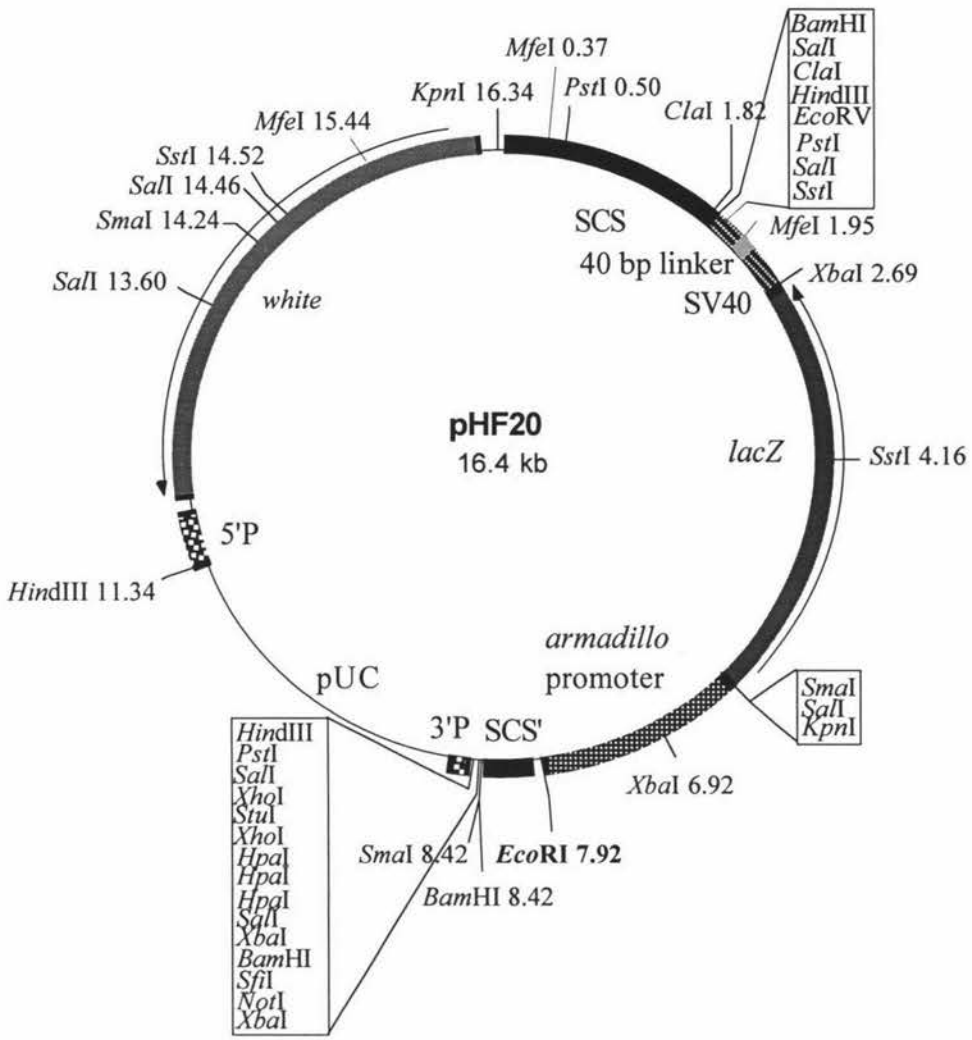
Physical Map of pHF18

16.53 kb, pHF18 was created by isolation of 6.4 kb *Eco*RI/*Pst*I (blunt) *arm-lacZ* fragment from pHF14 containing a 170 bp *runt* PCR product in the *Mfe*I site. This fragment was inserted into *Eco*RI and *Spe*I (blunt) sites of pHF10. pHF18 contains the *runt* PCR product in the orientation that produces poly A stretches



Physical Map of pHF19

16.4 kb, pHF19 was created by isolation of 6.4 kb *Eco*RI/*Pst*I (blunt) *arm-lacZ* fragment from pHF15 containing a 40 bp linker in the *Mfe*I site. This fragment was inserted into *Eco*RI and *Spe*I (blunt) sites of pHF10. pHF19 contains the *linker* in the orientation that produces *Sxl* binding sites



Physical Map of pHF20

16.4 kb, pHF20 was created by isolation of 6.4 kb *Eco*RI/*Pst*I (blunt) *arm-lacZ* fragment from pHF16 containing a 40 bp linker in the *Mfe*I site. This fragment was inserted into *Eco*RI and *Spe*I (blunt) sites of pHF10. pHF20 contains the *linker* in the orientation that produces poly A stretches

APPENDIX 2. β-Galactosidase Activities of Transgenic Lines

β-Galactosidase activity was expressed as Δabsorbance(abs)/min/mg or Δabs/min/mg wet weight x 10^2. The male to female (M/F) ratios and the average β-galactosidase activity were calculated for each line. The microinjection recipient strain, #4 *(yw)*, was assayed to measure the activity of endogenous β-galactosidase in the gut.

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F
#4 (*y w*)		0.0641	M	0.0372	M
		0.0901	F	0.0637	F
		0.3333	M	0.2263	M
		0.1234	F	0.1564	F
		0.5070	M	0.3185	M
		0.1218	F	0.1715	F
		0.2593	F	0.0707	M
		0.0714	M	0.0635	F
		0.2963	M	0.1023	M
		0.2534	F	0.5212	M
		0.2300	M	0.1333	M
		0.2283	F	0.1194	F
		0.0794	M	0.0589	M
		0.1587	M	0.0731	F
		0.1449	F	0.0697	M
		0.0721	M	0.0699	F
		0.1171	F	0.1525	M
		0.0476	M	0.1525	F
		0.0417	F	0.1170	M
				0.1274	F
Average activity		0.1705		0.1403	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
arm-lacZ #1	1	2.731		1.923	
		2.530	1.08	1.807	1.06
		4.527		1.923	
		5.144	0.88	1.939	0.99
		1.961		3.625	
		1.869	1.05	3.228	1.12
				2.730	
				2.121	1.29
Average activity		3.127		2.410	
arm-lacZ #1	2	13.50		4.141	
		11.11	1.22	3.654	1.13
		1.951		8.226	
		1.778	1.10	6.774	1.21
				2.188	
				1.861	1.18
				1.404	
				1.087	1.29
Average activity		7.085		3.995	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
arm-lacZ #2	1	1.366		1.658	
		1.389	0.98	1.378	1.20
		1.318		1.550	
		1.296	1.02	1.486	1.04
		1.616		1.433	
		1.400	1.15	1.338	1.07
		1.500		1.573	
		1.356	1.11	1.280	1.23
Average activity		1.405		1.462	
arm-lacZ #2	2	2.090		3.373	
		1.851	1.13	2.860	1.18
		2.456		3.372	
		2.032	1.21	2.944	1.15
		2.614		1.928	
		2.388	1.09	1.737	1.11
		2.614		2.632	
		2.346	1.11	2.275	1.16
Average activity		2.295		2.640	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
arm-lacZ #3	1	5.224		4.077	
		4.938	1.06	3.373	1.21
		5.267		3.774	
		5.110	1.02	3.573	1.06
		2.292		1.033	
		2.278	1.01	1.065	0.97
				2.426	
				2.000	1.21
Average activity		4.185		2.220	
arm-lacZ #3	2	4.786		6.689	
		4.444	1.08	7.835	0.85
		5.741		8.079	
		6.528	0.88	9.186	0.88
		3.626		4.677	
		3.676	0.99	4.619	1.01
Average activity		4.800		6.848	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
arm-lacZ X#1	M1/F1	2.924		4.722	
		1.818	1.61	2.671	1.77
		3.167		4.635	
		1.378	2.26	2.201	2.11
		2.543		4.764	
		1.285	2.00	2.111	2.25
Average activity	Male	2.878		4.707	
	Fem.	1.494		2.328	
arm-lacZ X#1	M1/F2	5.768		5.265	
		6.583	0.88	5.347	0.98
		6.465		6.189	
		6.373	1.01	6.730	0.92
				4.415	
				4.622	0.95
				4.383	
				4.778	0.91
Average activity		6.297		5.216	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
arm-lacZ X#2	M1/F1	5.000		4.079	
		2.765	1.81	2.189	1.86
		4.469		4.994	
		2.373	1.86	2.828	1.77
				5.285	
				2.936	1.8
Average activity	Male	4.735		4.785	
	Fem.	2.569		2.651	
arm-lacZ X#2	M1/F2	5.571		5.722	
		5.556	1.00	4.897	1.17
		5.109		7.291	
		4.769	1.07	6.370	1.14
		4.714		4.661	
		4.813	0.98	4.552	1.02
		5.206		3.966	
		4.492	1.06	4.194	0.95
				4.893	
				5.032	0.97
Average activity		5.029		5.158	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
arm-lacZ X#3	M1/F1	1.300		1.622	
		0.715	1.87	0.825	1.97
		1.234		1.659	
		0.735	1.68	0.892	1.86
		3.906		7.075	
		2.222	1.76	3.452	2.06
		4.715		4.388	
		2.892	1.63	2.389	1.84
Average activity	Male	2.789		3.686	
	Fem.	1.641		1.890	
arm-lacZ X#3	M1/F2	5.556		5.000	
		6.830	0.83	5.467	0.91
		2.189		1.474	
		2.715	0.81	1.634	0.90
		1.276		1.545	
		1.476	0.83	1.763	0.88
		1.302		4.333	
		1.414	0.92	4.452	0.97
		3.966		5.528	
		4.056	0.97	5.104	1.08
		3.956			
		4.506	0.88		
Average activity		3.270		3.630	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
HF11 #1	1	2.567		2.776	
		2.418	1.06	2.349	1.18
		2.741		2.576	
		2.651	1.03	2.196	1.17
		2.696		1.795	
		2.236	1.21	1.795	1.00
		2.533			
		2.571	0.99		
Average activity		2.552		2.248	
HF11 #1	2	7.642		5.733	
		7.073	1.08	7.794	0.70
		6.582		5.319	
		6.184	1.06	4.647	1.14
		5.842			
		5.064	1.15		
		5.263			
		4.775	1.10		
Average activity		6.053		5.873	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
HF11 #2	1	5.909		3.906	
		6.054	0.98	4.191	0.93
		4.629		3.752	
		4.491	1.03	3.798	0.98
		5.167			
		5.556	0.93		
		5.095			
		5.032	1.01		
Average activity		5.242		3.912	
HF11 #2	2	16.97		14.41	
		16.62	1.02	11.97	1.20
		14.13		13.48	
		12.89	1.10	12.63	1.07
		12.38			
		11.88	1.04		
		12.38			
		11.39	1.09		
Average activity		13.58		13.12	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
HF11 #3	1	6.566		7.139	
		5.850	1.12	6.092	1.17
		1.939		2.181	
		2.000	0.96	2.035	1.07
		1.811		2.468	
		2.045	0.97	2.262	1.09
				2.316	
				1.995	1.16
				2.048	
				1.751	1.17
Average activity		3.369		3.029	
HF11 #3	2	5.323		5.970	
		4.906	1.08	5.576	1.07
		4.106		4.473	
		3.991	1.03	4.623	0.97
		5.095		4.532	
		5.032	1.01	4.538	1.00
				3.752	
				3.798	0.98
Average activity		4.742		4.658	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
HF11 X #1	M1/F1	3.844		3.924	
		2.246	1.71	2.208	1.78
		3.295		3.060	
		2.008	1.64	1.751	1.74
		3.137		2.714	
		1.911	1.64	1.164	1.66
Average activity	Male	3.425		3.233	
	Fem.	2.055		1.708	
HF11 X #1	M1/F2	4.496		3.014	
		3.956	1.14	3.352	0.90
		3.454		5.285	
		3.860	0.90	4.975	1.06
		4.667		3.755	
		4.528	1.03	3.677	1.02
				3.943	
				3.686	1.07
				3.822	
				3.504	1.09
				4.210	
				3.329	1.26
Average activity		4.160		3.879	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
123B12 9.0 #1	1	4.867		4.712	
		4.476	1.09	4.099	1.15
		4.955		3.952	
		4.409	1.12	3.477	1.14
		3.962		2.856	
		3.843	1.03	3.001	0.95
		3.041			
		2.865	1.06		
Average activity		4.052		3.683	
123B12 9.0 #2	1	4.752		6.863	
		4.605	1.03	5.933	1.15
		6.978		4.044	
		6.346	1.10	3.814	1.05
		5.895		6.980	
		6.101	0.97	6.251	1.12
				4.676	
				5.056	0.92
Average activity		5.012		4.712	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
144A7 12.8 #1	1	3.269		3.376	
		2.947	1.11	2.874	1.18
		2.319		2.184	
		1.935	1.20	1.749	1.25
		2.000		1.861	
		1.866	1.07	1.875	0.99
				2.215	
				1.716	1.30
Average activity		2.089		2.231	
144A7 12.8 #2	1	3.519		3.235	
		4.031	0.87	2.881	1.16
		3.561		2.589	
		3.447	1.03	2.537	1.02
		3.864			
		3.741	1.03		
		1.981			
		2.276	0.87		
Average activity		3.303		2.240	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
185B11 6.6 #1	1	2.211		2.965	
		2.394	0.93	3.006	0.99
		2.994		2.891	
		2.928	1.01	2.630	1.10
		2.908		1.400	
		2.872	1.01	1.327	1.05
		6.082			
		6.629	0.92		
Average activity		3.627		2.370	
185B11 6.6 #2	1	1.301		1.344	
		1.239	1.05	1.236	1.09
		0.938		1.300	
		0.865	1.10	1.132	1.12
		1.222		1.535	
		1.094	0.95	1.539	1.00
		1.474			
		1.556	0.95		
Average activity		1.211		1.348	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
185B11 6.6 #3	1	2.624		2.195	
		2.315	1.13	1.928	1.14
		2.868		2.002	
		2.482	1.16	1.606	1.14
		1.760		2.116	
		1.538	1.14	2.090	1.01
		1.630			
		1.389	1.17		
Average activity		2.075		1.990	
11E3 6.2 #1	1	3.267		3.724	
		2.957	0.92	3.131	1.19
		5.938		1.113	
		5.440	1.09	1.040	1.09
		2.326		1.844	
		2.213	1.05	1.597	1.15
		2.326		1.772	
		2.132	1.09	1.670	1.06
Average activity		3.690		1.986	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
11E3 6.2 #2	1	1.151		1.167	
		1.255	0.92	1.229	0.95
		0.344		0.656	
		0.247	1.37	0.533	1.23
		1.169		0.879	
		1.298	0.90	0.952	0.92
		1.321		0.999	
		1.210	1.09	0.788	1.27
Average activity		0.999		0.900	
185B11 5.0 #1	1	2.083		2.121	
		2.005	1.04	2.256	0.94
		2.739		2.175	
		2.390	1.15	2.094	1.04
		2.348		2.683	
		2.375	0.99	2.683	1.00
		2.665			
		2.652	1.01		
Average activity		2.407		2.335	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
185B11 5.0 #3	1	1.978		2.235	
		1.910	1.03	2.152	1.04
		2.647		2.177	
		2.689	0.98	2.212	0.98
		2.593		2.581	
		2.451	1.06	2.498	1.03
		2.462			
		2.429	1.01		
Average activity		2.395		2.309	
11E3 15.4 #1	1	8.456		3.568	
		8.006	1.06	3.267	1.09
		7.857		3.285	
		9.015	0.87	3.568	0.92
		4.444		2.389	
		4.171	1.07	2.042	1.17
		3.981		2.579	
		3.803	1.05	2.367	1.09
Average activity		6.217		2.883	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
11E3 15.4 #2	1	4.559		1.949	
		4.575	1.00	1.792	1.09
		4.297		1.681	
		4.390	0.98	1.866	0.90
		2.170		1.455	
		2.045	1.06	1.206	1.21
		1.971		1.563	
		1.992	0.99	1.474	
Average activity		3.250		1.623	
23E12 7.7	1	1.293		1.206	
		1.209	1.07	0.877	1.06
		1.700		1.086	
		1.686	1.01	1.090	1.00
		1.402		0.808	
		1.486	0.94	0.749	1.08
		0.970		0.662	
		0.991	0.98	0.652	1.02
Average activity		1.342		0.891	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
144A7 12.8 #2	2	7.417		5.812	
		7.435	1.00	5.553	1.05
		6.719		6.273	
		6.944	0.97	6.061	1.03
		6.198		3.418	
		6.037	1.03	3.467	1.02
		8.340		5.202	
		9.317	0.90	4.845	1.07
Average activity		7.301		5.079	
HF17 #1	1	3.495		3.970	
		2.680	1.31	3.073	1.29
		4.816		3.845	
		3.368	1.43	2.933	1.31
		4.444		5.161	
		3.382	1.31	3.701	1.39
		5.937			
		4.086	1.26		
Average activity	Male	4.673		4.325	
	Fem.	3.379		3.236	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
HF17 #2	1	1.734		1.899	
		1.385	1.25	1.329	1.43
		2.507		2.499	
		1.852	1.35	1.925	1.30
		3.040		2.696	
		2.360	1.29	1.964	1.37
		2.702		2.168	
		1.984	1.36	1.541	1.42
Average activity	Male	2.496		2.316	
	Fem.	1.895		1.690	
HF17 #3	1	2.763		2.302	
		2.008	1.38	1.603	1.43
		3.060		2.514	
		1.984	1.54	1.654	1.52
		2.754		2.744	
		1.852	1.49	1.916	1.43
		2.915			
		2.121	1.37		
Average activity	Male	2.873		2.520	
	Fem.	1.991		1.724	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
HF19 #1	1	1.444		0.947	
		1.356	1.04	0.885	1.07
		0.856		0.593	
		0.949	0.90	0.658	0.90
		1.212		0.673	
		1.149	1.05	0.657	1.02
		0.944		0.580	
		0.942	1.00	0.556	1.04
Average activity		1.106		0.694	
HF19 #2	1	2.107		0.932	
		1.780	1.17	0.864	1.06
		1.331		1.229	
		1.132	1.03	1.174	1.05
		1.847		0.893	
		1.794	1.03	0.766	1.17
		1.465			
		1.346	1.09		
Average activity		1.602		0.976	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
HF19 #3	1	4.561		3.206	
		4.591	0.99	2.970	1.08
		2.863		2.111	
		2.787	1.03	1.997	1.06
		4.140		2.745	
		4.204	0.98	2.619	1.05
		3.300		2.043	
		3.111	1.06	1.854	1.10
Average activity		3.695		2.443	
HF17 #2	2	6.006		3.950	
		4.732	1.28	2.933	1.35
		7.075		4.498	
		5.400	1.31	3.084	1.46
		7.453		4.631	
		5.729	1.30	3.398	1.36
		6.311		4.504	
		5.091	1.24	3.394	1.33
Average activity	Male	6.711		4.396	
	Fem.	5.238		3.202	

Line	Dose	β-galactosidase activity Δabs/min/mg protein	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^2	M/F Ratio
HF17 #3	2	6.275		4.123	
		4.344	1.44	2.808	1.47
		5.526		3.527	
		4.094	1.35	2.633	1.34
		6.620		3.629	
		4.638	1.43	2.510	1.45
		5.525		3.420	
		3.707	1.49	2.348	1.46
Average activity	Male	5.987		3.675	
	Fem.	4.196		2.575	

APPENDIX 3. Genes Containing Three or More *Sxl* Binding Sites in their 3' UTRs

These genes were extracted from Genbank by a search for genes containing three or more *Sxl* binding sites in their 3' UTRs. (Kelley *et al.*, 1995). The sequences of the *Sxl* bindings sites and distances between them is shown. Also displayed is the distance from the polyA site to the nearest *Sxl* binding site and the distance from the stop codon to the nearest *Sxl* binding site. polyA sites that were not specified were selected as the first AATAAA or ATTAAA.

Database Name	*Sxl* binding sites	distances between *Sxl* binding sites (bp)	distance to polyA site (bp)	distance to stop codon (bp)
DROACS2	$A(U)_7$ (5' UTR)		38	108
	$A(U)_9$			
	$A(U)_7$	13		
	$A(U)_7$	75		
DMZESTE	$A(U)_7$		545	155
	$(U)_8$	7		
	$(U)_8$	175		
DMIRCRGHA	$(U)_{15}$		239	294
	$A(U)_8$	1030		
DROHELHELA	$A(U)_7$		347	305
	$(U)_{11}$	8		
	$(U)_{11}$	47		
DMCUT	$A(U)_7$ (5' UTR)		37	166
	$A(U)_7$			
	$(U)_9$	1		
	$(U)_{10}$	339		
	$A(U)_7$	676		
	$A(U)_{10}$	118		
DROFSHB	$(U)_9$		588	34
	$(U)_{13}$	504		
	$A(U)_{11}$	437		
	$(U)_{11}$	2		
DROINTBETN	$(U)_{14}$ (5' UTR)		186	11
	$(U)_{10}$			
	$(U)_{19}$	128		

APPENDIX 3. Continued

DMOTD	$(U)_{10}$		undetermined	462
	(5 ' UTR)			
	$(U)_{20}$			
	$A(U)_9$	16		
DMGS2	$(U)_9$		90	225
	$(U)_8$	1		
	$(U)_{10}$	30		
DROTDPTP10D	$A(U)_7$		332	1542
	$A(U)_{11}$	280		
	$A(U)_7$	19		
DROHSC3A	$A(U)_7$		404	309
	(5 ' UTR)			
	$A(U)_{10}$			
	$A(U)_{10}$	19		
	$A(U)_9$	114		
DMDISCO	$A(U)_{11}$		95	64
	$(U)_{13}$	235		
	$A(U)_7$	315		
DMBJ6	$A(U)_{11}$		20	721
	$A(U)_{11}$	120		
	$A(U)_7$	592		
DMCS14D	$A(U)_7$		97	268
	(5 ' UTR)			
	$(U)_{13}$			
	$(U)_9$	55		
	$A(U)_{10}$	297		
	$A(U)_{10}$	90		
DROANTPS2	$A(U)_{11}$		43	369
	$A(U)_8$	336		
	$A(U)_{11}$	479		
DROSFP	$A(U)_{21}$		169	69
	$A(U)_{12}$	123		
	$A(U)_{10}$	140		

APPENDIX 3. Continued

DROBARH2	$(U)_8$		37	134
	(5 ' UTR)			
	$(U)_{11}$			
	$(U)_{11}$	514		
	$A(U)_8$	95		
	$A(U)_{11}$	14		
	$A(U)_8$	3		
DMRUNTR	$(U)_{11}$		64	495
	$A(U)_7$	8		
	$A(U)_7$	69		
DMU03717	$(U)_9$		87	272
	$A(U)_7$	554		
	(5 ' UTR)			
	$A(U)_9$			
	$(U)_8$	83		
	$(U)_9$	139		

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