A STUDY OF *CIS*-ACTING ELEMENTS REQUIRED FOR DOSAGE COMPENSATION IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Dosage compensation (the equalisation of X-linked gene products) occurs in *Drosophila melanogaster* by a two fold transcriptional up-regulation of X-linked gene expression in males. This involves the binding of five proteins, MSL-1, MSL-2, MSL-3, MLE, MOF, and potentially an RNA (*roX1* or *roX2*), to hundreds of sites along the male X chromosome. The *cis*-acting X-linked DNA sequences required for dosage compensation (called dosage compensation regulatory elements or DCREs) remain elusive, despite numerous attempts of identify them. An insulated reporter gene assay system has been developed to minimise problems previously encountered with identification of these elements. The reporter system consists of the constitutive *armadillo* promoter fused to the *lacZ* reporter gene (called *arm-lacZ*). This reporter construct is flanked by SCS/SCS' insulator elements to block potential repressive effects of an autosomal chromatin environment.

The role of the *roX* genes during dosage compensation was investigated. Initially both the *roX1* and *roX2* RNAs were expressed from within the *arm-lacZ* insulated system. Expression of either RNA lead to a significant increase in *lacZ* expression in males, although consistently less than two-fold. These results suggested that either the MSL complex was binding to the *roX* genes or the expression of the *roX* RNAs in *cis* lead to male-specific hypertranscription of *lacZ*. To test these possibilities *roX1* and *roX2* cDNAs were inserted into the *arm-lacZ* reporter. Insertion of either cDNA lead to a significant increase in *lacZ* expression in males, suggesting that the transcribed regions of the *roX* genes contain binding site(s) for the MSL complex. Interestingly the level of *lacZ* hypertranscription in males was significantly higher in homozygous *roX1* cDNA lines than homozygous *roX1* gene lines. This may indicate that too high a local concentration of *roX1* RNA has a dampening effect on the level of hypertranscription meditated by the MSL complex. In a set of experiments designed to identify the MSL binding site(s) in *roX1*, two regions of the cDNA sequence were amplified and inserted into the *arm-lacZ* system. One of these fragments, containing a proposed DNAseI hypersensitivity site and possible GAGA binding sites, increased *lacZ* expression in males, but to levels lower than the entire cDNA. This suggests there may be more than one MSL biding site in *roX1*.

A second method of dosage compensation is thought to occur in *Drosophila*, independently of the MSL proteins. The *arm-lacZ* insulated reporter system was used to investigate the hypothesis that some genes may be dosage compensated due to repression by *Sex-lethal (Sxl)* in females. Several genes have been found to contain three or more Sxl binding sites in their 3' UTRs, with some also carrying Sxl binding sites in the 5' UTR. Fragments from the *Sxl, Cut* and *Small Forked* genes, containing numerous Sxl binding sites from the 3' UTR, were inserted into the 3' UTR region of *arm-lacZ*. Males carrying autosomal insertions of the construct had on average 1.07 – 1.50 times the level of β-galactosidase in females. This suggests that some genes could be partially compensated through Sxl repression in females.

In addition to inserting 3' UTR fragments into *arm-lacZ,* a synthetic oligonucleotide containing a long Sxl binding site was inserted into the 5' region of an *arm-lacZ* construct already carrying the *Runt* 3' UTR fragment. Males carrying autosomal insertions of the construct had levels of β-galactosidase activity similar to those lines carrying autosomal insertions of the 3' UTR fragments alone. This suggests that other factors such as RNA binding proteins or RNA secondary structure may be required in order to obtain efficient translation repression by Sxl.

Finally three X-linked DNA fragments, from the 1C region, were inserted individually between the SCS' element and the *armadillo* promoter. If the X-linked fragment contained a DCRE then males carrying autosomal insertions of the construct would produce twice the β-galactosidase activity of females. However, males and females expressed the same levels of *lacZ*.

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ABBREVIATIONS

β	beta
Δ	delta
λ	lambda
°C	degrees Celsius
ATP	adenine triphosphate
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
DIG	digoxigenin
DNA	deoxyribose nucleic acid
DNAse	deoxyribonuclease
dNTPs	dinucleotide triphosphates
F	female
g	gram
L	litre
kb	kilobase pairs
μ	micro
m	milli
M	male or molar
mRNA	messenger RNA
nt	nucleotide pairs
OD	optical density
PCR	polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
U	unit
UTR	untranslated region
UV	ultra violet
v/v	volume per volume
w/v	weight per volume

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Errata

The data values listed in tables within this thesis are correct, however due to calculation difficulties values quoted within the main body of text may vary slightly from those in the tables.

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1.0 INTRODUCTION

1.1 DOSAGE COMPENSATION - AN OVERVIEW

Dosage compensation is the mechanism by which the expression of X-linked genes is equalised between males with one X chromosome and females with two. Different organisms have evolved unique mechanisms to achieve dosage compensation. In mammals one female X chromosome is randomly inactivated to equal the expression of the single male X chromosome (Lyon, 1961). In *Caenorhabditis elegans* the expression of both female X chromosomes is down regulated to equal the expression of the single male X chromosome (Hsu and Meyer, 1993). Mammals and *C. elegans* are both examples of organisms where the male is the heterogametic sex. In organisms where the female is heterogametic (ZW), eg. birds and butterflies, dosage compensation has been shown not to occur (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 the hypertransactivation (transcriptional up-regulation) of genes on the single male X chromosome to equal the level of expression from two female X chromosomes. Early support for this statement came from Offermann's, (1936) observation in squashes of polytene chromosomes, from larval salivary glands, that the male X chromosome is wider and more diffuse in appearance than both female X chromosomes. This 'puffier' appearance indicates an increase in gene expression. Following this observation Mukherjee and Beermann, (1965) demonstrated that incorporation of tritiated (^{3}H) uridine into nascent salivary gland transcripts was significantly higher in the single unpaired male X chromosome than one female X chromosome. Results from these experiments pointed to an enhancing effect occurring on the male X chromosome rather than a repressing effect on the female.

1.2.2 Histone Acetylation, Transcriptional Activity and Dosage Compensation

The core particle of the nucleosome consists of four histones H2A, H2B, H3 and H4. Acetylation of the histones occurs at specific lysine residues in the N-terminal domain and is a ubiquitous post-translational modification found in all animal and plant species (Turner, 1991).

Histone acetylation has been stated as being potentially a major influence on transcription and DNA packaging through the cell cycle (Turner, 1991). Histones in actively transcribing genes are rapidly acetylated and deacetylated, which proposes a link between transcriptional activation and histone acetylation (reviewed by Turner, 1991). Neutralisation of positive charges by acetylation of histone H4 is thought to play a primary role in altering interactions between the DNA and histones, which may mediate enhanced binding of transcription factors to their DNA target sequences (Vettese-Dadey *et al.*, 1996).

Acetylation of ε-amino groups of lysine residues, present in the N-terminal domain of the core histones, is most strongly linked with transcriptional activity (Turner, 1991). Vettese-Dadey *et al.*, (1996) demonstrated that the highly acetylated histone H4 in nucleosome cores has the highest affinity for transcription factors USF and GAL-4H. Studies of the *Saccharomyces cerevisiae* mating type (MAT) locus (Johnson *et al.*, 1990) indicates that the repression of the silent mating loci requires histone acetylation. When lysine 16 is mutated to an arginine, which retains the positive charge, the regulation of the MAT locus is unaffected. But, when lysine 16 is mutated to a glutamine (a neutral amino acid mimicking acetylation) derepression of the locus occurs (Johnson *et al.*, 1990). A specific isoform of histone H4 acetylated at lysine 16 (H4Ac16) is also predominantly associated with the male X chromosome in *Drosophila* (Turner *et al.*, 1992). The acetylation of histone H4 on lysine 16 (H4Ac16) may play a role in loosening the chromatin structure and increasing the accessibility of transcription factors associated with the male X chromosome in *Drosophila* (Bone *et al.*, 1994).

1.3 THE TRANS-ACTING MALE SPECIFIC LETHALS

A simple model for dosage compensation in *Drosophila* would predict that increases in X-linked gene transcription result from the action of *trans*-acting factors upon target *cis*-acting sequences localised to the X chromosome (Palmer *et al.*, 1993). It has been rationalised that mutations inactivating regulatory genes responsible for dosage compensation could result in sex specific lethality (Lucchesi and Manning, 1987). A mutation that prevents normal compensation could cause the death of an individual with a single X chromosome due to a deficiency of X-linked gene products. Belote and Lucchesi, (1980a) carried out a large screen for ethyl methanesulfonate (EMS) induced sex-specific lethals on the 2nd and 3rd chromosomes. Three male-specific lethal mutations *male-specific lethal-1 (msl-1), male-specific lethal-2 (msl-2) and maleless (mle)* were discovered. Temperature sensitive 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 discovered *male-specific lethal-3 (msl-3)* (Lucchesi *et al.*, 1982) and *males-absent on the first (mof)* (Hilfiker *et al.*, 1997) have been collectively named the *male-specific lethals* or *msls.* Males mutant in any of these genes exhibit prolonged posthatching development and eventually die during the late larval or early pupal stages (Belote, 1983). These mutations have been shown to have no discernible effect on the viability and development of females (Belote and Lucchesi, 1980a).

Males homozygous for *msl-1, msl-2* or *mle* show a significant reduction in X-linked enzyme activities, while the levels of autosomal enzymes are not affected (Belote and Lucchesi, 1980b).

The MSL proteins bind to hundreds of sites along the entire length of the male X chromosome (Kuroda *et al.*, 1991; Palmer *et al.*, 1993). Immunolocalisation experiments show that the MSLs bind to the same sites along the X chromosome (Bone *et al.*, 1994), and the native X chromosome binding of any MSL protein requires the wildtype function of the other four MSLs (Bashaw and Baker, 1995; Gorman *et al.*, 1993; Gu *et al.*, 1998; Hilfiker *et al.*, 1994; Kelley *et al.*, 1995; Palmer *et al.*, 1994,). This dependent binding suggests that the MSL proteins form a heteromultimeric complex.

1.3.1 Maleless

As discussed above the *msl* genes have all recently been cloned. The *mle* gene (Kuroda *et al.*, 1991) encodes a polypeptide containing several short motifs characteristic of a superfamily of DNA and RNA helicases. MLE shows the highest sequence homology to a subfamily of RNA helicases containing DEAH box motifs (Schwer and Guthrie, 1991; Nakajima *et al.*, 1997). MLE shares 50% identity with human RNA helicase A (RHA) which mediates the interaction of CBP (CREB Binding Protein) with RNA polymerase II (Nakajima *et al.*, 1997). It has been proposed that the recruitment of CBP complexes may promote local unwinding of promoter DNA via RHA and allow access of transcriptional apparatus (Nakajima *et al.*, 1997). A study by Lee *et al.*, (1997) showed that MLE possesses NTPase and both RNA and DNA helicase activities and that these activities are essential functions of MLE for dosage compensation. Preliminary studies by Nakajima and Montminy (unpublished data cited by Nakajima *et al.*, 1997) have observed MLE associating with a 250 kDa CBP with histone acetylase activity. The unpublished data along with evidence that MLE appears to co-localise with acetylated histone H4 (Bone *et al.*, 1994), and has NTPase/helicase activity (Lee *et al.*, 1997) suggests that MLE may be involved in initiation of transcription, perhaps via chromatin remodelling of X-linked genes.

1.3.2 Male-Specific Lethal-1

The cloning and characterisation of the *msl-1* gene (Palmer *et al.*, 1993) showed that the MSL-1 protein is not closely related to any proteins in the current databases. It does however contain acidic regions in the N-terminus consisting of two extended aspartate and glutamate clusters, characteristic of proteins involved in chromatin modelling and transcription (Palmer *et al.*, 1993). The acidic regions of these proteins may provide a region of interaction with histones to mediate nucleosome assembly or release and thereby promote changes in chromatin structure and transcription (Palmer *et al.*, 1994; Turner *et al.*, 1992). MSL-1 protein is present in *mle* and *msl-3* mutant larvae, but is undetectable in *msl-2* mutant male larvae (Palmer *et al.*, 1994). This finding plus other genetic tests carried out by Palmer *et al.*, (1994) suggests that *msl-2* expression positively regulates the translation or stability of MSL-1 in males. MSL-1 also contains regions rich in proline, serine, threonine and glutamic acid which are residues (PEST sequences) associated with rapidly degraded proteins (Palmer *et al.*, 1993).

1.3.3 Male-Specific Lethal-2

MSL-1 and MSL-2 have been shown to co-immunoprecipitate from male nuclear extracts (Kelley *et al.*, 1995). MSL-2 (Zhou *et al.*, 1995), contains a RING finger, which is a C_3HC_4 zinc finger (Lovering *et al.*, 1993). Using a two-hybrid system Copps *et al.*, (1998) found that the RING finger domain of MSL-2 binds MSL-1. When residues clustered around the first zinc-binding site of the RING finger domain in MSL-2 were mutated interaction with MSL-1 was lost. In addition to the RING finger motif the MSL-2 protein also contains a positively and a negatively charged amino acid residue cluster and a coiled coil domain that may be involved in protein-protein interactions (Zhou *et al.*, 1995). Zhou *et al.*, (1995) hypothesise that MSL-2 may be a transcription regulator, with the positively charged amino acid cluster contributing to a DNA binding domain and the negatively charged cluster being part of a transcription *trans*-activator domain. Copps *et al.*, (1998) propose that the RING finger domain interaction with MSL-1, through the first zinc-binding site, may be an important prerequisite for subsequent protein-protein interactions and that the second zinc-binding site may have a second, but as yet unidentified activity.

MSL-1 and MSL-2 appear to form a core complex within the MSL complex. When either is removed through mutation the remaining MSL proteins fail to bind any site along the X chromosome (Gorman *et al.*, 1993; Gorman *et al.*, 1995; Palmer *et al.*, 1994; Lyman *et al.*, 1997). Conversely if MSL-3, MLE or MOF are removed MSL-1 and MSL-2 remain bound to 30 - 40 'high affinity' binding sites along the X chromosome (Palmer *et al.*, 1994; Gorman *et al.*, 1995; Gu *et al.*, 1998; Lyman *et al.*, 1997)

1.3.4 Male-Specific Lethal-3

Cloning and characterisation of the *msl-3* gene found that it encodes a novel protein (Gorman *et al.*, 1995). MSL-3 contains two chromatin organisation modifier (chromo) domains that are 30 - 50 amino acid domains conserved in several eukaryotic chromatin-binding proteins such as *Drosophila* hetereochromatin protein 1 (HP1) and Polycomb (PC) (Koonin *et al.*, 1995). Chromodomains have been implicated in the delivery of both positive and negative transcription regulators to chromatin targets.

1.3.5 Males-Absent on the First

Experimental results have identified an additional gene *males-absent on the first (mof)*, which encodes a putative histone acetyl transferase thought to be crucial for dosage compensation. This fifth male lethal gene was isolated by screening the X chromosome of *Drosophila melanogaster* for EMS-induced mutations, to identify genes carrying mutations that cause male specific lethality. Males mutant for *mof* die at the third instar larval stage of development, MSL-1, MSL-2 and MLE association with the X chromosome is reduced and the X-specific isoform of H4Ac16 is absent (Hilfiker *et al.*, 1997).

The *mof* mRNA encodes an 827 amino acid protein that contains a 250 amino acid domain common to many acetyl transferases and is shown to be required for binding of acetyl coenzyme A. This domain is found in proteins known to acetylate histones, such as histone acetyl transferase 1 of yeast (Kleff *et al.*, 1995) and histone acetyl transferase A of *Tetrahymena* (Brownell *et al.*, 1996). The mutation of *mof* results from a substitution of Gly691 (the most conserved residue in the 250 amino acid motif) for glutamic acid. This mutation leads to the absence of H4Ac16 on the male X chromosome and a male lethal phenotype. Lu *et al.*, (1996) showed that the mutation of the corresponding glycine to an aspartate, in the human spermidine/spermine acetyl transferase, abolishes enzyme activity. Recently it has also been demonstrated that MOF co-localises with the MSL complex on the male X chromosome using loss-of-function mutations (Gu *et al.*, 1998).

Immunolocalisation experiments have shown that all of the MSL proteins bind to hundreds of specific sites along the male X chromosome. Each of the MSLs is produced in both sexes except for MSL-2, which is absent in females (Zhou *et al.*, 1995). Henikoff and Meneely, (1993) suggest that MLE could catalyse the movement of the MSL complex along the nascent RNA. In *msl-1, msl-2* or *msl-3* mutant backgrounds MLE does not bind the X chromosome, but is still present, indicating that MSL-1, MSL-2 and MSL-3 are required for binding, but not for regulation of X chromosome expression (Gorman *et al.*, 1993). MOF, the fifth MSL protein, encodes a putative histone acetylase. Mutational studies provide strong evidence that MOF has histone acetyl transferase activity and is responsible for the histone acetylation involved in male specific hypertranscription of X-linked genes (Hilfiker *et al.*, 1997).

1.3.6 Histone Acetylation and MSL Localisation

The pattern of H4Ac16 distribution on the X chromosome is very similar to that of the MSLs suggesting a link between the signals required for localising these proteins to the male X chromosome (Bone *et al.*, 1994). Bone *et al.*, (1994) also observed that presence of this H4 isoform on the X chromosome requires the wildtype function of the *msl* genes. This suggests that the mechanism of dosage compensation involves histone acetylation through association with the MSL proteins.

1.4 NON-CODING RNA INVOLVEMENT IN DOSAGE COMPENSATION

Two new genes *roX1* and *roX2* (RNA on the X chromosome) have been isolated using an enhancer detector screen for β-galactosidase activity in the mushroom bodies of the *Drosophila* brain (Amrein and Axel, 1997; Meller *et al.*, 1997). Both genes are X-linked and each encodes an RNA without a significant open reading frame (ORF). Their expression is confined to the nucleus of male flies, which suggests that they may encode non-coding RNAs (Amrein and Axel, 1997; Meller *et al.*, 1997). Expression of *roX1* and *roX2* is dependent on the MSL complex (dosage compensation machinery) as neither of the genes are expressed in flies mutant for any of the *msls.* Additionally, expression of a *msl-2* transgene in females induces the expression of both *roX1* and *roX2* RNA (Amrein and Axel, 1997; Meller *et al.*, 1997). *In situ* hybridisation of *roX1* probes to late third-instar male larvae salivary gland X chromosomes displays a subcellular localisation of *roX1* RNA very similar to the localisation of the MSL complex binding the X chromosome (Amrein and Axel, 1997; Meller *et al.*, 1997).

Disruption of *roX1* produces no obvious phenotype, lethality, or developmental delay, which rules out *roX1* as an essential component of the dosage compensation complex. However the disrupted *roX1* mutant (the $roX1^{ex6}$ mutation removes the 5' half of the *roX1* gene and produces no stable RNA (Kelley *et al.*, 1999)) was used to show that *roX1* RNA could spread in *trans.* Kelley *et al.*, (1999) inserted a DNA fragment, containing the *roX1* gene, into either the second or third chromosome by P element mediated transformation. Males homozygous or hemizygous for the null $roX1^{ex6}$ mutation, but carrying one copy of the *roX1* transgene, were used for RNA *in situ* hybridisation to polytene chromosomes. These *in situ* experiments showed the

autosomally encoded RNA coating the entire X chromosome. This indicates that the *roX1* RNA still bound to the X despite being produced on another chromosome.

Kuroda *et al.*, (1991) proposed an RNA component of the dosage compensation system based on the observations that MLE contains RNA binding domains and is released from the X chromosome by RNase digestion (Richter *et al.*, 1996). However male flies mutant for *roX1* exhibit normal MLE binding and are fully dosage compensated (Meller *et al.*, 1997).

Franke and Baker, (1999) genetically produced a mutant with simultaneous loss of both *roX1* and *roX2*, which abolished binding of the MSL complex to the male X chromosome. They suggest this is a strong indication that the *roX* RNAs are integral components of a dosage compensation nucleoprotein complex and is consistent with the earlier proposal of Meller *et al.*, (1997) that there is a family of non-homologous and redundant genes including *roX1* and *roX2* that can compensate for the loss of one of its members. They also propose that *roX1* and its family members associate along the entire X chromosome to help change chromatin conformation and achieve hypertranscription, perhaps by associating with the MSLs, histone acetyl transferase or other chromatin constituents.

Comparisons have been made between the *roX* RNA and *Xist* RNA that coats the inactive mammalian X chromosome. *Xist* encodes a non-coding RNA expressed from the X-inactivation centre of the inactive X chromosome in mammals and is thought to 'spread' (in *cis*) along one of the female X chromosomes, remodelling chromatin to form a transcriptionally inactive Barr body (Lee *et al.*, 1996). There are some similarities between *roX1* and *Xist*; they are both nuclear and localised to a structurally modified X chromosome undergoing dosage compensation (Meller *et al.*, 1997).

1.5 REGULATION OF DOSAGE COMPENSATION

1.5.1 *Sex-Lethal*

The process of dosage compensation is one of several controlled by the 'master regulatory gene' *Sex-lethal (Sxl)*. Initially dosage compensation is controlled by the expression patterns of *Sxl,* which in turn is controlled by the ratio of X chromosomes (X) to autosomes (A). An X:A ratio of 1.0 (2X:2A) results in female development and a X:A ratio of 0.5 (1X:2A) results in male development. In *Drosophila* the X:A ratio acts to switch the *Sxl* gene into either the female mode which represents ON (functional) or the male mode which represents OFF (non-functional) (Cline, 1978). The X:A ratio itself is assessed by 'counting' genes, referred to as numerators and denominators (reviewed by Parkhurst and Meneely, 1994). These proteins are members of the helix-loop-helix (HLH) family of transcription factors (Parkhurst *et al.*, 1990). The numerators are a group of X chromosomal genes that behave as feminising elements because they increase the probability of activating *Sxl* expression. Lowering the number of numerators results in female lethality due to the lack of activated *Sxl,* whereas raising the number results in male lethality because *Sxl* is activated. Denominators are autosomally encoded genes acting as antagonists to the numerators by competing with numerators to form heterodimers. The heterodimers formed activate *Sxl* at the level of transcription (Keyes *et al.,* 1992).

The initial activation of *Sxl* results in production of *Sxl* mRNA transcripts from the early 'establishment' promoter P_E in females. These early *Sxl* mRNA protein products specify the production of active female-specific transcripts from the late 'maintenance' promoter P_L (Bell *et al.*, 1991) and thereby establish an autoregulatory feedback loop. Transcripts of *Sxl* are also produced in males from P_L, but these are truncated and inactive and maintained by default (Bell *et al.,* 1991; Keyes *et al.,* 1992).

In males a functional Sxl protein is missing, therefore male differentiation and dosage compensation occurs. In females, active Sxl protein acts upon the mRNA of *transformer* (*tra*), the next gene in the pathway. Sxl binding to *tra* RNA blocks a splice acceptor site, resulting in another female-specific splicing pattern occurring. The functional Tra protein is only produced in females and is involved in somatic sex

determination. *In vitro* studies by Samuels *et al.*, (1994) showed that Sxl protein binds to poly uridine (polyU) tracts in mRNA that consist of eight or more Us or AU_7.

1.5.2 *Sxl* Regulation of Dosage Compensation

Sxl loss-of-function mutations cause female lethality and gain-of-function mutations cause male lethality (Cline, 1978). Zhou *et al.*, (1995) demonstrated that the primary target of Sxl during dosage compensation is *msl-2*. The *msl-2* transcript is present in both males and females, with the same ORF, but the MSL-2 protein is present only in males (Bashaw and Baker, 1995; Kelley *et al.*, 1995; Zhou *et al.*, 1995). A small intron in the 5' UTR (untranslated region) of the *msl-2* transcript is spliced out in males and retained in females (Bashaw and Baker, 1995; Kelley *et al.*, 1995; Zhou *et al.*, 1995). Within this intron are poly(U) runs that resemble the Sxl binding sites found in *Sxl* and *tra* and are therefore spliced out in males. Four more Sxl binding sites are present in the 3' UTR that is retained in both sexes. Mutations of Sxl binding sites in either the 5' or the 3' regions result in ectopic expression of MSL-2 protein in females. This indicates that the sites in both the 3' and 5' UTRs are required for appropriate regulation of *msl-2* translation (Bashaw and Baker, 1997; Kelley *et al.*, 1997). Bashaw and Baker, (1997) suggest the possibility that *Sxl* binding at both ends of the *msl-2* transcript changes the structure of the RNA by circularisation and therefore prevents access of translational machinery.

1.5.3 A Second MSL Independent Method of Dosage Compensation

A second method of dosage compensation has been suggested in *Drosophila* that is independent of the *msls*. The first evidence for this second method was the observation by Cline, (1978) that females homozygous for a loss-of-function mutation for *Sxl* were not rescued if also homozygous for mutations in *msl-2, msl-1* or *mle*. *msl-3* is yet untested, but assumed to have the same phenotype due to the co-dependence of the *msls*. These findings suggest that *Sxl* and the *msls* may act on different loci to direct dosage compensation.

Dosage compensation of the X-linked *Runt* gene has been shown to be dependent on *Sxl*, but independent of the *msls* (Gergen, 1987; Bernstein and Cline, 1994). Wildtype *Runt* is required for the normal segmentation of *Drosophila* embryos (Gergen and Wieshaus, 1986) and is active at the blastoderm stage. Gergen, (1987) studied the

dosage compensation of *Runt* at the blastoderm stage of development and found that *Runt* expression was not affected by mutations in *msl-1, msl-2* and *mle*. Examination of the *Runt* gene revealed three *Sxl* binding sites in the 3' UTR (Kelley *et al.*, 1995). *Sxl* expression from the early *Sxl* promoter (Sxl_E) occurs at the same stage of development as *Runt* expression, which supports the idea that early dosage compensation begins at mid-stage four and that *Runt* expression is *Sxl* dependent and *msl* independent (Bernstein and Cline, 1994; Gergen, 1987). The regulation of *Runt* by *Sxl* is probably due to repression of expression in females as indicated by only female-specific lethal alleles of *Sxl* affecting dosage compensation at the blastoderm stage (Gergen, 1987).

Two models have been suggested for the relationship between MSL dependent and *Sxl* mediated dosage compensation. The first involves *Sxl* controlling 'early' dosage compensation during embryogenesis, while the MSLs mediate 'late' dosage compensation during the larval and pupal stages. Evidence for this model is that *msl* mutant males complete embryogenesis, but die as late larvae or pupae (Belote and Lucchesi, 1980a; Fukunaga *et al.*, 1975) and *Runt* is expressed before MSLs become functional (Gergen, 1987). *Sxl* and *Runt* expression is detected at mid-stage four of embryogenesis, but MSL binding to the X chromosome does not occur until the end of stage five (blastoderm stage). The *Sxl* 'early' dosage compensation process may have evolved to satisfy a need for dosage compensation before the MSLs become functional and therefore the two systems are operating sequentially (Franke *et al.*, 1996).

The second model is that *Sxl* and MSL mediated dosage compensation pathways act in parallel (Rastelli *et al.*, 1995) during development on separate sets of genes (Kelley *et al.*, 1995). Recent data suggests that *Sxl* may reduce the stability or translation of a subset of X-linked transcripts in females (Kelley *et al.*, 1995). Kelley *et al.*, (1995) suggest that this second dosage compensation system may upregulate X-linked genes in males, while a subset of X-linked genes are down regulated in females. A computer search scanning all available 3' UTRs of *Drosophila* genes produced 21 genes containing three or more 3' poly(U) sites. 20 of these genes are on the X chromosome. The only autosomal gene found was *msl-1* (*msl-2* is also autosomal) (Kelley *et al.*, 1995). Kelley *et al.*, (1995) proposed that *Sxl* directly regulates dosage compensation of many genes through their 3' UTRs. Bernstein and Cline, (1994) suggest that *Sxl* mediated dosage compensation is not limited to embryonic development through studies

of partial loss-of-function *Sxl* mutants, while Rastelli *et al.*, (1995) suggest that *msl*-dependent dosage compensation is not limited to larval development.

1.6 *CIS*-ACTING ELEMENTS CONTROLLING DOSAGE COMPENSATION

Relatively little is known about the *cis*-acting sequence characteristics of the X chromosome which identify it as a target for dosage compensation regulators (ie. MSLs and Sxl). These dosage compensation regulatory elements (DCREs) are thought to be distributed throughout the X chromosome. Evidence suggests that DCREs exert their efforts locally on individual genes or small groups of genes. When fragments from the X chromosome are transposed to an autosome the X-linked genes within the fragment remain dosage compensated (Ghosh *et al.*, 1989; Hazelrigg *et al.*, 1984; Krumm *et al.*, 1985; Levis *et al.*, 1985; McNabb and Beckendorf, 1986; Pirrotta *et al.*, 1985; Spradling and Rubin, 1983). Also when cloned X-linked genes are translocated to autosomal sites they remain at least partially dosage compensated (reviewed by Baker *et al.*, 1994; Lucchesi and Manning, 1987).

Conversely when autosomal fragments are translocated to X chromosome sites the genes within the fragment remain non-compensated. But, when cloned autosomal genes are translocated to the X chromosome they are compensated in males (Baker *et al.*, 1994). These observations suggest that dosage compensation in *Drosophila* is controlled by *cis*-acting sequences both distant and close to the genes. Supporting this suggestion is the observation that not all genes on the X chromosome are dosage compensated. These non-compensated genes can be in close proximity to genes that are.

Support for the hypothesis that *cis*-acting elements confer transcriptional upregulation only onto nearby sequences is the finding that *LSP-1α* is an X-linked gene but is not compensated. *LSP-1α* codes the alpha subunit of larval serum protein-1. *LSP-1α* has a transcription unit named *L12* immediately adjacent to it that is compensated (Ghosh *et al.*, 1989). Females exhibit twice the amount of gene product found in males (Brock and Roberts, 1982; Roberts and Evans-Roberts, 1972). This phenomenon could be explained by assuming that the *LSP-1α* gene has only relatively recently been

translocated to the X chromosome. Ghosh *et al.*, (1989) determined that *LSP-1α* is inherently capable of dosage compensation by relocating the *LSP-1α* gene to ectopic X chromosome sites. The results of this experiment showed steady state levels in males (one dose) are equivalent to females (two doses).

1.6.1 DCREs Are Still Unidentified

For the last ten years the search for *cis*-acting sequences involved in dosage compensation has been fruitless. Two X-linked genes (*white* and *Sgs-4*) have been extensively studied using genetic and molecular techniques in an attempt to localise the DCREs. The studies predominantly involved inserting X-linked transgenes, which contained progressive deletions, into autosomes to isolate a possible consensus sequence for dosage compensation.

Levis *et al.*, (1985) analysed the *cis*-acting sequences involved in regulating the *white* gene. Varying lengths of both 3' and 5' flanking sequences were deleted from the *white* gene. Flanking sequences 420 bp upstream and 160 bp downstream of the gene were found to be sufficient for dosage compensation to occur. Pirrotta *et al.*, (1985) further delimited the required sequence to 200 bp upstream of the gene.

As sequences in the *white* gene are gradually removed from the 5' end a progressive decline in dosage compensation is observed (Qian and Pirrotta, 1995). Qian and Pirrotta, (1995) concluded that *cis*-acting DCREs consist of multiple elements present near and within the promoter and some within the coding region of the gene. Despite these observations no DCRE consensus sequence has been identified.

Transformation experiments involving the *Sgs-4* gene demonstrated that 840 bp upstream and 130 bp downstream of the gene are sufficient for proper activity and regulation when relocated to autosomal sites (McNabb and Beckendorf, 1986). Sequence comparisons between compensated and non-compensated alleles failed to show any base substitutions specific to the non-compensated alleles (Hofmann and Korge, 1987).

1.6.2 Mono and Dinucleotide Repeats Correlate with Dosage Compensation

Evidence has been presented that suggests the X chromosome has unique structural features that may be related to dosage compensation. Two dinucleotide repeats $(CA/GT)_n$ and $(CT/GA)_n$ and one mononucleotide repeat $(C/G)_n$ have been reported to be found at twice the level on the X chromosome as on autosomes (Huijser *et al.*, 1987; Lowenhaupt *et al.*, 1989; Pardue *et al.*, 1987). Chromosomal arms from autosomes translocated to the X chromosome acquire the ability to dosage compensate in several *Drosophila* species. The newly translocated arm also gains a higher density of $(CA/GT)_n$ similar to the other X chromosomes. The pattern of $(CA/GT)_n$ sequences shows several correlations with general chromosomal functions such as dosage compensation (Pardue *et al.*, 1987). Pardue *et al.*, (1987) suggests that the acquisition of dosage compensation ability and higher density of $(CA/GT)_n$ repeats reflects a relationship between the two processes.

These repeats are all able to adopt a non B form of DNA when subjected to negative supercoiling *in vitro* and may be involved in the adoption or maintenance of a decondensed X chromatin structure required for dosage compensation (Lowenhaupt *et al.*, 1989). Other than their enrichment on the X chromosome there is no evidence that these repeats are involved in dosage compensation as the repeats are also found on autosomes at significant levels.

1.6.3 Why Have DCREs Not Been Identified?

A major limitation of previous attempts to identify DCREs is that X-linked genes on autosomes are only partially compensated.

All studies (excluding *white* studies) used Northern Blots or RNase Protection Assays to quantitate gene expression levels in males and females. These methods experience technical difficulties when quantitating two fold differences in expression. Studies of the *white* gene used spectrophotometric eye pigment assays that must take into account the non-linearity of the pigmentation response to gene dose (Qian and Pirrotta, 1995). Early indications of this non-linearity were demonstrated when transgenes at autosomal sites exhibited full dosage compensation - males with one dose produce twice as much pigment as one dose females. However females with two copies of the white transgene

have a two to three fold higher level of pigmentation than males with one (Hazelrigg *et al.*, 1984; Levis *et al.*, 1985; Pirrotta *et al.*, 1985).

Hypotheses have been suggested to account for partial dosage compensation when X-linked transgenes are translocated to autosomes. Qian and Pirrotta, (1995) suggest that the requirement for a certain amount of DCREs associated with the gene is not being met and/or that the autosomal chromatin environment (more condensed than X chromosomes) is having an inhibitory effect upon the transgene. The use of insulator elements to flank the transgene supports this hypothesis (Roseman *et al.*, 1995).

1.7 A NEW APPROACH TO IDENTIFYING DCRES

Fitzsimons *et al.*, (1999) developed a reporter gene assay that can be used to screen X chromosomal DNA for DCREs. The components of this assay are the *E. coli lacZ* gene under the control of the constitutive promoter from the *armadillo* gene (this fusion is referred to as *arm-lacZ*). The *arm* promoter was chosen because it is constitutive and active in all tissues and all stages of development (Vincent *et al.*, 1994) in both males and females. *arm-lacZ* was flanked by SCS and SCS' insulator elements (specialised chromatin structures). SCS and SCS' sequences act as domain boundaries (Udvardy and Schedl, 1993). Domain boundaries establish a domain of independent gene activity by protecting against regulatory effects of surrounding chromosomal DNA. It has been found that *arm-lacZ* can respond to DCREs when on the X chromosome - one copy in males is expressed at twice the level of one copy in females.

A limitation in studying X-linked genes is that the coding region must remain intact enough so its product can be assayed for. Using this newly developed assay system allows X-linked sequences to be subdivided as a reporter gene is detected in the assay rather than the gene product. Fitzsimons *et al.*, (1999) placed portions of DNA from the *D. melanogaster* X chromosome immediately upstream of the *arm* promoter. The hypothesis for these experiments was that any X-linked sequence containing DCREs would confer dosage compensation onto *arm-lacZ* in males and thereby produce twice the *lacZ* activity in males over females. As yet the DCREs remain unidentified.

1.8 RESEARCH OBJECTIVES

This study has three main objectives. The first objective is to investigate the effect of *roX* genes on dosage compensation regulated by isolated DCREs. The second is to continue with the study begun by Fitzsimons *et al.*, (1999) and isolate the DCREs involved in dosage compensation. However our study will focus particularly on regions of the X chromosome (eg. 1C) known to contain 'high affinity' binding sites for the MSL-1/MSL-2 core complex (Lyman *et al.*, 1997). The final objective is to investigate further the possibility of *Sxl* regulating a second dosage compensation pathway throughout development.

1.8.1 Specific Objectives

Previous studies have been carried out to develop a new reporter gene assay system that can be used to screen X chromosomal DNA for DCREs. Fitzsimons *et al.*, (1999) developed and used this assay system on many constructs containing X-linked fragments of DNA in the attempt to isolate the elusive DCREs. This study will utilise the *arm-lacZ* assay system developed by Fitzsimons *et al.*, (1999) to investigate the role of various X-linked DNA fragments in dosage compensation.

The initial aim was to test if the *roX* genes are needed to be present in *cis* in order for a fragment containing a DCRE to cause a male specific increase in *lacZ* expression. It was found that the *roX* genes alone caused elevated *lacZ* expression ie. the *roX* genes contained DCREs. Consequently the initial objective was modified to test if *roX* cDNAs and fragments of *roX* genes contained DCREs.

The second aim of this study was to determine if DNA fragments from the tip of the X chromosome, in particular the region that shows "high affinity" binding with the MSL-1/MSL-2 core complex. The assay system will also be used to look for DCREs in these X-linked DNA fragments. The presence of DCREs would be confirmed by an increase in *lacZ* expression in males

The third aim was to investigate the role of *Sxl* in dosage compensation in females throughout development. The study aimed to determine if insertion of 3' UTR fragments from other X-linked genes (*Sxl, Small Forked, and Cut*) would cause a

decrease in female specific expression of *lacZ*. These 3' UTR fragments contained 3 or more Sxl binding sites and were inserted into the 3' UTR of *arm-lacZ*.

The fourth and final aim also looked at Sxl involvement in dosage compensation. Experiments with *msl-2* showed the Sxl binding sites were required in both the 3' and 5' UTR to get complete repression of translation. Previously Fitzsimons *et al.*, (1999) showed insertion of a *Runt* 3' UTR fragment into the *arm-lacZ* 3' UTR caused a modest decrease in female *lacZ* expression. This study aimed to determine if an additional Sxl site in the 5' UTR of the *arm-lacZ* construct, carrying the *Runt* 3' UTR fragment, would result in a more dramatic decrease in female *lacZ* expression.

2.0 MATERIALS AND METHODS

2.1 PLASMIDS AND COSMIDS

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

2.2 BACTERIAL STRAIN

The Bacterial strains used in this study are described in Table 1.

2.3 BACTERIAL MEDIA

2.3.1 Luria Broth (LB)

Luria Broth contained (g/L): tryptone, 10; yeast extract, 5; NaCl, 5 and NaOH, 1 ml. For LB agar 25 g/L of agar (Davis) was added to LB medium before autoclaving.

2.3.2 SOB

SOB contained (g/L): tryptone, 20; yeast extract, 5; NaCl, 0.6 and KCl, 0.2. After autoclaving the medium was supplemented with (g/L): $MgCl_2$, 0.2 and $MgSO_4$, 2.5. For SOB agar 15 g/L of agar (Davis) was added to SOB medium before autoclaving.

2.3.3 SOC

SOC contained (g/L): tryptone, 20; yeast extract, 5; NaCl, 0.6 and KCl, 0.2. After autoclaving the medium was supplemented with (g/L): $MgCl_2$, 0.2, $MgSO_4$, 2.5 and sterile glucose, 3.6.

2.3.4 2x YT

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

2.3.5 Terrific Broth (TB)

TB contained (g/L): tryptone, 12, yeast extract, 24 and 4 ml glycerol. This mixture was made up to 900 ml with sterile water. After autoclaving the medium was supplemented with 100 ml of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4.

2.3.6 Antibiotics and Media Additives

2.3.6.1 Ampicillin

A 50 mg/ml stock solution of ampicillin was made up and added to LB, LB agar (section 2.3.1) and 2xYT (section 2.3.4) to a final concentration of 50 μg/ml.

2.3.6.2 Kanamycin

A 10 mg/ml stock solution of kanamycin was made and added to LB agar (section 2.3.1) to a final concentration of 50 μg/ml

2.3.6.3 X-gal (5-bromo-4-chloro-3-indyol-β-D-galactosidase)

A 25 mg/ml stock solution of X-gal dissolved in N, N dimethyl formamide and added to LB agar (section 2.3.1) to a final concentration of 25 μg/ml.

2.3.6.4 IPTG (Isopropylthio-β-D-galactosidase)

A 25 mg/ml stock solution of IPTG was made up and added to LB agar (section 2.3.1) to a final concentration of 25 μg/ml.

2.3.6.5 Chloramphenicol

A 34 mg/ml stock solution of chloramphenicol was made up and added to LB agar (section 2.3.1) to a final concentration of 170 μg/ml.

2.4 MAINTENANCE OF BACTERIAL CULTURES

For long term storage, 3 ml of LB (section 2.3.1) was inoculated with a loopful of culture and grown overnight at 37°C or 30°C. 700 μl of the culture and 300 μl of sterile glycerol were combined in a sterile cryotube and stored at -70°C.

When required a loopful of the stored culture was streaked out onto an LB agar (section 2.3.1) plate containing the appropriate antibiotic (section 2.3.6) and incubated overnight at 37°C or 30°C. If necessary the plate was stored at 4°C for up to two weeks.

Table 1. Plasmid, Cosmid or Bacterial Strain

Plasmid, Cosmid or Bacterial Strain	Relevant Characteristic	Source
Plasmids		
pUChsπΔ2,3	7.3 kb, pUC118 containing 3.6 kb P-element coding sequences, *hsp70* promoter and *ry* 3' flanking region.	Rio and Rubin, (1985)
pHF10	10.22 kb, pCaSpeR4 with *EcoRI* and *SpeI* sites removed, containing 2.4 kb *KpnI/NotI* SCS-SCS' fragment from Elba 6 and *EcoRI-SpeI* linker.	Fitzsimons *et al.*, (1999)
pHF11	16.36 kb, pHF10 containing 6.2 kb *EcoRI/HindIII* (partial fill-in) *arm-lacZ* fragment.	Fitzsimons *et al.*, (1999)
pHF12	8.93 kb, pBSII KS- containing 6.2 kb *PstI/EcoRI arm-lacZ* fragment.	Fitzsimons *et al.*, (1999)
pHF13	9.10 kb, pBSII KS- containing 6.2 kb *PstI/EcoRI arm-lacZ* fragment with 170 bp *runt* PCR product (Sxl orientation) in *MfeI* site of *arm-lacZ*.	Fitzsimons *et al.*, (1999)
pUC118	3.2 kb, cloning vector with *EcoRI* site downstream of P_{lac}. Confers ampicillin resistance.	Vieira and Messing, (1987)
roX1 in pBSII SK-	pBSII SK- containing 3.7 kb 10.A2 *roX1* cDNA, inserted between *EcoRI* and *XhoI*.	H. Amrein[a]
roX1 in pBSII SK-	pBSII SK- containing 3.7 kb 10.A1 *roX1* cDNA, with single 68 nt intron, inserted between *EcoRI* and *XhoI*.	H. Amrein
roX2 in pBSII SK-	pBSII SK- containing 1.1 kb 78.13 *roX2* cDNA inserted between *EcoRI* and *XhoI*.	H. Amrein
roX2 in pBSII SK-	pBSII SK- containing 1.1 kb 78.2.2 *roX2* cDNA, with two introns - 141 nt and 250 nt, inserted between *EcoRI* and *XhoI*.	H. Amrein
hsp83 in pBSII KS-	pBSII KS- containing 0.8 kb *hsp83* fragment inserted into *EcoRV* site of polylinker.	J. Heinrich[b]

pGMroX5	pCaSpeR containing 4.9 kb genomic *D. melanogaster roX1 EcoRI* fragment from *l710E*.	M. Kuroda[c]
pBS2N	pBS KS containing a *NotI* and *NcoI* site insert between *XhoI* and *Asp718*.	J. Heinrich
pRH01	*hsp83* in pBSII KS- containing 1.1 kb *SmaI/XhoI roX2* fragment inserted between *SmaI* and *XhoI*.	This Study
pRH02	pHF11 containing 2.0 kb *NotI/PspOMI (ApaI) hsp83roX2* fragment from pRH01 inserted into *NotI* site upstream of the SCS' element.	This Study
pRH03	pHF11 containing 2.0 kb *NotI/PspOMI (ApaI) hsp83roX2* fragment from pRH01 inserted into *EcoRI* site between SCS/SCS' elements.	This Study
pRH04	pHF11 containing 4.9 kb *roX1 EcoRI* fragment from pGMroX5 inserted into *EcoRI* site.	This Study
pRH05	pBS2N containing 3.7 kb 10.A1 *roX1* cDNA, from *roX1* in BS II SK-,inserted into *BamHI/XhoI* sites of polylinker.	This Study
pRH06	pHF11 cut with *NotI* and treated with Klenow to blunt ends and religated to remove *NotI* site.	This Study
pRH07	pRH06 with *NotI* linker inserted into *EcoRI* site inside the SCS' element.	This Study
pRH08	pRH07 with 3.7 kb 10.A1 *roX1* cDNA from pRH05 inserted into *NotI* linker site.	This Study
pRH09	pRH07 with 1.1 kb 73.13 *roX2* cDNA *NotI/PspOMI* fragment from roX2 in BS SK- inserted into *NotI* linker site.	This Study
pRH10	pRH07 with 4.9 kb *roX1 EcoRI* fragment from pGMroX5 inserted into *EcoRI* site.	This Study
pRH11	pHF11 with 250 bp PCR fragment from 3' end (similar sequence to *roX2*) of *roX1* cDNA inserted into *EcoRI* site.	This Study
pRH12	pHF11 with 400 bp PCR fragment (DNAse hypersensitivity site) from *roX1* cDNA inserted into *EcoRI* site.	This Study

pRH13	pRH09 with 3.7 kb *roX1* cDNA *NotI* fragment inserted into *NotI* site (reconstituted by *roX2* insertion into *NotI* linker).	This Study
p34F3 5.0	pHF11 containing 5.0 kb *EcoRI* fragment from cosmid p34F3.	This Study
p34F3 8.0	pHF11 containing 8.0 kb *EcoRI* fragment from cosmid p34F3.	This Study
p34F3 17.0	pHF11 containing 17.0 kb *EcoRI* fragment from cosmid p34F3.	This Study
pRH17	pHF12 containing 1.2 kb PCR product of *Cut* 3' UTR, containing Sxl binding sites in polyT orientation, inserted into *MfeI* site in 3' UTR of *arm-lacZ*.	This Study
pRH18	pHF12 containing 0.77 kb PCR product of *Sxl* 3' UTR, containing Sxl binding sites in polyT orientation, inserted into *MfeI* site in 3' UTR of *arm-lacZ*.	This Study
pRH19	pHF12 containing 0.3 kb PCR product of *Forked* 3' UTR, containing Sxl binding sites in polyT orientation, inserted into *MfeI* site in 3' UTR of *arm-lacZ*.	This Study
pRH20	pHF12 containing 1.2 kb PCR product of *Cut* 3' UTR, containing Sxl binding sites in polyA orientation, inserted into *MfeI* site in 3' UTR of *arm-lacZ*.	This Study
pRH21	pHF12 containing 0.77 kb PCR product of *Sxl* 3' UTR, containing Sxl binding sites in polyA orientation, inserted into *MfeI* site in 3' UTR of *arm-lacZ*.	This Study
pRH22	pHF12 containing 0.3 kb PCR product of *Forked* 3' UTR, containing Sxl binding sites in polyA orientation, inserted into *MfeI* site in 3' UTR of *arm-lacZ*.	This Study
pRH23	pHF10 containing 7.33 kb *SpeI/EcoRI* fragment, from pRH17, inserted between SCS and SCS' elements.	This Study
pRH24	pHF10 containing 6.88 kb *SpeI/EcoRI* fragment, from pRH18, inserted between SCS and SCS' elements.	This Study

pRH25	pHF10 containing 6.47 kb *SpeI/EcoRI* fragment, from pRH19, inserted between SCS and SCS' elements.	This Study
pRH26	pHF10 containing 7.33 kb *SpeI/EcoRI* fragment, from pRH20, inserted between SCS and SCS' elements.	This Study
pRH27	pHF10 containing 6.88 kb *SpeI/EcoRI* fragment, from pRH21, inserted between SCS and SCS' elements.	This Study
pRH28	pHF10 containing 6.47 kb *SpeI/EcoRI* fragment, from pRH22, inserted between SCS and SCS' elements.	This Study
pRH29	pUC118 containing 6.3 kb *arm-lacZ* fragment from pHF13 with an additional Sxl binding site (oligo), in polyT orientation, inserted into the *KpnI* site in the 5' UTR.	This Study
pRH30	pUC118 containing 6.3 kb *arm-lacZ* fragment from pHF13 with an additional Sxl binding site (oligo), in polyA orientation, inserted into the *KpnI* site in the 5' UTR.	This Study
pRH31	pHF10 containing 6.3 kb *arm-lacZ* fragment from pRH29 inserted into *SpeI/EcoRI* sites between the SCS and SCS' elements.	This Study
pRH32	pHF10 containing 6.3 kb *arm-lacZ* fragment from pRH30 inserted into *SpeI/EcoRI* sites between the SCS and SCS' elements.	This Study
pRH33	pUC118 containing 6.2 kb *arm-lacZ* fragment from pHF13 inserted into *SpeI/EcoRI* sites.	This Study
Cosmids		
115C2	pLorist 6 containing genomic *D. melanogaster* DNA from region 1B/1C of the X chromosome.	*Drosophila* Genome Project[d]
118B3	pLorist 6 containing genomic *D. melanogaster* DNA from region 1C of the X chromosome.	*Drosophila* Genome Project
165D1	pLorist 6 containing genomic *D. melanogaster* DNA from region 1C of the X chromosome.	*Drosophila* Genome Project
40F6	pLorist 6 containing genomic *D. melanogaster* DNA from region 1C of the X chromosome.	*Drosophila* Genome Project

77G2	pLorist 6 containing genomic *D. melanogaster* DNA from region 1C of the X chromosome.	*Drosophila* Genome Project
34F3	pLorist 6 containing genomic *D. melanogaster* DNA from region 1C of the X chromosome.	*Drosophila* Genome Project
109H7	pLorist 6 containing genomic *D. melanogaster* DNA from region 1C/1D of the X chromosome.	*Drosophila* Genome Project
107G5	pLorist 6 containing genomic *D. melanogaster* DNA from region 1C/1D of the X chromosome.	*Drosophila* Genome Project
55G8	pLorist 6 containing genomic *D. melanogaster* DNA from region 1C/1D of the X chromosome.	*Drosophila* Genome Project
155B1	pLorist 6 containing genomic *D. melanogaster* DNA from region 1C of the X chromosome.	*Drosophila* Genome Project
Bacterial Strain		
Esherischia coli DH5α	-F80d*lacZD* M15D(*lacZ* YA-*arg* F)U169 *deo* R *rec* A1 *end* A1 *hsd* R17(r_{K-},m_{K+}) *pho* A *sup* E44 Γ *thi* -1 *gyr* A96 *rel* A1	Hanahan, (1983)
Max Efficiency STBL2 Competant Cells	F' *mcr* A D(*mcr* BC-*hsd* RMS-*mrr*) *rec* A1 *end* A1 *lon gyr* A96 *thi* -1 *sup* E44 *rel* A1 Γ D(*lac-pro* AB)	Hanahan, (1983)

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2.5 BUFFERS AND SOLUTIONS

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

2.5.1 Solutions for Gel Electrophoresis

2.5.1.1 10x Gel Loading Dye

10x 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.

2.5.1.2 1x TAE Buffer (Tris Acetate EDTA Buffer)

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

2.5.1.3 1x TBE buffer (Tris Borate EDTA Buffer)

1x TBE contained 89 mM Tris-HCl, 89 mM Boric acid and 2.5 mM Na_2EDTA.

2.5.2 Solutions for Preparation of Plasmid and Cosmid DNA

2.5.2.1 GTE (Glucose/Tris/EDTA)

GTE contained 50 mM glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA (pH 8.0). GTE buffer was autoclaved and stored at room temperature.

2.5.2.2 TE Buffer 10/1 (Tris EDTA Buffer)

TE buffer 10/1 contained 10 mM Tris-HCl and 1 mM Na_2EDTA. TE buffer was autoclaved and stored at room temperature.

2.5.2.3 Phenol (Tris Equilibrated)

Phenol crystals were heated to 65°C until melted. The bottle was filled with deionized water and mixed to form a fine emulsion between the organic and aqueous phases. The bottle was then placed at 4°C until the phases separated (8 - 16 hours). The upper aqueous layer was removed and the remaining phenol was aliquoted for standard use.

An aliquot of this water-saturated phenol was taken and an equal volume of 0.5 M Tris-HCl (pH 8.0) was added. This was mixed and the phases allowed to separate at 15 - 30°C. After phase separation the upper aqueous phase was removed and an equal volume of 0.1 M Tris-HCl (pH 8.0) was added. The solution was mixed and the upper aqueous phase removed. This step was repeated until the aqueous phase had a pH of 8.0. The phenol was overlayed with an equal volume of 0.1 M Tris-HCl (pH 8.0) and stored at 4°C in a foil covered bottle.

2.5.2.4 Potassium Acetate (pH 4.8)

Potassium acetate contained 5 M KAc and 11.5% glacial acetic acid. Potassium acetate was not autoclaved.

2.5.3 Solutions for Transformation of DNA

2.5.3.1 DnD

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

2.5.3.2 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.

2.5.3.3 Transformation Buffer (TFB)

TFB contained 10 mM K-MES; 45 mM $MnCl.4H_2O$; 10 mM $CaCl.2H_2O$; 3 mM $HCoCl_3$ and 100 mM KCl. TFB was filter sterilised and stored at 4°C.

2.5.3.4 Transformation Buffer 1 (Tfb1)

Tfb1 contained 100 mM $RbCl_2$; 50 mM $MnCl_2$; 30 mM KAc; 10 mM $CaCl_2$ and 15 % glycerol. Tfb1 was adjusted to pH 5.8 with 0.2 M CH_3COOH, before the glycerol was added, filter sterilised and stored at room temperature.

2.5.3.5 Transformation Buffer 2 (Tfb2)

Tfb2 contained 10 mM RbCl2; 80 mM CaCl2; 0.2 % MOPS and 15 % glycerol. Tfb2 was adjusted to pH 7.0, before the glycerol was added, filter sterilised and stored at room temperature.

2.5.4 Solutions used for Fluorometric Quantitation of DNA

2.5.4.1 1x TNE buffer

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

2.5.4.2 Working Dye Solution A

Working dye solution A contained 1x TNE and 0.1 µg/ml Hoechst 33258 solution. Working dye solution A was not autoclaved.

2.5.5 Solutions for Southern Blotting and Immunological Detection (DIG)

2.5.5.1 Denaturation Solution

Denaturation solution contained 1.5 M NaCl and 0.5 M NaOH. Denaturation solution was stored at room temperature.

2.5.5.2 Neutralisation Solution

Neutralisation solution contained 1.5 M NaCl and 0.5 M Tris-HCl (pH 7.0). Neutralisation solution was stored at room temperature.

2.5.5.3 20x SSC (Sodium Standard Citrate)

20x SSC contained 3 M NaCl and 300 mM Sodium citrate (pH 7.0).

2.5.5.4 Standard Hybridisation Buffer

Standard hybridisation buffer contained 5x SSC; 0.1 % (w/v) Sarcosyl; 0.02 % (w/v) SDS and 1 % Blocking reagent.

2.5.5.5 Maleic Acid Buffer (DIG Detection) pH 7.5

Maleic Acid buffer contained 100 mM Maleic acid and 150 mM NaCl.

2.5.5.6 Washing Buffer (DIG Detection)

Washing buffer contained Maleic acid buffer and 0.3 % (v/v) Tween® 20.

2.5.5.7 Blocking Solution (DIG Detection)

Blocking solution contained 1 % blocking reagent dissolved in Maleic acid buffer.

2.5.5.8 Detection Buffer (DIG Detection) pH 9.5

Detection buffer contained 100 mM Tris-HCl; 100 mM NaCl and 50 mM $MgCl_2$.

2.5.6 Solutions for Microinjection

2.5.6.1 Injection Buffer

Injection buffer contained 0.1 mM NaH_2PO_4; 0.1 mM Na_2HPO_4 and 5 M KCl. Injection buffer was stored in 1 ml aliquots at -20°C.

2.5.7 Solutions for β-galactosidase Assays

2.5.7.1 β-galactosidase Assay Buffer

β-galactosidase assay buffer contained 50 mM KH_2PO_4 and 50 mM K_2HPO_4. 1 mM $MgCl_2$ was added after autoclaving. β-galactosidase assay buffer was stored at 4°C.

2.5.7.2 CPRG (Chlorophenol red-β-galactopyranoside monosodium salt)

A 10 mM stock solution of CPRG was made up by adding 250 mg of CPRG powder to 41.16 ml of β-galactosidase assay buffer (section 2.5.7.1). 15 ml aliquots of the 10 mM stock were stored at -20°C. The stock solution was diluted in β-galactosidase assay buffer to give a final concentration of 1 mM.

2.6 PREPARATION OF PLASMID DNA

2.6.1 Large Scale Preparation of Plasmid or Cosmid DNA

Plasmid and cosmid DNA were prepared using the Qiagen Plasmid Midi/Maxi Kit with the Qiagen-tip 100 or 500 respectively or using the Concert Nucleic Acid Purification System Midi prep (Life Tech).

Alternatively, unstable or low yield plasmid DNA was prepared by following Unit 1.7.1 of Current Protocols in Molecular Biology, (1989). 1 ml of LB (section 2.3.1) was inoculated with a loopful of bacteria, from a freshly streaked plate, and grown for 5 - 6 hours at 37°C. This was used to inoculate a 2 L flask containing 500 ml of LB or 2x YT (section 2.3.4) supplemented with ampicillin (section 2.3.6.1). This flask was shaken overnight at 37°C. Cells were harvested in GSA bottles by centrifugation at 6000 rpm for 10 minutes at 4°C in a Sorvall RC5C centrifuge. The pelleted cells were resuspended in 4 ml of GTE (section 2.5.2.1) and 1 ml of 25 mg/ml hen egg white lysozyme (Sigma) in GTE was added, mixed gently and allowed to stand for 10 minutes at room temperature. 15 ml of freshly prepared 0.2 M NaOH/1 % SDS was added, mixed gently by inversion until the solution became homogenous and clear and stood on ice for 10 minutes. 7.5 ml of 5 M KAc (section 2.5.2.4) was then added and the solution mixed by gentle inversion until the viscosity was reduced and a thick precipitate had formed. The solution was incubated on ice for 10 minutes and then centrifuged at 13 000 rpm for 10 minutes at 4°C. The supernatant was poured through sterile cheesecloth into a sterile SS34 tube, 0.6 ml of isoproponal was added per ml of collected filtrate, mixed and left to stand at room temperature for 10 minutes. The pellet was recovered by centrifugation at 11 500 rpm for 10 minutes at room temperature. The supernatant was removed and the pellet dried under vacuum in a SpeedVac Concentrator (Savant).

Purification of the plasmid DNA was based on Unit 1.4.2 of Sambrook et al., (1989). The pellet was resuspended in 4 ml of TE and transferred to a 50 ml polypropylene tube (Greiner). 4.4 g of caesium chloride and 400 µl of ethidium bromide were then added and the solution mixed by inversion. After centrifuging at 4100 rpm for 5 minutes at room temperature, the clear red solution under the flocculant precipitate was transferred to a 6 ml ultracentrifuge tube (Sorvall). If necessary the tube was topped with additional $CsCl_2$/TE solution then sealed with plastic plugs and crimped with a metal cap. Centrifugation was carried out at 55 000 rpm for 18 hours in a Sorvall Combi Ultracentifuge at a water temperature of no less than 15°C. The closed circular plasmid band (second band) was removed using a syringe needle, under UV light if necessary. Repeating the ultracentrifugation steps further purified the plasmid. The ethidium bromide was removed from solution by adding an equal volume of TE saturated

isoamyl alcohol (IAA) and shaking for 2 minutes. After the phases had separated completely the upper organic phase was removed. This process was repeated until no red colour remains in the aqueous phase. The DNA solution was diluted three-fold in TE to prevent precipitation of the $CsCl_2$ then ethanol precipitated (section 2.7.3).

2.6.2 Small Scale Preparation of Plasmid DNA

Small scale plasmid DNA preparations were carried out according to the method of Birnboim and Doly, (1979) and Birnboim, (1983). 3 ml of sterile LB (section 2.3.1) was inoculated with a single bacterial colony and grown to saturation overnight at 37°C, with moderate shaking. 1.5 ml of the overnight cultures were centrifuged for 1 minute at 13 000 rpm in a Heraeus Biofuge 13 microcentrifuge. The supernatant was poured off and the pellet resuspended in 100 µl GTE (section 2.5.2.1). 200 µl 0.2 M NaOH/1 % SDS was added and mixed by inversion and then placed on ice for 5 minutes. 150 µl 5 M KAc (section 2.5.2.4) was added and the solution vortexed at maximum speed for 2 seconds then placed on ice for a further 5 minutes. A second centrifugation for 3 minutes at 13 000 rpm was carried out to pellet cellular debris and chromosomal DNA. The supernatant was transferred to a new microcentrifuge tube and mixed with 800 µl absolute ethanol. Plasmid DNA was then pelleted by centrifugation at 13 000 rpm for 1.5 minutes. The pellet was washed with 70 % ethanol and air dried at 37°C. The pellet was resuspended in 30 µl TE and stored at -20°C.

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

2.7 PURIFICATION OF DNA

2.7.1 Purification of DNA after PCR

Following amplification by Polymerase Chain Reaction (section 2.13), DNA was purified using the Qiagen QIAquick PCR Purification Kit according to the manufacturer's instructions.

2.7.2 Phenol/Chloroform Extraction of DNA

The DNA solution was made up to 200 µl with TE (section 2.5.2.2.) and 10 % NaAc was added. To this solution equal volumes of Tris equilibrated phenol (section 2.5.2.3) and chloroform were added and shaken for two minutes. The solution was centrifuged at 13 000 rpm for 5 minutes. The upper aqueous phase was transferred to a new microcentrifuge tube and ethanol precipitated (section 2.7.3).

2.7.3 Ethanol Precipitation of DNA

Two volumes of absolute ethanol were added to the DNA solution and mixed. After 2 - 3 hours or overnight at -20°C or 30 minutes at -70°C the DNA was pelleted by centrifugation at 13 000 rpm for 15 minutes. The pellet was washed with 70 % ethanol, air dried and resuspended in TE (section 2.5.2.2) to the desired concentration.

2.8 DETERMINATION OF DNA CONCENTRATION

2.8.1 Determination by Comparison to a DNA Ladder

The λ *HindIII/SstII* ladder was run alongside DNA of an unknown concentration. The concentration was estimated by comparison to the intensity of the bands of the ladder. For smaller fragments eg. PCR fragments, DNA molecular weight marker XIII (Boehringer Mannheim) was used for comparison.

2.8.2 Spectrophotometric Determination of DNA Concentration

The DNA of unknown concentration was diluted in TE (section 2.5.2.2.) and the absorbance read at 260 nm after zeroing the spectrophotometer with TE. An OD_{260} of 1 corresponds to 50 µg/ml of double stranded DNA. The absorbance reading was multiplied by 50 and the dilution factor to determine the DNA concentration in µg/ml.

2.8.3 Fluorometric Determination of DNA Concentration

The Hoefer Scientific TKO 100 Fluorometer was adjusted to zero with 2 ml of Working Dye Solution A (section 2.5.4.2). 2 µl of 100 µg/ml Calf Thymus DNA was added to 2 ml Working Dye Solution A to give a reading of 100. The scale was altered to 100 if not exact first time. 2 µl of sample DNA was added to 2 ml Working Dye solution A

and measured. The measurements were taken in duplicate to ensure accuracy. The reading was the concentration of the DNA in ng/µl.

2.9 AGAROSE GEL ELECTROPHORESIS

DNA was size fractionated by electrophoresis on agarose gels in 1x TBE buffer (section 2.5.1.3). The concentration of agarose ranged from 0.8 % for separation of high molecular weight fragments, to 2 % for separation of small fragments. The appropriate amount of agarose was added to 1x TBE buffer and microwaved until melted. When the agarose had cooled it was poured into a casting tray and left to set for 15 - 45 minutes. DNA fragments containing 1x loading dye (section 2.5.1.1) were loaded into wells in the set gel alongside 10 µl of *HindIII/SstII* digested λ DNA or 4 µl of DNA molecular weight marker XIII (Boehringer Mannheim). Gel electrophoresis was carried out in Horizon or Biorad minigel apparatus for 1 - 2 hours at 80 - 100 Volts. The gel was then stained for 15 minutes in 2 µg/ml ethidium bromide and destained in water. The gel was visualised under short wave UV light and photographed using the Gel Documentation System (Alpha Innotech).

2.9.1 DNA Extraction from Seaplaque Agarose

Following an appropriate restriction enzyme digest (section 2.10.2), DNA was size fractioned on a 0.8 – 1 % TAE (section 2.5.1.2) seaplaque agarose gel. The gel was stained in ethidium bromide and the required fragment was cut out with a scalpel under UV light. The DNA was purified from the agarose using the Qiagen QIAquick Gel Extraction Kit or the GibcoBRL Concert Gel Extraction System.

2.10 RESTRICTION DIGESTION OF DNA

2.10.1 Restriction Enzymes

All enzymes were either from New England Biolabs or Boehringer Mannehim.

70 Units/µl: *PspOMI*

20 Units/µl: *BamHI, BstBI, EcoRI, HindIII, PstI, SacII, SmaI, XbaI, XhoI*

10 Units/µl: *AflII, AseI, Asp718, BglII, Bsp120I, HincII, MfeI, NotI, SpeI, XbaI*

5 Units/µl: *HpaI, NheI, SphI*

2.10.2 Analytical Restriction Enzyme Digestion

Restriction enzyme digestions for plasmid analysis after transformation were carried out in commercially prepared buffer. Three µl of DNA was used and 10 units of the appropriate restriction enzyme(s) (section 2.10.1), constituting less than 1/10 of the final volume. When required BSA was added to a final concentration of 0.1 mg/ml. 0.1 µl of RNase was added to each digest or 1 µl was added to a master-mix of buffer and enzyme for multiple digests. Digestions were incubated at 37°C (unless other wise specified ie. *SmaI* at 25°C and *BstBI* at 65°C) for 2-3 hours or overnight, using only 5 units of enzyme. Each digest was mixed with 1x gel loading dye (section 2.5.1.1), then fractionated on an agarose gel (section 2.9).

2.10.3 Preparative Restriction Digestion

2-10 µg of DNA was digested using 10 units of the appropriate enzyme(s) (section 2.10.1) per µg of DNA. Digested DNA was run on a agarose gel (section 2.9) to determine whether complete digestion has occurred. If digestion is not complete more enzyme was added and the DNA digested overnight. Once digestion is complete the enzyme was heat inactivated, if possible, at 65°C of 20 minutes or a phenol/chloroform extraction (section 2.7.2) and ethanol precipitation (section 2.7.3) was carried out to remove all trace of the enzyme.

2.11 SOUTHERN BLOTTING

The Southern Blotting protocol from Ausubel *et al.*, (1997) was followed and a Nylon membrane used. The Whatman 3MM filter paper wick method was performed instead of the sponge method. One addition to the protocol after disassembling the transfer pyramid was sealing the blot in 3MM filter paper and baking it at 80°C for two hours, then wrapping the blot in gladwrap and UV crosslinking by placing on a transilluminator for 1 minute.

2.11.1 Preparation of DIG Labelled Probe

The first step for labelling the DNA probe was to denature the DNA. 16 µl of DNA (about 500 ng) was heated for 10 minutes at 95°C in a boiling water bath. The tube was cooled immediately on ice to prevent reannealing. Once the tube was cooled 4 µl of

DIG-High Prime mix (Boehringer Mannheim) was added and mixed, centrifuged briefly and incubated at 37°C for 60 minutes. The reaction was stopped by adding 2.5 µl of 4M LiCl and 75 µl of cold 95 % ethanol and mixed well. The DNA was left to precipitate on ice for at least 5 minutes. The DNA was pelleted in a microcentrifuge at 4°C for 15 minutes. The supernatant was decanted and the pellet washed with 100 µl of cold 70 % ethanol and centrifuged for a further 5 minutes at 4°C. The supernatant was removed and the pellet dried by placing in a speedvac for 5 minutes. The labelled DNA pellet was resuspended in 50 µl of TE (section 2.5.2.2) to give a final concentration 10 µg/ml.

2.11.2 Hybridisation and Post Hybridisation Washes

Pre-hybridisation and hybridisation was carried out using DIG High Prime (Boehringer Mannheim) standard hybridisation buffer (section 2.5.5.4). For pre-hybridisation the membrane was placed in a hybridisation tube and 100 ml of 2x SSC (section 2.5.5.3) was added to wash away any excess salt. The 2x SSC was then replaced with approximately 25 ml of DIG High Prime standard hybridisation buffer. The hybridisation tube was placed in a 68°C rotary oven for at least one hour. Following pre-hybridisation denatured DIG labelled DNA probe was added to 25 ml of standard hybridisation buffer and mixed. The DNA containing hybridisation buffer was then used to replace the pre-hybridisation buffer. The hybridisation tube and its contents were then placed back in the rotary oven overnight. The next day the blot was removed from the hybridisation tube and washed as follows: 2x 5 minutes in 2x SSC/0.1 % SDS at room temperature, 2x 15 minutes in 0.1x SSC/0.1 % SDS at 68°C under gentle agitation.

2.11.3 Chemiluminescent Detection of DIG Labelled Probe

After hybridisation and stringency washes (section 2.11.2) the membrane was rinsed briefly with washing buffer (section 2.5.5.6). The membrane was then incubated in blocking solution (section 2.5.5.7) for 30 minutes. Anti-DIG-AP conjugate (Boehringer Mannheim) was diluted 1:10 000 in blocking solution and the membrane incubated in this solution for 30 minutes. The membrane was then washed 2x 15 minutes in 100 ml of washing buffer. Prior to detection the membrane was equilibrated in detection buffer (section 2.5.5.8) for 2-5 minutes. Detection involved incubating the blot with about 20

drops of lumigen CSPD® (Boehringer Mannheim), between two sheets of acetate. This was left at room temperature for 5 minutes and then incubated at 37°C for 15 minutes. This was then exposed to X-ray film for 15-25 minutes at room temperature and then the film was developed.

After detection the membrane was stripped by washing 2x 15 minutes with 0.2 M NaOH/0.1 % SDS to remove the probe and then rinsed in 2x SSC (section 2.5.5.3). The membrane was then wrapped in plastic wrap and stored at room temperature until needed for future probing.

2.12 SYNTHETIC OLIGONUCLEOTIDES

Synthetic oligonucleotides used in this study are described in Table 2

2.13 AMPLIFICATION OF DNA BY THE POLYMERASE CHAIN REACTION

Two separate mixes were prepared on ice, one containing 1 µl of genomic DNA, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 1 µl of 10 mM dNTP mix and 16 µl sterile water. The other mix contained 4 µl of 5x Buffer A, 6 µl of 5x Buffer B (both buffers combine to give desired Mg^{2+} concentration), 1 µl of ELONGASE Enzyme Mix (Life Technologies) and 19 µl sterile water. The two mixes were combined in an amplification tube on ice. A negative control containing no genomic DNA was also prepared. The amplification tubes were placed in a thermal cycler and the following cycles were used to amplify the desired DNA:

Small forked - Cycle 1, denaturation at 94°C for 4 minutes; cycles 2 - 6, denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 68°C for 1 minute; cycles 7 - 36, denaturation at 94°C for 30 seconds, extension at 68°C for 1.5 minutes; cycle 37 extension at 68°C for 7 minutes; hold at 4°C.

Cut - Cycle 1, denaturation at 94°C for 4 minutes; cycles 2 - 6, denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 68°C for 1.5 minutes; cycles 7 - 36, denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 68°C for 1.5 minutes; cycle 37, extension at 68°C for 7 minutes; hold at 4°C

Sex lethal - Cycle 1, denaturation at 94°C for 4 minutes; cycles 2 - 6, denaturation at 94°C for 30 seconds, annealing at 45°C for 30 seconds, extension at 68°C for 1.5 minutes; cycles 7 - 36, denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 68°C for 1.5 minutes; cycle 37 extension at 68°C for 7 minutes; hold at 4°C.

DNAse I roX1 and 3' roX1 - Cycle 1, denaturation at 94°C for 4 minutes; cycles 2 - 6, denaturation at 94°C for 30 seconds, annealing at 40°C for 30 seconds, extension at 68°C for 1 minute; cycles 7 - 36, denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, extension at 68°C for 1 minute; cycle 37, extension at 68°C for 7 minutes; hold at 4°C.

2.14 SUBCLONING

2.14.1 Filling in 5' Overhangs with DNA Polymerase I, Large (Klenow) Fragment

After restriction enzyme digestion (section 2.10.3) and heat inactivation, 1 U/μg Klenow (New England Biolabs) and 33 μM 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. If the restriction enzyme could not be heat inactivated a phenol/chloroform extraction (section 2.7.2) was performed and the ethanol precipitated (section 2.7.3) DNA resuspended at 50 μg/ml in 1x EcoPol buffer (NEB) before Klenow and dNTPs were added.

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

After restriction enzyme digestion (section 2.10.3) and heat inactivation, 1U/μg Mung Bean Nuclease (NEB) and 1 mM $ZnSO_4$ were added to DNA with a concentration of 100 μg/ml. The solution was incubated at 30°C for 30 minutes and then heat inactivated at 75°C for 10 minutes. If the restriction enzyme could not be heat inactivated a phenol/chloroform extraction (section2.7.2) was performed and the ethanol precipitated (section 2.7.3) DNA resuspended to 100 μg/ml in 1x Mung Bean Nuclease buffer (NEB) before the addition of Mung Bean Nuclease and $MgSO_4$.

Table 2. Synthetic Oligonucleotides

Oligonucleotide[a]	Sequence 5' to 3'	Use
Sequencing Primers		
T7	GTAATAGGACTCACTATAG GGC	Sequencing of *Sxl, Forked,* and *Cut* PCR inserts in pHF12.
Sxl Top	GTCGAATTCATTTTAGATA CTAACCTACAACTAA	Sequencing of *Sxl* PCR insert in pHF12.
Sxl Bottom	GTTCCAATTGGTAGTTGTT GTCGCTGGTAGAGAT	Sequencing of *Sxl* PCR insert in pHF12.
arm forward	TCAAACACTGGTCTTTAAT TTCTT	Sequencing inserts in *EcoRI* site of pHF11.
PCR Primers		
Forked Top	GTCGAATTCTTCAATCGAC TCCTAAGCGGTGCA	PCR amplification of S*mall Forked* 3' UTR.
Forked Bottom	GTTCCAATTGCTAAATAAA TTCAAGCCAAGTCACA	PCR amplification of S*mall Forked* 3' UTR.
Cut Top	GTCGAATTCGCACTTAGGC TCTAGAAAACTATA	PCR amplification of *Cut* 3' UTR.
Cut Bottom	GTCGAATTCACGTTGTAAA CTTAAGATCTAGGC	PCR amplification of *Cut* 3' UTR.
Sxl Top	GTCGAATTCATTTTAGATA CTAACTACAACTAA	PCR amplification of *Sxl* 3' UTR.
Sxl Bottom	GTTCCAATTGGTAGTTGTT GTCGCTGGTAGAGAT	PCR amplification of *Sxl* 3' UTR.
DNAseI *roX1* Top	GTCGAATTCGAACGAAAG AGACAAATGAACCC	PCR amplification of DNAseI site in *roX1* .
DNAseI *roX1* Bottom	GTCGAATTCTTATGGCGAT TCTACGCTCCTG	PCR amplification of DNAseI site in *roX1* .
3' *roX1* Top	GTCGAATTCGAAAAACACA TTTACTAACAAATAA	PCR amplification of *roX1* 3' UTR.
3' *roX1* Bottom	GTCGAATTCCCCAAAGAAA TCCACATAACAT	PCR amplification of *roX1* 3' UTR.

Oligonucleotide Linkers		
Sxl 5' Insert Top	GTACCTTTTTTTTTTTGCTA GCC	Forms Sxl binding site linker with *Sxl* 5' insert Bottom.
Sxl 5' Insert Bottom	GTACGGCTAGCAAAAAAA AAAAG	Forms Sxl binding site linker with *Sxl* 5' insert Top.
NotI Top	AATTCATCTGCGGCCGCAG ATC	Forms single *NotI* site linker with *NotI* Bottom.
NotI Bottom	AATTGATCTGCGGCCGCAG ATC	Forms single *NotI* site linker with *NotI* Top.

[a] All oligonucleotides were custom made from Life Technologies, except T7 (Stratagene), DNAseI *roX1* Top and Bottom and 3' *roX1* Top and Bottom (Sigma Genosys)

2.14.3 Removal of 5' Phosphate Groups from DNA with Calf Intestinal Phosphatase

Removal of 5' phosphate groups prior to ligation prevents vector self-ligation. 1 U/pmol DNA ends of Calf Intestinal Alkaline Phosphatase was added and incubated at 37°C for 30 minutes. The enzyme was then heat inactivated at 75°C for 10 minutes. CIP is active in all NEB buffers.

2.14.4 Annealing Oligonucleotides

Oligonucleotides were resuspended to 100 μM in sterile TE (section 2.5.2.2). 25 μg of the two oligonucleotides (section 2.12) was combined with 15 μl of 5M NaCl and the volume made up to 500 μl with sterile water. The solution was heated to 95°C, removed from the heat and allowed to cool to room temperature.

2.14.5 Addition of 5' Phosphates to Oligonucleotides by T4 Polynucleotide Kinase

15 μl of annealed synthetic oligonucleotides (section 2.12) was added to 1x ligase buffer (contains 1 mM ATP) and 10 Units T4 polynucleotide kinase (NEB). The solution was incubated at 37°C for 30 minutes then heat inactivated at 65°C for 20 minutes.

2.15 DNA LIGATION

Ligation of DNA fragments was carried out in 1 x ligase buffer, with 1 μl of T4 DNA ligase (NEB), in a final volume of 10-25 μl. The ligation mixture contained 200 ng of vector DNA and a 3 fold molar excess of insert DNA to vector. Prior to ligation vector DNA was treated with calf intestinal alkaline phosphatase (section 2.14.3) to prevent self ligation. Ligations were incubated overnight at 18°C.

2.16 TRANSFORMATION OF PLASMID DNA

Plasmids (except p34F3 17.0) were transformed into *Esherischia coli* DH5α cells (section 2.2) by the method of Hanahan, (1983). DH5α cells were streaked out for single colonies from a glycerol stock onto a SOB agar (section 2.3.2) plate and incubated at 37°C overnight. Several 2-3 mm diameter colonies were picked off the

plate and dispersed in 1 ml SOB medium (section 2.3.2) by vortexing. The cells were then inoculated into a 1 litre flask containing 30 ml of SOB medium and incubated at 37°C, with moderate shaking, until the cell density reached 4 - 7 x 10^7 viable cells/ml (OD_{550} of 0.45 - 0.55). Once the density is obtained the culture was collected in an SS34 centrifuge 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 was removed and the cells resuspended in 1/3 the original culture volume of TFB (section 2.5.3.3). The cells were chilled on ice and pelleted as before and resuspended in TFB to 1/12.5 of the original culture volume. 7 µl of DnD (section 2.5.3.1) was added per 200 µl of cell suspension and mixed immediately by swirling the tube for several seconds. The cells were chilled on ice for 10 minutes before a second equal aliquot of DnD was added and the cells were chilled for a further 10 minutes. 210 µl aliquots of cells were transferred into chilled 15 ml polypropylene tubes (Falcon). 1 µl of ligation solution was added, swirled and chilled on ice for 30 minutes. Vector alone DNA was used as a positive control. The cells were heat shocked in a 42°C water bath for 90 seconds and then cooled on ice for two minutes before 800 µl of SOC (section 2.3.3) was added to each tube. The cells were incubated at 37°C with moderate shaking for 60 minutes. 100 µl of each culture was spread onto LB (section 2.3.1) plus ampicillin (section 2.3.6.1) plates. The remaining culture was transferred to a microcentrifuge tube and the cells pelleted at 13 000 rpm for 1 minute. Most of the supernatant was removed and the pellet resuspended in the remaining drops. 100 µl of this concentrated culture was spread onto LB plus ampicillin plates. The plates were incubated overnight at 37°C.

An alternative transformation method was also used. This method allowed for the storage of competent cells at -70°C, which could then be thawed and used when required. A single DH5α colony (section 2.2) from a freshly streaked SOB agar plate (section 2.3.2) or 200 µl of a fresh overnight culture, was added to 100 ml of SOB and grown at 37°C until an OD_{550} of about 0.55 was reached. The cells were harvested at 4°C for 10 minutes at 3000g, resuspended in 28 ml of Tfb1 (section 2.5.3.4.) and left to stand on ice for 20 minutes. The cells were pelleted again at 4°C for 8 minutes at 2500g and resuspended in 8 ml of Tfb2 (section 2.5.3.5). The suspension was divided into 200

µl aliquots and either used directly or flash frozen in liquid air, then stored at -70°C. Before use frozen cells were allowed to thaw on ice. ≤ 100 ng of DNA was added to each tube. This corresponded to 2 - 6 µl of a 10 - 15 µl ligation mix. These cells plus DNA were incubated on ice for 20 - 40 minutes. The cells were then heat shocked for 90 seconds at 42°C, cooled on ice for 1 minute, added to 1 ml of SOC (section 2.3.3) and incubated at 37°C for 1 hour. The samples were spun for 30 seconds a 13 000 rpm to concentrate the cells. 100 µl of the supernatant was saved and used to resuspend the pellet. This 100 µl was plated onto LB agar (section 2.3.1) plus ampicillin (section 2.3.6.1) plates and incubated overnight at 37°C.

The plasmid p34F3 17.0 was transformed into STBL2™ competent cells (Life Tech) (section 2.2). These cells have been prepared by modification of the procedure of Hanahan, (1983), and are suitable for cloning of unstable inserts. The protocol for transformation of the competent cells was basically the same as the previous protocols except the cells were only heat shocked for 25 seconds and cooled for two minutes on ice. The tubes containing ligation reactions in SOC medium (section 2.3.3) were incubated at 30°C for 90 minutes to optimise transformation efficiency. The culture was spread on LB (section 2.3.1) amp (section 2.3.6.1) plates, but incubated overnight at 30°C instead of 37°C.

2.17 FLY STOCKS AND TRANSGENIC FLY STOCKS

Fly stocks and transgenic fly stocks used in this study are described in Table 3.

2.18 FLY MEDIA

2.18.1 Cornmeal Agar

Cornmeal agar contained (g/L): agar (Davis), 10.7; cornmeal, 107.1; yeast, 32. These were combined with 1 L of H_2O and stirred over a medium heat until boiling. Once boiling 128.3 g sugar and 3.3 g Mouldex (Methyl paraben) dissolved in 37 ml 95 % ethanol were added and stirred in. Once mixed the cornmeal agar was poured into 20 ml vials (Labserv), 100 ml Schott bottles or 60 x 15 mm tissue culture dishes (Corning or Falcon).

Table 3. *Drosophila melanogaster* Fly Stocks and Transgenic Fly Stocks

Fly Strain	Use/Chromosomal Linkage	Source
***Drosophila melanogaster* Fly Stocks**		
y w	Microinjection recipient strain.	J. Lucchesi[a]
w; In(3LR)TM3, Sb ry^{rk} e/In(3LR)TM6, Tb e	Determination of chromosomal linkage.	J. Lucchesi
y w; L^{2}/CyO, Cy pr cn^{2} y^{+}	Determination of chromosomal linkage.	J. Lucchesi
Transgenic Fly Stocks		
arm-lacZ X #3: *{w+} y w*	X	Fitzsimons *et al.*, (1999)
p34F3 5.0 #1: *{w+} y w*	3rd	This Study
p34F3 8.0 #1: *{w+} y w*	3rd	This Study
pRH02 #1: *{w+} y w*	2nd	This Study
pRH02 #2: *{w+} y w*	3rd	This Study
pRH03 #1: *{w+} y w*	2nd	This Study
pRH03 #2: *{w+} y w*	2nd	This Study
pRH03 #3: *{w+} y w*	2nd	This Study
pRH04 #1: *{w+} y w*	3rd	This Study
pRH04 #2: *{w+} y w*	2nd	This Study
pRH24 #1: *{w+} y w*	2nd	This Study
pRH24 #2: *{w+} y w*	2nd	This Study
pRH24 #3: *{w+} y w*	3rd	This Study
pRH25 #1: *{w+} y w*	3rd	This Study
pRH23 #1: *{w+} y w*	2nd	This Study
pRH23 #2: *{w+} y w*	2nd	This Study

pRH23 #3: *{w+} y w*	3rd	This Study
pRH31 #2: *{w+} y w*	2nd	This Study
pRH08 #1: *{w+}y w*	3rd	This Study
pRH08 #2: *{w+}y w*	2nd	This Study
pRH09 #1: *{w+}y w*	3rd	This Study
pRH09 #2: *{w+}y w*	3rd	This Study
pRH09 #3: *{w+}y w*	2nd	This Study
pRH11 #1: *{w+} y w*	3rd	This Study
pRH11 #2: *{w+} y w*	2nd	This Study
pRH11 #3: *{w+} y w*	3rd	This Study
pRH12 # 1: *{w+}y w*	2nd	This Study
pRH12 # 2: *{w+}y w*	3rd	This Study
pRH12 # 3: *{w+}y w*	3rd	This Study
pRH13 #1: *{w+} y w*	2nd	This Study
pRH13 #2: *{w+} y w*	3rd	This Study
pRH13 #3: *{w+} y w*	X	This Study

[a] J. Lucchesi, Department of Biology, 1510 Clifton Road, Emory University, Atlanta, Georgia 30322, USA.

2.18.2 Formula 4-24 (Instant Fly Food)

7 ml of H_2O was added to 1.5 g of Formula 4-24 in a vial and mixed.

2.19 MAINTENANCE OF FLY STOCKS

All stocks were kept in 20 ml cornmeal agar vials (section 2.18.1) at 18°C and or 22°C. Every four weeks (22°C stocks) or six weeks (18°C stocks) flies were turned into new vials. When in use, flies were bred in 100 ml bottles, and kept at 25°C, to expand the stock.

2.19.1 Setting Fly Crosses

Five virgin females (section 2.19.2) and five males of required strains were mated in a cornmeal agar vial (section 2.18.1). To expand the stock approximately 20 virgin females and 20 males were mated in 100 ml bottles. Adult flies were removed after one week and the progeny emerged three to five days later.

2.19.2 Collection of Virgin Females

Virgins were collected from bottles in which flies were beginning to ecclose. The bottles were cleared of flies in the morning and incubated at 25°C for six to eight hours. Female flies were then collected in the afternoon and held in vials until needed. Eight hour old flies have not reached sexual maturity, which ensured that female flies collected were virgins.

2.20 MICROINJECTION OF *D. MELANOGASTER* EMBRYOS

2.20.1 Co-Precipitation of Plasmid DNA

20 µg of plasmid DNA and 6 µg of pUChsπΔ2,3 (Rio and Rubin, 1985) (section 2.1) were added together and the volume was made up to 100 - 200 µl with TE (section 2.5.2.2). The DNA was then ethanol precipitated (section 2.7.3) and resuspended in injection buffer (section 2.5.6.1) to 0.5 µg/µl. Before use the DNA solution was centrifuged at 13 000 rpm for 20 minutes to remove any particles that may block the microinjection needle. 3 µl of coprecipitated DNA was loaded into a Femtotip using a microloader (Eppendorf).

2.20.2 Collection of Embryos

Approximately 200 flies were placed in a plastic tripour beaker and inverted onto a 60x 15 mm plate of cornmeal agar (section 2.18.1), with a small amount of yeast paste in the centre. These chambers were placed at 15 - 20°C in the dark for three days to allow the flies to acclimatise. When embryos are being collected for injection the cornmeal agar plates are changed every 30 - 40 minutes and embryos transferred from the plate to a microscope slide with a wet paintbrush. The microscope slide had double sided tape on it to help with removal of the chorion and hold the embryos in place during injection.

2.20.3 Dechorination of Embryos

Using fine forceps under a dissecting microscope, the chorion of the collected embryos was teased away by rolling the embryos along the double sided tape. Dechorionated embryos were lined up along one edge of the tape. Five minutes per slide was allowed for dechorionation of as many embryos as possible (usually about 30). All the tape except the strip containing the embryos was removed before dehydration.

2.20.4 Dehydration of Embryos

The dechorionated embryos were dehydrated by placing the microscope slide into a large glass petri dish containing silica gel. Dehydration was carried out for 0 - 5 minutes depending on temperature, humidity and any other factors that may affect the firmness or softness of the embryos. The embryos needed to be flaccid enough so as not to burst during microinjection, but firm enough not to buckle and possibly break the needle. After dehydration the embryos were covered with halocarbon oil (Series 700, Halocarbon Products Corporation) or paraffin oil (Witerex 334, Pauling Industries Ltd) to stop further 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). An injection pressure of 50 - 600 kPa was used to inject the DNA into the posterior pole of the embryos (collected on microscope slides) where the germ cells eventually would form. Following microinjection the slides were placed on a rack in a plastic container lined with moist paper towels (to increase humidity). The

container was incubated at 18°C for 24 hours and then at 22°C for 24 hours. Surviving larvae were transferred to a vial containing Formula 4-24 food (section 2.18.2) (approximately 40 per vial) and incubated at 25°C until the flies emerge. These first flies are termed the G0 generation for future crosses.

Alternatively microinjection was carried out using a syringe attached to the micromanipulator via a plastic tube filled with paraffin oil. Femtotips could still be used attached to the micromanipulator. Pressure was applied by moving the syringe plunger. This created an appropriate flow of DNA out of the needle.

2.20.6 Crossing Adult Survivors

After hatching the G0 flies were mated to the *y w* recipient stock individually. Each G0 female was mated with two *y w* males and each G0 male was mated with five *y w* virgin females (section 2.19.2) and incubated at 25°C. The adults were removed after one week and vials incubated at 25°C until the G1 progeny hatched.

2.20.7 Identification of Transformants and Establishment of a Transformant Stock

G1 progeny were examined twice a day for transformants. Transformant flies (w^+) had eye colours ranging from pale orange to wildtype. Transformant males were crossed with five *y w* virgin females (section 2.19.2). If no transformant males were found a transformant female was mated with two *y w* males. Heterozygous G2 males and virgin *y w* females (or heterozygous G2 virgin females and *y w* males) were crossed to produce heterozygous G3 progeny for use in β-galactosidase and total protein assays. Heterozygous G2 males and G2 virgin females were also crossed, then a single homozygous male and virgin female were crossed to establish 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^+ males 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 2.19.2). *Sb* w^+ and *Cy* w^+ progeny from these crosses were mated with *y w* virgin females. If the insert was on the 2nd chromosome, all the non-*Cy*

progeny would be w^+. Alternatively if the insertion was on the 3rd chromosome, all non-*Sb* progeny would be w^+.

To determine if the insertion 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 would w^+, due to females receiving their X chromosome from the male parent.

2.22 β-GALACTOSIDASE ASSAYS

β-galactosidase assays were based on the method of Simon and Lis, (1987). 12 male flies and 9 female flies were hemisected by cutting between the abdomen and thorax. The heads and thoraxes were transferred to weighed microcentrifuge tubes and weighed again. The heads and thoraxes were then transferred to a 1 ml homogeniser (Kontes) and ground in 500 µl of assay buffer (section 2.5.7.1). The homogenate was transferred back to the microcentrifuge tubes 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 975 µl or 950 µl respectively of assay buffer containing 1 mM Chlorophenol red-β-galactopyranoside monosodium salt (CPRG) (section 2.5.7.2). Assays were performed in triplicate for each sample. The samples were incubated at 37°C. After 30 minutes each sample was transferred to a 1 ml quartz cuvette and the absorbance read at 574 nm using a Shimadsu spectrophotometer. The samples were returned to the microcentrifuge tubes and incubated for a further 30 minutes after which the absorbance was read again. A graph of absorbance versus time (minutes) was drawn and a straight line through zero was plotted for each sample. The difference in amounts of protein in each sample was standardised by measuring the wet weight of the flies and by carrying out total protein assays (section 2.22.1). β-galactosidase activity was measured as Δabsorbance/min/µg protein, and Δabsorbance/min/mg fly weight. Male:Female ratios of β-galactosidase activity were calculated using these measurements.

2.22.1 Total Protein Assays

Total protein assays were carried out following the Bio-Rad Protein Microassay procedure according to the manufacturer's instructions. 0 - 15 μg/ml of bovine gamma immunoglobulin (Biorad) was used as the protein standard for the generation of a standard curve. The standard curve plotted absorbance at 595 nm against μg/15μl protein. 15 μl of each fly homogenate was assayed in triplicate. The average absorbance was calculated and the μg protein/15 μl determined using the standard curve.

3.0 RESULTS

3.1 DETERMINATION OF THE EFFECT OF *ROX* GENES IN *CIS* ON REPORTER GENE EXPRESSION

3.1.1 GENERATION OF *HSP83-ROX2* RNA EXPRESSING CONSTRUCTS

hsp-83-roX2 constructs described below were designed to determine the extent to which *roX2* RNA expression under the control of the *hsp83* promoter affects dosage compensation in males and females. The *hsp83-roX2* fragment was inserted both upstream of the SCS' insulator element and within the region bounded by the SCS and SCS' insulator elements.

3.1.1.1 Insertion of *roX2* cDNA into *hsp83* in pBS

Both *roX1* and *roX2* cDNA sequences in pBluescript (pBS) were obtained from Dr. Hubert Amrein (Table 1). These cDNAs came in two forms, with and without introns, inserted between *EcoRI* and *XhoI* (5' - 3') in pBluescript SK- (Stratagene). To generate larger DNA stocks the resuspended DNA was transformed into *E. coli* (section 2.16) and a large scale DNA preparation (section 2.6.1) was carried out.

Plasmids were digested with *EcoRI* and *XhoI* (section 2.10.2) to determine if these restriction enzymes could be used to excise the *roX* cDNA inserts from pBS SK-. *EcoRI* did not cut the *roX1* cDNA (10.A2) plasmid, suggesting a problem with the *EcoRI* site reconstitution in the *roX1* cDNA (10.A2). This digest also found that *roX2* contained an extra *EcoRI* site 292 base pairs into the 5' end of the insert, which eliminated the use of *EcoRI* as a cloning enzyme. This made subcloning of the *roX* cDNAs more difficult that initially anticipated.

The 1.1 kb *roX2* cDNA fragment was excised out (section 2.9.1) of pBS SK- using *SmaI* and then *XhoI* separately (both in the polylinker of pBS) (section 2.10.3) and ligated (section 2.15) with *hsp83* in pBS, cut with *HincII* then *XhoI* separately. *HincII* and *SmaI* both generate blunt ends, which enabled a straightforward ligation. Transformation of this ligation mix (section 2.16) into *E. coli* was carried out and

plasmid DNA was isolated from transformation colonies (section 2.6.2). The correct clone was identified by digestion with *HincII* and *XhoI,* which generated a 3.9 kb *hsp83* in pBS fragment and 1.3 kb *roX2* fragment. This plasmid was called pRH01.

3.1.1.2 Insertion of *hsp83-roX2* into pHF11 Upstream of the SCS' Insulator Element

pRH01 had a *NotI* site present at the 5' end of *hsp83* and an *ApaI* (compatible end with *NotI*) site 3' of *roX2*. These two sites allowed for excision (section 2.9.1) of *hsp83-roX2* out of pBS SK-, cut with *NotI and PspOMI* (*ApaI* site) (section 2.10.3), and subsequent insertion of the fragment into the *NotI* site of pHF11 (section 2.15). The *NotI* site in pHF11 is upstream of the SCS' element, resulting in *hsp83-roX2* being outside of the region protected by the SCS and SCS' insulators.

hsp83-roX2 was initially digested with *NotI*, phenol/chloroform treated (section 2.7.2) and ethanol precipitated (section 2.7.3), then digested with *PspOMI.* This digestion resulted in a 2.2 kb *hsp83-roX2* fragment which was gel extracted and prepared for ligation into pHF11, which had been cut with *NotI* and treated with Calf Intestinal Phosphatase (CIP) (section 2.14.3). Transformation into *E. coli* (section 2.16) of the ligation mixture and subsequent small-scale DNA preparations (section 2.6.2), digested with *XbaI,* showed bands at 10.63 kb, 4.23 kb, and 1.5 kb consistent with vector pHF11. A second attempt at small scale DNA preparations found three colonies showing additional 1.2 kb and 0.8 kb fragments (Figure 1A). A digest with *AseI and Asp718* (4.05 kb and 2.42 kb bands) confirmed these plasmids as having the *hsp83-roX2* insert in the opposite orientation to *arm-lacZ* (Figure 1B). This plasmid was called pRH02.

3.1.1.3 Insertion of *hsp83-roX2* into pHF11 Within the SCS/SCS' Insulated Region

As noted previously the single *NotI* site of pHF11 is upstream of the SCS' insulator element. To insert *hsp83-roX2* within the region bounded by the insulator elements, an alternative cloning strategy was used. This alternative strategy involved digesting pRH01 with *NotI* and *PspOMI* (section 2.10.3) and treating both ends with DNA polymerase I (Klenow) to generate blunt ends (section 2.14.1). This fragment was then ligated with pHF11 (section 2.15), digested with *EcoRI* and treated with DNA Polymerase I (Klenow). Cutting pHF11 with *EcoRI* allowed for the insertion of the fragment into the vector downstream of the SCS'

Figure 1. Restriction Enzyme Digestions of *hsp83-roX2* containing Constructs to confirm Insertion and Orientation

A. *XbaI* digestion of pRH02 to confirm the insertion of *hsp83-roX2* into pHF11.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *XbaI* digestion of plasmid pRH02.

B. *Asp718* and *AseI* digestion of pRH02 to confirm the orientation of the *hsp83-roX2* insert.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *Asp718/AseI* digestion of plasmid pRH02.

C. *XbaI* digestion of pRH03 to confirm the insertion of *hsp83-roX2* into pHF11.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. XbaI digestion of plasmid pRH03.

D. *BamHI* digestion of pRH03 to confirm the orientation of the *hsp83-roX2* insert.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *BamHI* digestion of plasmid pRH03.

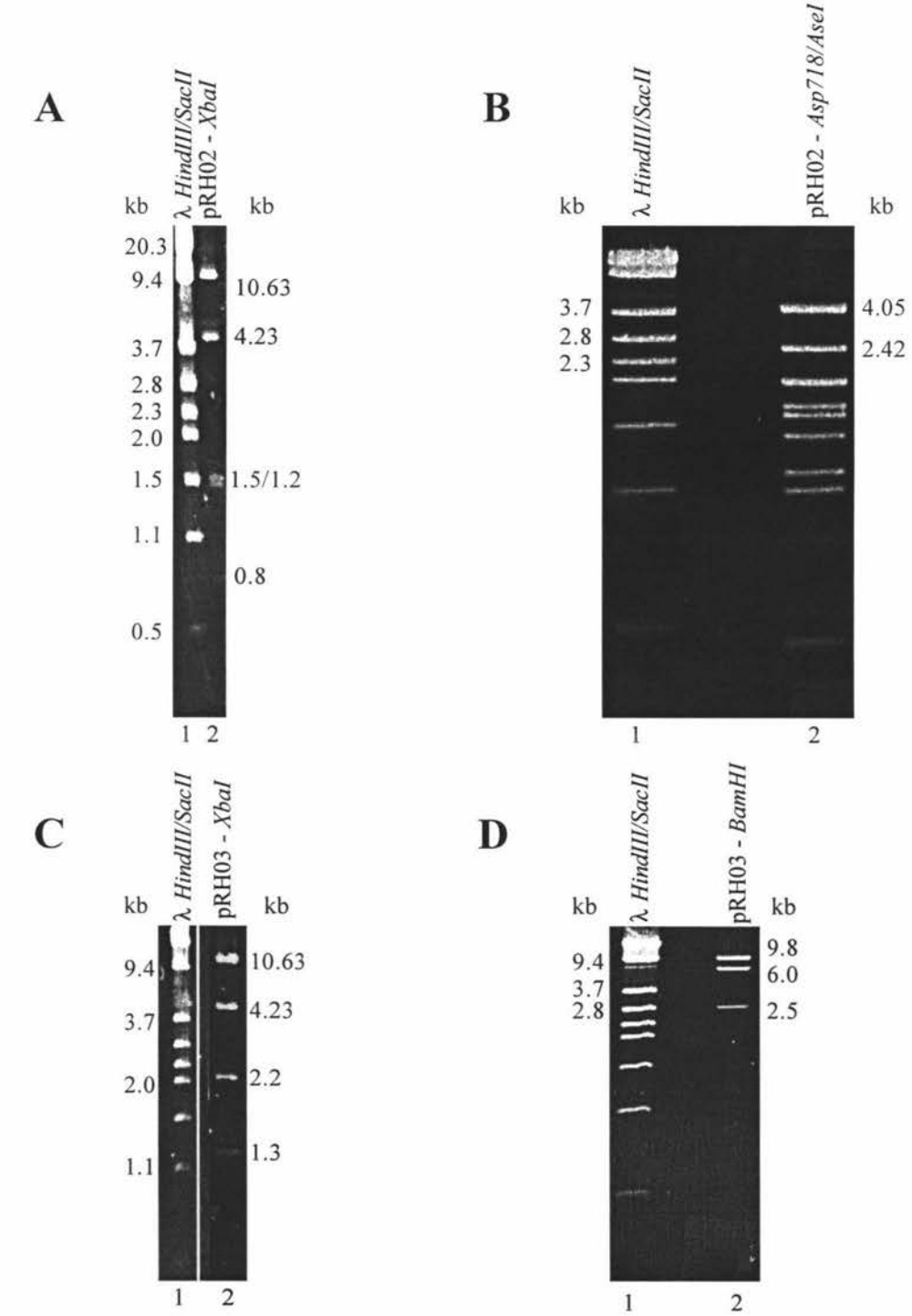

A
λ HindIII/SacII
pRH02 - XbaI
kb
kb
20.3
9.4
3.7
2.8
2.3
2.0
1.5
1.1
0.5
10.63
4.23
1.5/1.2
0.8
1 2
B
λ HindIII/SacII
pRH02 - Asp718/AseI
kb
kb
3.7
2.8
2.3
4.05
2.42
1
2
C
λ HindIII/SacII
pRH03 - XbaI
kb
kb
9.4
3.7
2.0
1.1
10.63
4.23
2.2
1.3
1
2
D
λ HindIII/SacII
pRH03 - BamHI
kb
kb
9.4
3.7
2.8
9.8
6.0
2.5
1
2

Table 4. β-galactosidase Assays of Transgenic Flies carrying Autosomal Insertions of pRH02 and pRH03

Five transgenic lines were assayed from both constructs. For each line the expression of one dose males and females was compared, as was expression of two dose males and females for three lines. Each assay was performed a minimum of four times and standardised using total protein measurements and total wet weight. The standard error represents the variability between the experiments.

Line	Dose	Chromosome	M:F Ratio		Standard Error
pRH02 - Insertion of *hsp83-roX2* into pHF11 upstream of the SCS' element					
pRH02 #1	1	2	Protein	1.34	0.04
			Wet weight	1.51	0.08
pRH02 #2	1	3	Protein	1.59	0.03
			Wet weight	1.70	0.07
	2		Protein	1.44	0.04
			Wet weight	1.65	0.03
pRH03 - Insertion of *hsp83-roX2* into pHF11 within the SCS/SCS' insulated					
pRH03 #1	1	2	Protein	1.65	0.04
			Wet weight	1.82	0.10
	2		Protein	1.60	0.04
			Wet weight	1.68	0.07
pRH03 #2	1	2	Protein	1.50	0.07
			Wet weight	1.54	0.05
pRH03 #3	1	2	Protein	1.56	0.07
			Wet weight	1.69	0.07
	2		Protein	1.22	0.05
			Wet weight	1.39	0.09

element. The ligation mix was transformed into *E. coli* (section 2.16) and small scale DNA preparations (section 2.6.2) checked for insertion by digestion with *XbaI* (section 2.10.2). A single preparation was found to contain the correct banding pattern, with fragments at 10.63 kb, 4.23 kb, 2.2 kb, and 1.3 kb (Figure 1C). A final check by digestion with *BamHI* confirmed (bands present at 9.8 kb, 6.0 kb, and 2.5 kb) the insertion orientation as having *hsp83-roX2* inserted in the opposite orientation to *arm-lacZ* in pHF11 (Figure 1D). This plasmid was called pRH03.

3.1.1.4 β-galactosidase Assays of Autosomal Insertions of *hsp83-roX2* Expressing Constructs – pRH02 and pRH03

Two lines carrying autosomal insertions of pRH02 and three lines carrying autosomal insertions of pRH03 were obtained via P-element mediated transformation (section 2.20). β-galactosidase assays (section 2.22) were performed on one dose (heterozygous) males and females and on two dose (homozygous) males and females. Reporter gene expression was significantly increased in heterozygous males carrying either pRH02 or pRH03. However homozygous males showed no further increase in reporter gene expression for pRH02 and a decrease in expression in one line for pRH03. Results of the assays are summarised in Table 4.

For flies with one copy of pRH02 the male:female ratios were 1.34 (se 0.04), 1.58 (se 0.03) (standardised with total protein) and 1.51 (se 0.08), 1.66 (se 0.10) (standardised with total fly weight). For flies carrying two copies of pRH02 the male:female ratios were 1.45 (se 0.03) (standardised with total protein) and 1.58 (se 0.07) (standardised with total fly weight).

For flies with one copy of pRH03 the male:female ratios were 1.69 (se 0.03), 1.51 (se 0.05), 1.62 (se 0.06) (standardised with total protein) and 1.86 (se 0.12), 1.56 (se 0.04), 1.70 (se 0.08) (standardised with total fly weight). For flies with two copies of pRH03 the male:female ratios were 1.60 (se 0.04), 1.22 (se 0.05) (standardised with total protein) and 1.68 (se 0.07), 1.39 (se 0.09) (standardised with total fly weight).

These results suggest that either the *hsp83-roX2* construct contains an MSL binding site or expression of *roX2* RNA in *cis* was required for increased expression of the *arm-lacZ*

reporter gene. The noted lack of a further increase in homozygous males will be discussed later.

3.1.2 GENERATION OF 4.9 KB GENOMIC *ROX1* EXPRESSING CONSTRUCT

The *hsp83-roX2* constructs (pRH02 and pRH03) displayed an increase in *lacZ* expression in males compared to females (section 3.1.1.4)). To determine the extent to which *roX1* RNA expression affects reporter gene expression in males and females, a genomic fragment containing the *roX1* gene was inserted into the *arm-lacZ* reporter construct.

The *roX1* genomic sequence was received from Dr. Mitzi Kuroda as pGMroX5 (Table 1). This plasmid consisted of pCasPeR with the 4.9 kb *roX1* genomic fragment inserted into the *EcoRI* site. Having the *roX1* sequence inserted as an *EcoRI* fragment indicated that there were no *EcoRI* sites within the *roX1* fragment. This allowed for relatively easy cloning into pHF11. pGMroX5 was transformed into *E. coli* (section 2.16) upon arrival to generate a glycerol stock and for generation of a large scale DNA preparation (section 2.6.1).

The *roX1* fragment was digested out of pCasPeR with *EcoRI* (section 2.10.3) resulting in a 4.9 kb *roX1* band and a 7.8 kb band corresponding to the vector (Figure 2A). The 4.9 kb *roX1 EcoRI* fragment was gel extracted (section 2.9.1) and column purified. pHF11 was cut with *EcoRI* and treated with Calf Intestinal Phosphatase (CIP) (section 2.14.3). The *roX1* 4.9 kb *EcoRI* fragment was ligated into pHF11 (section 2.15). *E. coli* transformation colonies were checked with an *EcoRI* digest (section 2.10.2) and correct colonies showing bands at 16.36 kb and 4.9 kb (Figure 2B) were kept as glycerol stocks (section 2.4). The new plasmid clone was called pRH04.

3.1.2.1 β-galactosidase Assays of Autosomal Insertions of *4.9 kb* Genomic *roX1* Expressing Construct – pRH04

Two lines carrying autosomal insertions of pRH04 were obtained via P-element mediated transformation (section 2.20). β-galactosidase assays (section 2.22) were performed on one dose (heterozygous) males and females, and two dose (homozygous) males and females from the two lines. Reporter gene expression was significantly

Figure 2. Restriction Enzyme Digestions of Genomic *roX1* Expressing Constructs to confirm Excision and Insertion

A. *EcoRI* digestion of pGMroX5 to isolate the 4.9 kb genomic *roX1* fragment.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *EcoRI* digestion of plasmid pGMroX5.

B. *EcoRI* digestion of pRH04 to confirm the insertion of the 4.9 kb *roX1* genomic fragment.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *EcoRI* digestion of plasmid pRH04.

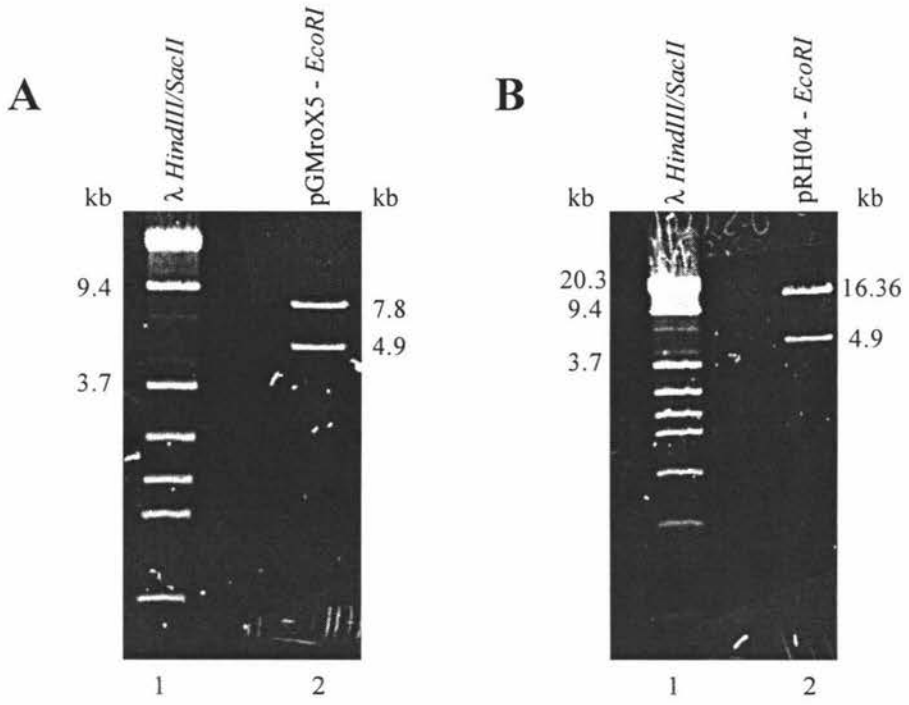

A
kb
λ HindIII/SacII
pGMroX5 - EcoRI
kb
9.4
3.7
7.8
4.9
1
2
B
kb
λ HindIII/SacII
pRH04 - EcoRI
kb
20.3
9.4
3.7
16.36
4.9
1
2

Table 5. β-galactosidase Assays of Transgenic Flies carrying Autosomal Insertions of pRH04

Two transgenic lines were assayed from this construct. For each line the expression of one dose males and females was compared, as was expression of two dose males and females. Each assay was performed a minimum of three times and standardised using total protein measurements and total wet weight. The standard error represents the variability between the experiments.

Line	Dose	Chromosome	M:F Ratio		Standard Error
pRH04 - Insertion of 4.9 kb genomic *roX1* sequence into pHF11					
pRH04 #1	1	3	Protein	1.41	0.03
			Wet weight	1.67	0.05
	2		Protein	1.21	0.05
			Wet weight	1.39	0.07
pRH04 #2	1	2	Protein	1.42	0.08
			Wet weight	1.44	0.08
	2		Protein	1.25	0.01
			Wet weight	1.31	0.03

increased in heterozygous males carrying pRH04, but the increase in homozygous males was not as large. Results of the assays are summarised in Table 5.

For flies with one copy of pRH04 the male:female ratios were 1.41 (se 0.03), 1.42 (se 0.08) (standardised with total protein) and 1.67 (se 0.05), 1.44 (0.08) (standardised with total fly weight). For flies with two copies of pRH04 the male:female ratios were 1.21 (se 0.05), 1.25 (se 0.01) (standardised with total protein) and 1.39 (se 0.07), 1.31 (0.03) (standardised with total fly weight).

These results suggest that the expression of the *roX1* RNA in *cis* is required for an increase in *arm-lacZ* reporter gene expression, but increased expression of *roX1* RNA in homozygous flies results in a decrease in the increase of *lacZ* expression in males. These results will be discussed in more detail later.

3.1.3 GENERATION OF 3.7 KB *ROX1* and 1.1 KB *ROX2* cDNA CONTAINING CONSTRUCTS

The previous experiments with *hsp83-roX2* (section 3.1.1.4) and 4.9 kb *roX1* (section 3.1.2.1) inserted into pHF11 (pRH02, pRH03 and pRH04) showed increased expression of the reporter gene in males. These results raised the question - was the RNA expression required for the increase in *lacZ* synthesis? This led to the generation of plasmids containing the *roX1* and *roX2* cDNA sequences inserted into the *arm-lacZ* reporter gene construct, both separately and together.

3.1.3.1 Insertion of a *NotI* Linker into pHF11

Because the *roX1* cDNA sequence had a *EcoRI* restriction site at one end that had not been reconstituted, an alternative cloning strategy was devised to enable the insertion of the *roX1* cDNA sequence into pHF11 downstream of the SCS' element. The cloning strategy involved first destroying the *NotI* site in pHF11 (found in the polylinker upstream of the SCS' element), followed by insertion of a *NotI* linker into the *EcoRI* site in pHF11 (found downstream of the SCS' element).

pHF11 was digested with *NotI* (section 2.10.3) and treated with DNA Polymerase I (Klenow) (section 2.14.1) to fill in the cohesive ends. This vector was then religated

(section 2.15). If the ligation was successful the *NotI* site would be lost. The religation was confirmed by digestion with *NotI* (section 2.10.2), of the plasmid DNA isolated from transformation colonies (section 2.6.2). This plasmid was called pRH06.

The *NotI* linker was created so it had *EcoRI* sites at either end. One of the *EcoRI* sites would not reconstitute when the linker was inserted. This means that the *EcoRI* site can be utilised in conjunction with the *NotI* site for future cloning. pRH06 (religated pHF11) was cut with *EcoRI* and treated with Calf Intestinal Phosphatase (CIP) (section 2.14.3). The single *EcoRI* site in pHF11 is present between the *armadillo* promoter and the SCS' element. The annealed *NotI* linker was treated with T4 Polynucleotide Kinase (section 2.14.5) to phosphorylate the ends for ligation into the *EcoRI* site. Plasmid DNA isolated from *E. coli* transformation colonies (section 2.16) was checked with a *NotI* digest. The vector would be cut once if the *NotI* linker has inserted resulting in a single band of 16.36 kb. Digestion with *EcoRI* confirmed that at least one site was reconstituted.

This plasmid was called pRH07 and was used for cloning both *roX1* and *roX2* cDNA sequences upstream of the *arm* promoter, but downstream of SCS' insulator element.

3.1.3.2 Insertion of 3.7 kb *roX1* cDNA Sequence into pBS2N

Plasmid DNA containing the 3.7 kb *roX1* cDNA sequence was obtained from Dr. Hubert Amrein (Table 1). The cDNA sequence was inserted into *EcoRI* and *XhoI* sites in the polylinker of pBS SK- (Stratagene). The original cloning strategy had been to cut *roX1* cDNA out of pBS using *EcoRI* and *XhoI*. However this method of insertion of *roX1* cDNA into pHF11 was hindered by the discovery that the *EcoRI* site was not cutting.

A alternative cloning strategy involved using pBS + 2 x *NotI* (pBS2N), a pBS KS plasmid which has an extra *NotI* site inserted in the polylinker between *XhoI* and *Asp718* (*Kpn*I) (received from Dr. Jörg Heinrich). The 3.7 kb *roX1* cDNA was cut out of pBS using *XhoI* and *BamHI* (section 2.10.3), separated on an agarose gel (section 2.9), excised from the gel (section 2.9.1), column purified and ligated (section 2.15) with pBS2N that had also been cut with *XhoI* and *BamHI*. A high number of *E. coli* transformant colonies (section 2.16) were checked by digestion with *NotI* (section

2.10.2) and none were found to contain an insertion of the *roX1* cDNA. It was suspected that the large number of non-recombinants might be because either *XhoI* or *BamHI* did not digest pBS2N completely. Consequently the 2.97 kb *XhoI/BamHI* fragment from pBS2N was separated on a gel, extracted from the gel, column purified and ligated with the 3.7 kb *roX1 XhoI/BamHI* fragment. Plasmid DNA from *E. coli* transformants was checked with a *NotI* digest. Correct recombinants showed bands at 2.96 kb and 3.7 kb. A final digest with *NotI* and *HindIII* (bands at 2.97 kb, 2.63 kb and 1.0 kb) confirmed the 3.7 kb *roX1* cDNA sequence was inserted into pBS2N. This plasmid was called pRH05.

NotI was used to excise the 3.7 kb *roX1* cDNA from pRH05, since two *NotI* sites in pBS2N are present on either side of the *XhoI* and *BamHI* insertion sites. The fragment was separated on an agarose gel, excised from the gel and column purified before being ligated with pRH07, also cut with *NotI*. Plasmid DNA isolated from *E. coli* transformant colonies was checked for insertion by digestion with *NotI*. Insertion of the 3.7 kb fragment was confirmed by banding at 16.36 kb and 3.7 kb (Figure 3A), and orientation confirmed by digestion with *HindIII* (bands at 7.13 kb, 6.88 kb, 3.13 kb and 2.92 kb) (Figure 3B). The *HindIII* digest showed the *roX1* cDNA was in the same 5' – 3' direction as *arm-lacZ* in pRH07. This plasmid was called pRH08.

3.1.3.3 Insertion of 1.1 kb *roX2* cDNA into pHF11 + *NotI* Linker

Plasmid DNA containing the 1.1 kb *roX2* cDNA sequence was obtained from Dr. Hubert Amrein (Table 1). Due to the presence of an *EcoRI* site within the cDNA, this fragment had to be excised from pBS SK- using *NotI* and *PspOMI* (section 2.10.3) and the 1.1 kb *roX2* fragment separated by gel electrophoresis from 2.96 kb pBS SK-, then gel extracted (section 2.9.1). pRH07 was digested with *NotI* and treated with Calf Intestinal Phosphatase (CIP) (section 2.14.3). Following ligation (section 2.15) and transformation (section 2.16), plasmid DNA isolated from *E. coli* colonies (section 2.6.2) was checked for insertion using *SpeI* (section 2.10.2) (bands at 16.46 kb and 1.0 kb) (Figure 3C) and *EcoRI* (bands at 16.65 kb and 0.81 kb) (Figure 3D) separately. The *EcoRI* digest confirmed the 1.1 kb *roX2* cDNA was in the opposite (3' – 5') orientation to *arm-lacZ* in pRH07. This plasmid was called pRH09.

Figure 3. Restriction Enzyme Digestions of *roX1* and *roX2* cDNA Containing Constructs to confirm Insertion and Orientation

A. *NotI* digestion of pRH08 to confirm the insertion of the *3.7* kb roX1 cDNA into pRH07.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *NotI* digestion of plasmid pRH08.

B. *HindIII* digestion of pRH08 to confirm the orientation of the 3.7 kb *roX1* cDNA.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *HindIII* digestion of plasmid pRH08.

C. *SpeI* digestion of pRH09 to confirm the insertion of the 1.1 kb *roX2* cDNA into pRH07.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *SpeI* digestion of plasmid pRH09.

D. *EcoRI* digestion of pRH09 to confirm the orientation of the 1.1 kb *roX2* cDNA.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *EcoRI* digestion of plasmid pRH09.

E. *NotI* digestion of pRH13 to confirm the insertion of the 3.7 kb *roX1* cDNA into pRH09.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *NotI* digestion of plasmid pRH13.

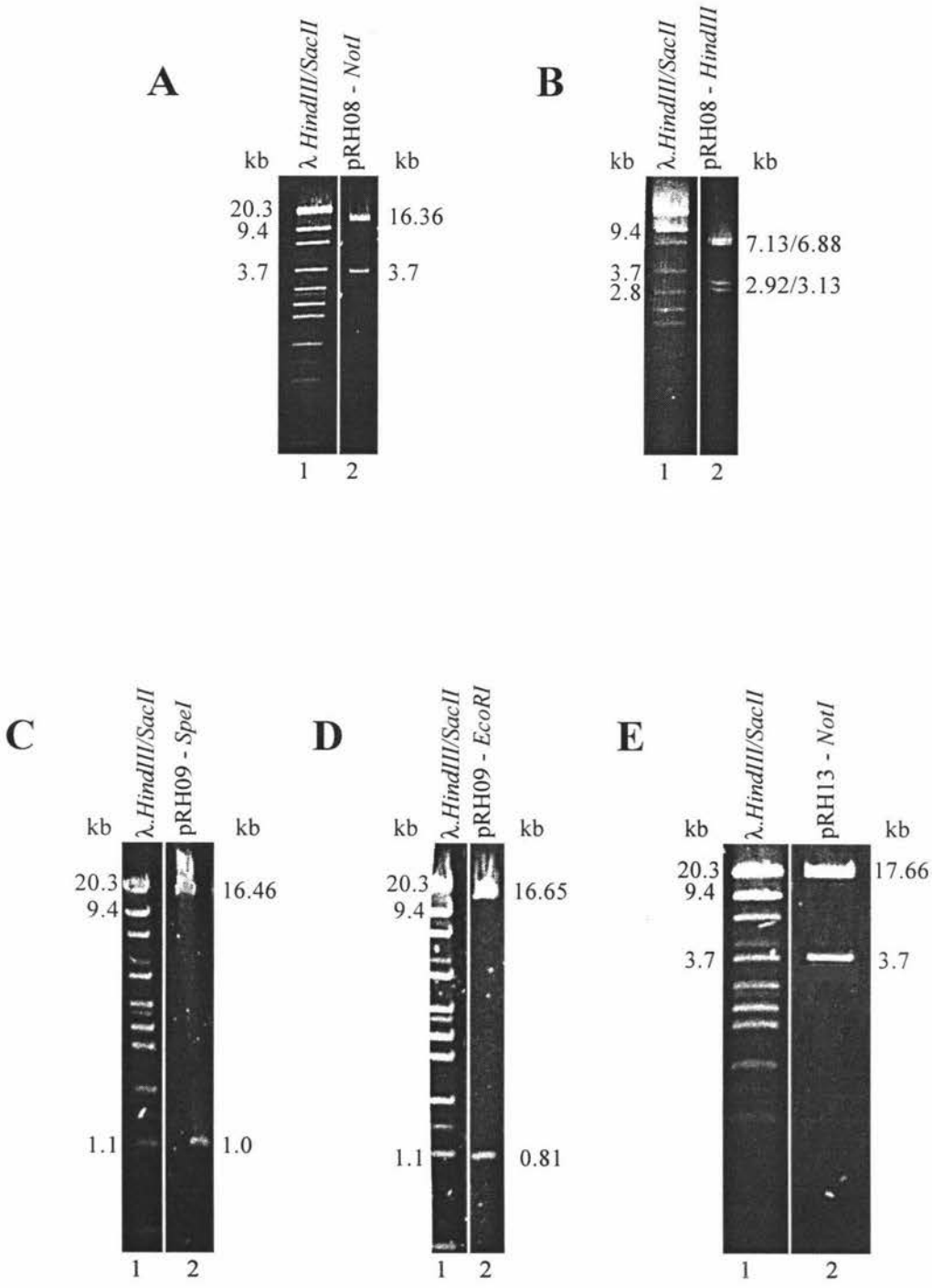

A
λ HindIII/SacII
pRH08 - NotI
kb
kb
20.3
9.4
3.7
16.36
3.7
1
2
B
λ.HindIII/SacII
pRH08 - HindIII
kb
kb
9.4
3.7
2.8
7.13/6.88
2.92/3.13
1
2
C
λ.HindIII/SacII
pRH09 - SpeI
kb
kb
20.3
9.4
1.1
16.46
1.0
1
2
D
λ.HindIII/SacII
pRH09 - EcoRI
kb
kb
20.3
9.4
1.1
16.65
0.81
1
2
E
λ.HindIII/SacII
pRH13 - NotI
kb
kb
20.3
9.4
3.7
17.66
3.7
1
2

Table 6. β-galactosidase Assays of Transgenic Flies carrying Autosomal Insertions of pRH08, pRH09, and pRH13

Eight transgenic lines were assayed from the three constructs. For each line the expression of one dose males and females was compared, as was expression of two dose males and females for seven of the eight lines. Each assay was performed a minimum of three times and standardised using total protein measurements and total wet weight. The standard error represents the variability between the experiments.

Line	Dose	Chromosome	M:F Ratio		Standard Error
pRH08 - Insertion of 3.7 kb roX1 cDNA sequence into HF11 + *NotI* Linker					
pRH08 #1	1	3	Protein	1.54	0.03
			Wet weight	1.57	0.04
	2		Protein	1.81	0.10
			Wet weight	2.03	0.10
pRH08 #2	1	2	Protein	1.57	0.07
			Wet weight	1.58	0.03
	2		Protein	1.73	0.07
			Wet weight	1.90	0.07
pRH09 - Insertion of 1.1 kb *roX2* cDNA sequence into pHF11 + *NotI* linker					
pRH09 #1	1	3	Protein	1.23	0.05
			Wet weight	1.38	0.04
	2		Protein	1.38	0.04
			Wet weight	1.50	0.03
pRH09 #2	1	3	Protein	1.61	0.09
			Wet weight	1.68	0.05
pRH09 #3	1	2	Protein	1.74	0.07
			Wet weight	1.93	0.06
	2		Protein	1.74	0.05
			Wet weight	1.97	0.09
pRH13 - Insertion of 3.7 kb *roX1* cDNA sequence into 1.1 kb *roX2* in HF11 + *NotI* linker					
pRH13 #1	1	2	Protein	1.41	0.03
			Wet weight	1.54	0.02
	2		Protein	1.33	0.03
			Wet weight	1.41	0.01

pRH13 #2	1	3	Protein	1.39	0.04
			Wet weight	1.42	0.03
	2		Protein	1.40	0.09
			Wet weight	1.39	0.07
pRH13 #3	1	X	Protein	2.02	0.09
			Wet weight	2.29	0.09
	2		Protein	1.17	0.03
			Wet weight	1.09	0.03

3.1.3.4 Insertion of 3.7 kb *roX1* cDNA into 1.1 kb *roX2* in pHF11 + *NotI* Linker

The previous experiments with the individual *roX* cDNA sequences (section 3.1.3.5) showed increased *lacZ* synthesis in males. These results raised the question – would insertion of both *roX* cDNA sequences result in higher expression of the *arm-lacZ* reporter gene in males?

The 4.9 kb *roX1* genomic DNA sequence was excised from pGMroX5 with *EcoRI* and the fragment separated by gel electrophoresis (section 2.9) and then gel purified (section 2.9.1). pRH07 was cut with *EcoRI* (section 2.10.3) and treated with Calf Intestinal Phosphatase (CIP) (section 2.14.3). The 4.9 kb fragment was ligated (section 2.15) into pRH07 and plasmid DNA isolated from *E. coli* transformants (section 2.6.2) checked with an *EcoRI* digest (section 2.10.2). Insertion was confirmed with the presence of bands at 16.36 kb and 4.9 kb. Several large-scale preparations (section 2.6.1) of the resulting 21.26 kb plasmid (pRH10) were required due to low yields from each preparation. Despite repeated attempts a complete *NotI* digestion of pRH10 could not be obtained. Nevertheless ligation was carried out anyway with the 2.2 kb *hsp83-roX2* and 1.1 kb *roX2* cDNA fragments both excised from pBS with *NotI* and *PspOMI*.

The first attempt at ligation resulted in the *hsp83-roX2* transformants showing fragment sizes expected for either pBS or pRH10. The incomplete digestion of the vector with *NotI* was thought to be the problem, therefore pRH10 was digested again and the linearised plasmid band separated by gel electrophoresis and gel purified. Ligations were carried out again, but the plasmid DNA small scale preparations from transformant colonies all showed a pBS banding pattern. It appeared that pBS was being co-purified with the *roX2* fragments, religating and resulting in a large number of non-recombinant clones.

An alternative strategy was developed in which *hsp83-roX2* and 1.1 kb *roX2* fragments would be obtained by PCR (section 2.13) using M13 pUC forward and reverse primers. The products were separated by gel electrophoresis and the correct sized bands were gel extracted, digested with *NotI* and *PspOMI*, and column purified. However the yield after two purifications was very low. A second alternative was to excise *hsp83-roX2* and 1.1 kb *roX2* from pBS with *NotI*, *PspOMI* and *SacII*. This *SacII* digestion would cut the *NotI* site off one end of the pBS fragment and reduce religation. Following

ligation and transformation no *E. coli* transformant colonies were obtained. It is not known why this experiment failed, but it is possible that *NotI* may not have cut the vector completely before the *SacII* digestion leaving a *SacII* end, which would not have ligated with *NotI* ends in pRH07.

It was at this stage that one more alternative was devised. This alternative was to insert the 3.7 kb *roX1* cDNA into the single *NotI* site in pRH09. This construct would then have both cDNA sequences together. pRH09 was digested with *NotI* and treated with Calf Intestinal Phosphatase (CIP). The 3.7 kb *roX1* cDNA was excised from pBS2N with *NotI*, separated by gel electrophoresis, then gel extracted and purified. Following ligation and transformation, plasmid DNA from *E. coli* transformant colonies was checked for insertion with individual *NotI* and *PspOMI* digests. DNA from one colony showed the *NotI* banding pattern expected for a recombinant clone (bands at 17.66 kb and 3.7 kb) (Figure 3E). This plasmid was called pRH13.

3.1.3.5 β-galactosidase Assays of Autosomal Insertions of 3.7 kb *roX1* and 1.1 kb *roX2* cDNA Containing Constructs – pRH08, pRH09, pRH13

Two lines carrying autosomal insertions of pRH08, three lines carrying autosomal insertions of pRH09, and two lines carrying autosomal insertions of pRH13 were obtained via P-element mediated transformation (section 2.20). β-galactosidase assays (section 2.22) were performed on one dose (heterozygous) males and females and two dose (homozygous) males and females from the three constructs. Reporter gene expression was increased in heterozygous males carrying pRH08, pRH09 and pRH13, and was significantly increased in homozygous males carrying pRH08. Results of the assays are summarised in Table 6.

For flies with one copy of pRH08 the male:female ratios were 1.54 (se 0.03), 1.57 (se 0.07) (standardised with total protein) and 1.57 (se 0.04), 1.58 (se 0.03) (standardised with total fly weight). For flies with two copies of pRH08 the male:female ratios were 1.81 (se 0.10), 1.73 (se 0.07) (standardised with total protein) and 2.03 (se 0.10), 1.90 (se 0.07) (standardised with total fly weight).

For flies with one copy of pRH09 the male:female ratios were 1.23 (se 0.05), 1.60 (se 0.09), 1.74 (se 0.07) (standardised with total protein) and 1.38 (se 0.04), 1.68 (se 0.05),

1.93 (0.06) (standardised with total fly weight). For flies with two copies of pRH09 the male:female ratios were 1.38 (se 0.04), 1.74 (se 0.07) (standardised with total protein) and 1.50 (se 0.03), 1.97 (se 0.09) (standardised with total fly weight).

For flies with one copy of pRH13 the male:female ratios were 1.41 (se 0.03), 1.39 (se 0.04), 2.02 (se 0.09) (standardised with total protein) and 1.54 (se 0.02), 1.42 (se 0.03), 2.30 (se 0.09) (standardised with total fly weight). For flies with two copies of pRH13 the male:female ratios were 1.33 (se 0.03), 1.40 (se 0.09), 1.17 (se 0.03) (standardised with total protein) and 1.41 (se 0.02), 1.39 (se 0.07), 1.09 (se 0.03) (standardised with total fly weight).

These results suggest that the presence of *roX* cDNA sequences is as important as the expression of *roX* RNA for dosage compensation. This will be discussed further later.

To compare the signifacance of the difference between the increase in *lacZ* expression in homozygous males carrying the 4.9 kb genomic *roX1* fragment and the 3.7 kb *roX1* cDNA a two sample t-procedure was carried out. This statistical procedure calculates the 95 % confidence interval for the difference between two means. It was found, with 95 % confidence, that the β-galactosidase activity increase in homozygous 3.7 kb *roX1* cDNA males is significantly higher than the increase in homozygous 4.9 kb genomic *roX1* males.

3.1.4 GENERATION OF CONSTRUCTS CONTAINING *ROX1* PCR FRAGMENTS

The previous experiments have shown insertion of either the *roX1* or *roX2* cDNAs is sufficient to achieve an increase in reporter gene expression (section 3.1.3.5). The question these results raise is – which region(s) of the cDNAs are important? Two regions of *roX1* have been suggested to be important for the function.

Both *roX1* and *roX2* share a 30 base pair conserved sequence located near the 3' end of each RNA (Franke and Baker, 1999) suggesting this element has an important function. Another region that may be important is about 100 base pairs upstream of the P-element insertion site, in *roX1*, and contains several possible GAGA binding sites. A male-

specific DNAseI hypersensitivity site has been mapped to this region (Dr. R. Kelley, personal communication). These regions will be defined as 3' *roX1* and DNAseI *roX1* respectively.

PCR primers (Table 2) were designed to amplify both regions from the *roX1* cDNA sequence. The primers were designed with *EcoRI* sites at both ends, for easy insertion into the *EcoRI* site in pHF11. A *BstBI* site was also placed in the forward primer to determine orientation of the insert. Following amplification (section 2.13), the DNA fragments were separated by gel electrophoresis (section 2.9) and products of the expected size (410 bp for DNAseI *roX1* and 250 bp for 3' *roX1*) were gel extracted (section 2.9.1) and column purified (Figure 4A).

Purified fragments were digested with *EcoRI* (section 2.10.3) to generate cohesive ends for ligation (section 2.15) into pHF11, which had been cut with *EcoRI* and treated with Calf Alkaline Phosphatase (CIP) (section 2.14.3). Following ligation and transformation into *E. coli* (section 2.16), plasmid DNA from the resulting transformant colonies (section 2.6.2) was checked for insertion by digestion with *BstBI* (section 2.10.2). Although there are many *BstBI* sites in pHF11, the bands generated when an insert was present were distinguishable from vector alone. For the 3' *roX1* PCR fragment inserted into pHF11 (called pRH11) a band at 0.55 kb indicated the fragment was inserted and in the same 5' – 3' orientation as *arm-lacZ*, while a band at 0.3 kb indicated the fragment was in the opposite orientation (Figure 4B). For the DNAseI *roX1* PCR fragment inserted into pHF11 (called pRH12) a band at 0.7 kb indicated the insert was in the same 5' – 3' direction as *arm-lacZ*, and a band at 0.3 kb showed the insert in the opposite orientation (Figure 4C). Plasmid DNA containing each fragment in both orientations was obtained. A second *NotI* and *Asp718* digestion confirmed the insertion of both fragments. 3' *roX1* insertion had a band at 2.45 kb and DNAseI *roX1* insertion had a band at 2.6 kb (Figure 4D).

Orientation of the fragments was confirmed by PCR using the forward primers from the initial reactions and a primer complimentary to sequences at the 5' end of the *armadillo* promoter (Figure 4E). Controls of both original PCR primers generated fragment sizes of 0.25 kb for 3' *roX1* and 0.4 kb for DNAseI *roX1*. The combinations with the *arm*

primer confirmed that the *roX1* fragments were in the same 5' – 3' orientation as *arm-lacZ*.

3.1.4.1 β-galactosidase Assays of Autosomal Insertions of *roX1* PCR Fragment Containing Constructs – pRH11, pRH12

Three lines carrying autosomal insertions of pRH11 and three lines carrying autosomal insertions of pRH12 were obtained via P-element mediated transformation (section 2.20). β-galactosidase assays (section 2.22) were performed on males and females with one dose (heterozygous) and two dose (homozygous) males and females from one pRH12 line. Reporter gene expression was increased in males carrying pRH12, but not in males carrying pRH11. Results of the assays are summarised in Table 7.

For flies with one copy of pRH11 the male:female ratios were 1.04 (se 0.02), 1.04 (se 0.03), 1.06 (se 0.04) (standardised with total protein) and 1.12 (se 0.03), 1.13 (se 0.03), 1.06 (se 0.03) (standardised with total fly weight).

For flies with one copy of pRH12 the male:female ratios were 1.18 (se 0.03), 1.16 (se 0.04), 1.25 (se 0.03) (standardised with total protein) and 1.28 (se 0.03), 1.24 (se 0.03), 1.33 (se 0.03) (standardised with total fly weight). For flies with two copies of pRH12 the male:female ratios were 1.25 (se 0.01) (standardised with total protein) and 1.34 (se 0.05) (standardised with total fly weight).

These results indicate that the 3' fragment of *roX1* chosen does not contain sequences for dosage compensation of *arm-lacZ*, however the DNAseI fragment of *roX1* does. The DNAseI *roX1* fragment had an increase in *lacZ* expression lower than both genomic *roX1* and the *roX1* cDNA. The implications of this result will be discussed later.

Figure 4. Restriction Enzyme Digestions of *roX1* PCR Fragment Containing Constructs to confirm PCR Amplification, Insertion and Orientation

A. PCR amplified fragments from the *roX1* cDNA sequence.

Lane 1. 50 bp ladder (Boehringer Mannheim).
Lane 2. 410 bp DNAseI *roX1* PCR product.
Lane 3. 250 bp 3' *roX1* PCR product.

B. *BstBI* digestion of pRH11 to confirm the orientation of the 250 bp 3' *roX1* PCR product insertion into pHF11.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *BstBI* digestion of plasmid pRH11 containing the 3' *roX1* PCR product in the same (5' – 3') orientation as *arm-lacZ.* This construct was used for micro-injection.
Lane 3. *BstBI* digestion of plasmid pRH11 containing the 3' *roX1* PCR product in the opposite orientation to *arm-lacZ.*

C. *BstBI* digestion of pRH11 to confirm the orientation of the 410 bp DNAseI *roX1* PCR product insertion into pHF11.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *BstBI* digestion of plasmid pRH11 containing the DNAseI *roX1* PCR product in the same (5' – 3') orientation as *arm-lacZ.* This construct was used for micro-injection.
Lane 3. *BstBI* digestion of plasmid pRH11 containing the DNAseI *roX1* PCR product in the opposite orientation to *arm-lacZ.*

D. *NotI and Asp718* digestion of pRH11 and pRH12 to confirm the insertion of the PCR fragments.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *NotI/Asp718* digestion of plasmid pRH12.
Lane 3. *NotI/Asp718* digestion of plasmid pRH11.

E. PCR amplified products from combinations of primers to confirm the insertion of 3'*roX1*and DNAseI *roX1* PCR fragments into pHF11.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. 375 bp PCR product from *arm* forward and 3' *roX1* Top primers.
Lane 3. 250 bp PCR product from 3' *roX1* Top and Bottom primers - control.
Lane 4. 575 bp PCR product from *arm* forward and DNAseI *roX1* Top primers.
Lane 5. 410 bp PCR product from DNAseI *roX1*Top and Bottom primers - control.

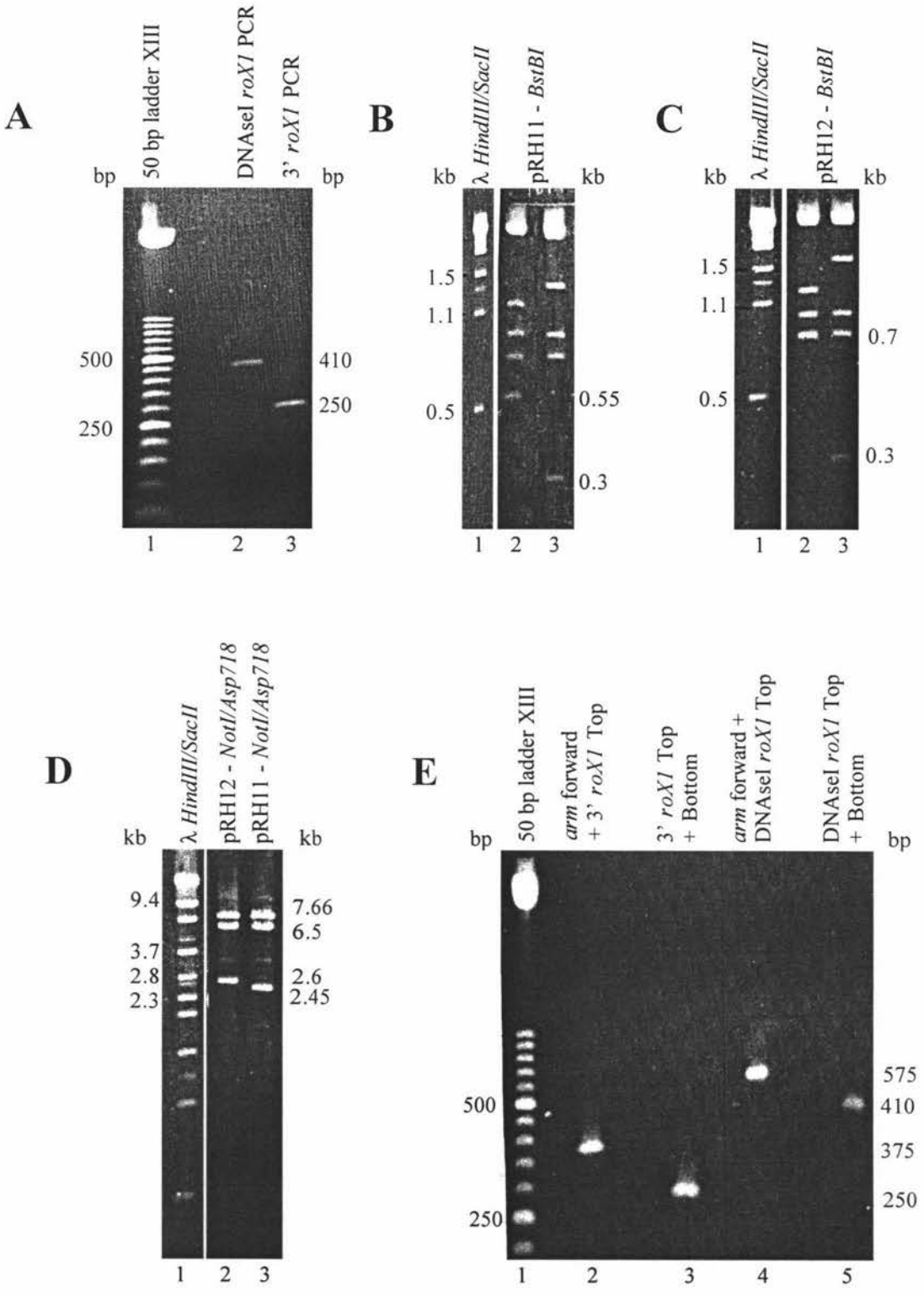

A
50 bp ladder XIII
DNAseI roX1 PCR
3' roX1 PCR
bp
bp
500
250
410
250
1
2
3
B
λ HindIII/SacII
pRH11 - BstBI
kb
kb
1.5
1.1
0.5
0.55
0.3
1
2
3
C
λ HindIII/SacII
pRH12 - BstBI
kb
kb
1.5
1.1
0.5
0.7
0.3
1
2
3
D
λ HindIII/SacII
pRH12 - NotI/Asp718
pRH11 - NotI/Asp718
kb
kb
9.4
3.7
2.8
2.3
7.66
6.5
2.6
2.45
1
2
3
E
50 bp ladder XIII
arm forward + 3' roX1 Top
3' roX1 Top + Bottom
arm forward + DNAseI roX1 Top
DNAseI roX1 Top + Bottom
bp
bp
500
250
575
410
375
250
1
2
3
4
5

Table 7. β-galactosidase Assays of Transgenic Flies carrying Autosomal Insertions of pRH11 and pRH12

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

Line	Dose	Chromosome	M:F Ratio		Standard Error
pRH11 - Insertion of 3' *roX1* PCR fragment into pHF11					
pRH11 #1	1	3	Protein	1.04	0.02
			Wet weight	1.12	0.03
pRH11 #2	1	3	Protein	1.04	0.03
			Wet weight	1.13	0.03
pRH11 #3	1	2	Protein	1.06	0.04
			Wet weight	1.06	0.03
pRH12 - Insertion of DNAseI *roX1* PCR fragment into pHF11					
pRH12 #1	1	2	Protein	1.18	0.03
			Wet weight	1.28	0.03
	2		Protein	1.25	0.01
			Wet weight	1.34	0.05
pRH12 #2	1	3	Protein	1.16	0.04
			Wet weight	1.24	0.03
pRH12 #3	1	3	Protein	1.25	0.03
			Wet weight	1.33	0.03

3.2 DETERMINATION OF THE EFFECT OF SXL BINDING SITES ON THE *ARM-LACZ* REPORTER GENE EXPRESSION

3.2.1 GENERATION OF SXL BINDING SITES IN THE 3' UTR OF pHF11

Fitzsimons *et al.,* (1999) carried out preliminary experiments, investigating the involvement of Sxl in dosage compensation in *Drosophila melanogaster.* That study found that the insertion of *Runt* 3' UTR (containing Sxl binding sites) downstream of *arm-lacZ* in the reporter gene resulted in a decrease in *lacZ* expression in females. This study continues Fitzsimons *et al.,* (1999) work, but will concentrate on other genes found to contain Sxl binding sites. The aim is to determine if the number or length of Sxl binding sites affects dosage compensation (down regulation of some X-linked genes) in females.

3.2.1.1 Insertion of PCR Fragments Containing Sxl Binding Sites into pHF12

The *Runt* 3' UTR contains three predicted Sxl binding sites (Kelley *et al.,* 1995). Three other genes were chosen which also contain at least three Sxl binding sites in their respective 3' UTRs. These genes were *Sex Lethal, Cut*, and *Small Forked. Sxl* itself has nine Sxl protein binding sites in its 3' UTR, *Cut* has five sites and *Small Forked* has three longer sites.

Primers were designed to amplify a segment of the 3' UTRs of these three genes, using PCR, from genomic DNA (section 2.13). Either *MfeI* or *EcoRI* restriction sites (*MfeI* and *EcoRI* digested DNA have compatible cohesive ends) were added to the ends of the primers to allow for insertion of the PCR fragments into a *MfeI* site in the 3' UTR of pHF11 (downstream of *arm-lacZ*). For *Sxl* and *Small Forked* primers, a *MfeI* site was placed at one end to reconstitute a *MfeI* site and an *EcoRI* site at the other end. The *EcoRI* site was to identify orientation. *Cut* primers were designed with *EcoRI* sites at both ends due to the presence of an internal *MfeI* site. This *MfeI* site was used for determining orientation. Due to the presence of more than one *MfeI* site in pHF11 the PCR fragments were initially inserted into the unique *MfeI* site within the *arm-lacZ* 3' UTR of pHF12.

PCR products of the expected sizes (*Sxl* 0.77 kb, *Cut* 1.2 kb and *Small Forked* 0.356 kb) (Figure 5A) were column purified, digested with *EcoRI* and *MfeI* (*Sxl, forked*) or *EcoRI* (*Cut*) to generate cohesive ends (section 2.10.3) and column purified again for insertion into pHF12. pHF12 was cut with *MfeI* (a unique cloning site in the 3' UTR) and treated with Calf Intestinal Phosphatase (CIP) (section 2.14.3).

Following ligation (section 2.15) and transformation (section 2.16), isolated plasmid DNA (section 2.6.2) was digested with *MfeI* and *XbaI* (*Sxl, Cut* and *Forked*) to check insertion and with *XbaI* (*Cut*) (Figure 5C) or *XbaI* and *AflII* (*Sxl, forked*) (Figure 5B) for orientation (section 2.10.2). *XbaI* or *AflII* sites were present at the end of each PCR fragment. These digests confirmed the orientation of the insert as being in either the binding (poly T) orientation (banding at 0.7 kb and 1.37 kb for *Cut*, 0.9 kb and 0.26 kb for *Small Forked*, 0.87 kb and 0.77 for *Sxl*), or the non-binding (poly A) orientation (banding at 1.9 kb and 0.18 kb for *Cut*, 0.79 kb and 0.4 kb for *Small Forked*, 1.4 kb and 0.24 kb for *Sxl*). These plasmids were called pRH17 (*Cut*), pRH18 (*Sxl*) and pRH19 (*Forked*) when in the poly T orientation and pRH20, pRH21 and pRH22 respectively when in the poly A orientation.

3.2.1.2 Insertion of *arm-lacZ* + Sxl Binding Sites into pHF10

The PCR fragments containing the expected Sxl binding sites were cloned into pHF12 first because it contained a unique *MfeI* site in the 3' UTR of the reporter gene. Next the arm-*lacZ* + Sxl sites was cloned into pHF10 to allow for insulation by the SCS/SCS' insulator elements.

pRH17, pRH18, pRH19, pRH20, pRH21 and pRH22 were digested with *SpeI* and *EcoRI* (section 2.10.3). This digest excises the *arm-lacZ + Sxl*, *Cut* or *Forked* PCR fragment out of pHF12. The 7.33 kb (*Cut*), 6.88 kb (*Sxl*) and 6.47 kb (*Forked*) fragments were separated by gel electrophoresis (section 2.9), extracted from the gel (section 2.9.1), and column purified. pHF10 was digested with *SpeI* and *EcoRI* and treated with Calf Intestinal Phosphatase (CIP) (section 2.14.3). Following ligation into pHF10 (section 2.15) and transformation into *E. coli* (section 2.16), isolated plasmid DNA (section 2.6.2) was digested with *SpeI* and *EcoRI* (section 2.10.2) to check for insertion. The *SpeI* and *EcoRI* digest generated a 10.22 kb fragment (corresponding to pHF10) and fragments at 7.33 kb (*Cut*), 6.88 (*Sxl*), and 6.47 (*Forked*) (Figure 5D).

Insertions of all three fragments were confirmed with a *PstI* digest, showing fragments at 8.32 kb, 7.83 (*Cut*), 7.48 kb (*Sxl*) or 7.07 kb (*Forked*) and 1.3 kb (Figure 5E). These plasmids were called pRH23 (*Cut*), pRH24 (*Sxl*), pRH25 (*Small Forked*) when in the poly T orientation and pRH26 (*Cut*), pRH27 (*Sxl*), pRH28 (*Small Forked*) when in the poly A orientation.

3.2.1.3 β-galactosidase Assays of Autosomal Insertions of 3' UTR Sxl Binding Site Containing Constructs– pRH23, pRH24, pRH25

Three lines carrying autosomal insertions of pRH24, one line carrying an autosomal insertion of pRH25 and three lines carrying autosomal insertions of pRH23 were obtained via P-element mediated transformation (section 2.20). β-galactosidase assays (section 2.22) were performed on one dose (heterozygous) males and females from the three constructs. Reporter gene expression was not significantly decreased in females carrying pRH24, but was decreased more in females carrying pRH23 and pRH25. Results of the assays are summarised in Table 8.

For flies with one copy of pRH24 the male:female ratios were 1.07 (se 0.05), 1.22 (se 0.03), 1.08 (se 0.02) (standardised with total protein) and 1.14 (se 0.06), 1.31 (se 0.02), 1.20 (se 0.05) (standardised with total fly weight).

For flies with one copy of pRH25 the male:female ratios were 1.26 (se 0.04) (standardised with total protein) and 1.34 (se 0.04) (standardised with total fly weight).

For flies with one copy of pRH23 the male:female ratios were 1.31 (se 0.07), 1.36 (se 0.02) 1.40 (se 0.05) (standardised with total protein) and 1.48 (se 0.06), 1.48 (se 0.06), 1.50 (se 0.05) (standardised with total fly weight)

These results indicate that the insertion of Sxl binding sites into the 3' UTR of *arm-lacZ* was not enough to achieve a dramatic decrease in females, as expected. The 3' UTR of *Sxl* contains nine Sxl binding sites and still this is not enough to facilitate a significant decrease in females. The implications of these results will be discussed later.

Figure 5. Restriction Enzyme Digestions of 3' UTR, Sxl Binding Site Containing Constructs to confirm PCR Amplification, Insertion and Orientation

A. PCR amplification of 3' UTRs of *Sxl, Small Forked,* and *Cut* genes

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. 0.77 kb *Sxl* 3' UTR PCR product.
Lane 3. 0.356 kb *Small Forked* 3' UTR PCR product.
Lane 4. 1.2 kb *Cut* 3' UTR PCR product.

B. *XbaI/AflII* digestion of pRH18, pRH19, pRH21, and pRH22 to confirm the orientation of the *Sxl* and *Forked* PCR products insertion into pHF12.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *XbaI/AflII* digestion of plasmid pRH19.
Lane 3. *XbaI/AflII* digestion of plasmid pRH22.
Lane 4. *XbaI/AflII* digestion of plasmid pRH18.
Lane 5. *XbaI/AflII* digestion of plasmid pRH21.

C. *XbaI* digestion of pRH17 and pRH20 to confirm the orientation of *Cut* PCR product insertion onto pHF12.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *XbaI* digestion of plasmid pRH020.
Lane 3. *XbaI* digestion of plasmid pRH017.

D. *SpeI* and *EcoRI* digestion of pRH23, pRH24, pRH25, pRH26, pRH27, and pRH28 to confirm the insertion of the *arm-lacZ + Sxl, Forked* or *Cut* PCR fragment into pHF10.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *SpeI/EcoRI* digestion of plasmid pRH23 or pRH26.
Lane 3. *SpeI/EcoRI* digestion of plasmid pRH24 or pRH27.
Lane 4. *SpeI/EcoRI* digestion of plasmid pRH25 or pRH28.

E. *PstI* digestion of pRH23, pRH24, pRH25, pRH26, pRH27, and pRH28 to confirm the insertion of the *arm-lacZ + Sxl, Forked* or *Cut* PCR fragment into pHF10.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *PstI* digestion of plasmid pRH25 or pRH28.
Lane 3. *PstI* digestion of plasmid pRH23 or pRH26.
Lane 4. *PstI* digestion of plasmid pRH24 or pRH27.

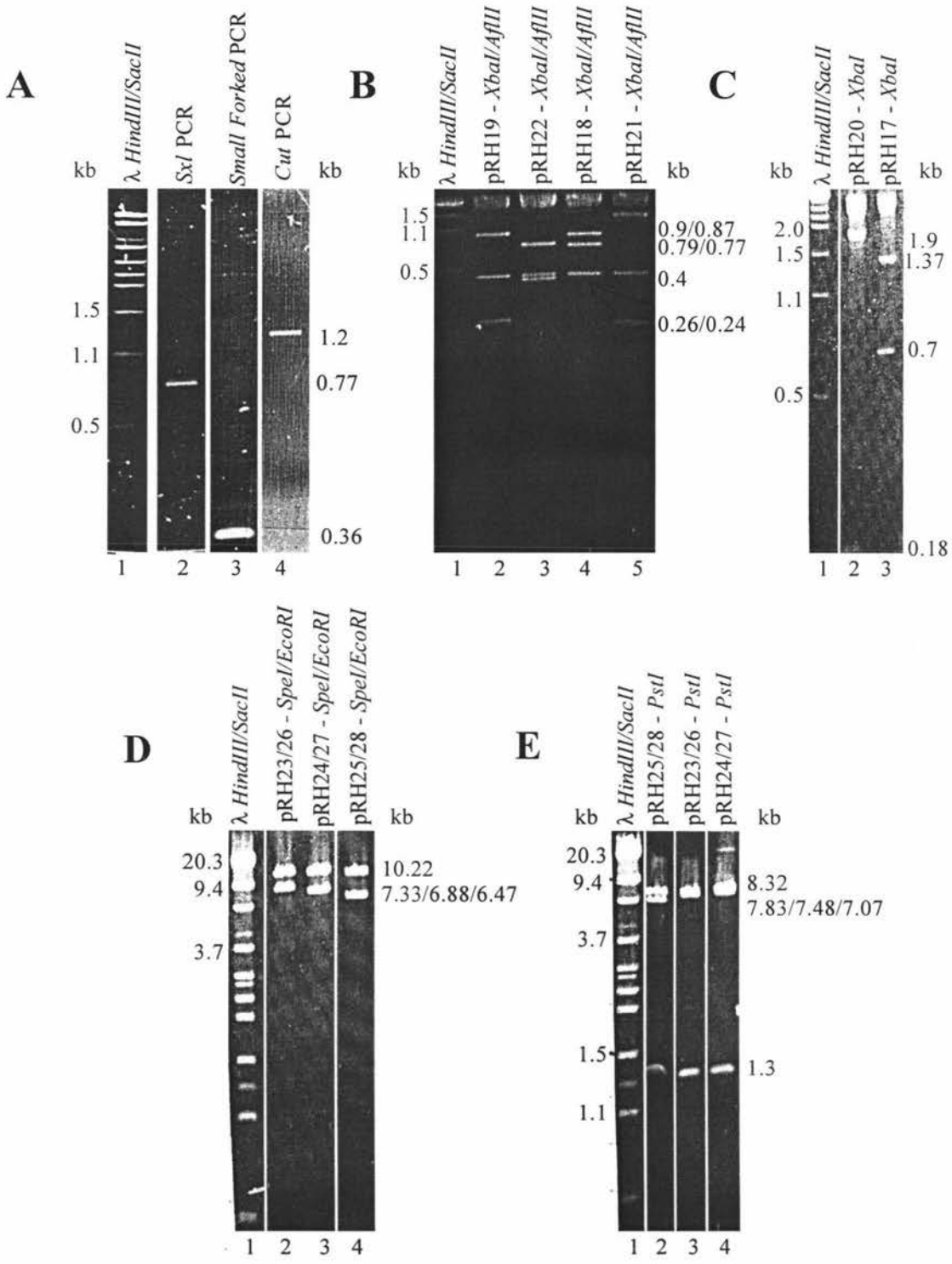

A
kb
λ HindIII/SacII
SxI PCR
Small Forked PCR
Cut PCR
kb
1.5
1.1
0.5
1.2
0.77
0.36
1 2 3 4
B
kb
λ HindIII/SacII
pRH19 - XbaI/AflII
pRH22 - XbaI/AflII
pRH18 - XbaI/AflII
pRH21 - XbaI/AflII
kb
1.5
1.1
0.5
0.9/0.87
0.79/0.77
0.4
0.26/0.24
1 2 3 4 5
C
kb
λ HindIII/SacII
pRH20 - XbaI
pRH17 - XbaI
kb
2.0
1.5
1.1
0.5
1.9
1.37
0.7
0.18
1 2 3
D
kb
λ HindIII/SacII
pRH23/26 - SpeI/EcoRI
pRH24/27 - SpeI/EcoRI
pRH25/28 - SpeI/EcoRI
kb
20.3
9.4
3.7
10.22
7.33/6.88/6.47
1 2 3 4
E
kb
λ HindIII/SacII
pRH25/28 - PstI
pRH23/26 - PstI
pRH24/27 - PstI
kb
20.3
9.4
3.7
1.5
1.1
8.32
7.83/7.48/7.07
1.3
1 2 3 4

Table 8. β-galactosidase Assays of Transgenic Flies carrying Autosomal Insertions of pRH23, pRH24, and pRH25

Seven transgenic lines were assayed from the three constructs. For each line the expression of one dose males and females was compared. Each assay was performed a minimum of four times and standardised using total protein measurements and total wet weight. The standard error represents the variability between the experiments.

Line	Chromosome	M:F Ratio		Standard Error
pRH24 - Insertion of *Sxl* PCR fragment into pHF10				
pRH24 #1	2	Protein	1.07	0.05
		Wet weight	1.14	0.06
pRH24 #2	2	Protein	1.22	0.03
		Wet weight	1.30	0.02
pRH24 #3	3	Protein	1.08	0.02
		Wet weight	1.20	0.05
pRH25 - Insertion of *Small Forked* PCR fragment into pHF10				
pRH25 #1	3	Protein	1.26	0.04
		Wet weight	1.34	0.04
pRH23 - Insertion of *Cut* PCR fragment into pHF10				
pRH23 #1	2	Protein	1.30	0.05
		Wet weight	1.44	0.05
pRH23 #2	2	Protein	1.36	0.02
		Wet weight	1.47	0.06
pRH23 #3	3	Protein	1.40	0.05
		Wet weight	1.51	0.05

3.2.2 GENERATION OF A SINGLE SXL BINDING SITE IN THE 5' UTR OF *ARM-LACZ*

Previous studies have shown that complete repression of translation of Msl-2 mRNA in females requires Sxl binding sites in both the 3' and 5' UTRs of the gene (Bashaw and Baker, 1997; Kelley *et al.*, 1997). Based on these studies it would be predicted that insertion of a Sxl site into the 5' UTR of *arm-lacZ* + *Runt* 3' UTR, would result in further inhibition of *lacZ* translation in females.

3.2.2.1 Insertion of *arm-lacZ* + *Runt* 3' UTR from pHF13 into pUC118

pHF13 carries the *arm-lacZ* construct with a 170 bp *Runt* fragment containing Sxl binding sites inserted into the 3' UTR. Due to pHF11 not having a unique *KpnI* site in the *arm-lacZ* 5' UTR, the *arm-lacZ* + *Runt* 3' UTR fragment (excised from pHF13) was ligated into pUC118 to allow for insertion of the oligonucleotide containing a Sxl site into the now unique *KpnI* site in the *arm-lacZ* 5' UTR. The *arm-lacZ* + *Runt* 3' UTR + 5' Sxl fragment was then ligated into pHF10 for insulation by the SCS/SCS' insulator elements.

pHF13 was digested with *PstI* (section 2.10.3), treated with Mung Bean Nuclease (section 2.14.2) to remove cohesive ends, then digested with *EcoRI*. This excises the *arm-lacZ* + *Runt* 3' UTR fragment from pHF13 and effectively removes the extra *KpnI* site, present in the polylinker. The 6.2 kb *arm-lacZ* + *Runt* fragment was separated by gel electrophoresis (section 2.9), excised from the gel (section 2.9.1) and column purified in preparation for ligation into pUC118.

pUC118 was also digested with *PstI*, treated with Mung Bean Nuclease, then cut with *EcoRI*. This digestion removes the *KpnI* site present in the pUC118 polylinker. Deletion of this site makes the *KpnI* site in the *arm-lacZ* 5' UTR unique, allowing for insertion of the Sxl 5' oligonucleotide.

The 6.2 kb *arm-lacZ* + *Runt* fragment was ligated into pUC118 (section 2.15) and transformed into *E. coli* (section 2.16). Transformant colonies were transferred to LB plates containing X-gal and IPTG for blue/white selection (section 2.3.6.3, section

2.3.6.4). Plasmid DNA isolated from blue colonies (section 2.6.2) displayed correct insertion of the fragment when digested with *EcoRI* and *PstI* (section 2.10.2) (bands at 6.3 kb, *arm-lacZ* + *Runt* PCR and 3.2 kb, pUC118) (Figure 6A). This plasmid was called pRH33 and used to mediate the insertion of the Sxl 5' oligonucleotide into *arm-lacZ*.

3.2.2.2 Insertion of Sxl 5' Oligonucleotide into *arm-lacZ* + *Runt* 3' UTR in pUC118

Oligonucleotides were designed to generate a single long Sxl binding site. This would then be used for insertion into the 5' UTR of pRH33. The oligonucleotides would generate a Sxl binding site consisting of 11 T's. When annealed (section 2.14.4) together the linker carried *Asp718* (isoschizomer of *KpnI*) 5' overhangs at each end for insertion into the unique *KpnI* site in pRH33. One site would not be reconstituted upon insertion allowing for orientation of the insert to be determined. The linker also contained a *NheI* site at the opposite end to the reconstituted *Asp718* site. The oligonucleotides were annealed and treated with T4 Polynucleotide Kinase (section 2.14.5). This new linker will be referred to as Sxl 5' insert.

pRH33 was cut with *Asp718* (to cut unique *KpnI* site in the 5' UTR of *arm-lacZ*) (section 2.10.3) and treated with Calf Intestinal Phosphatase (CIP) (section 2.14.3). Following ligation of the Sxl 5' insert into pRH33 (section 2.15) and transformation (section 2.16), plasmid DNA isolated from *E. coli* colonies (section 2.6.2) was digested with *XbaI* and *NheI* (section 2.10.2). If the linker was present, bands were seen at 5.24 kb, 3.6 kb and 0.69 kb (Figure 6B). DNA isolates found to carry the linker were digested with *Asp718* and *HpaI* to determine orientation (Figure 6C). This digest resulted in a number of small bands, but the two orientations were distinguishable from each other (band at 0.598 kb in poly T and 0.57 kb in poly A). Both orientations were found and the resulting plasmids named pRH29 with the insert in the poly T orientation (binding) or pRH30 with the insert in the poly A orientation (non-binding).

3.2.2.3 Insertion of *arm-lacZ* + *Runt* 3' UTR + Sxl 5' Insert into pHF10

The final cloning step involved digesting pRH29 or pRH30 with *PstI* (section 2.10.3), treating the linearised vector with DNA Polymerase I (Klenow) (section 2.14.1) and then cutting with *EcoRI*. The resulting 6.2 kb fragment was separated out by gel electrophoresis (section 2.9), gel extracted (section 2.9.1) and column purified.

pHF10 was digested with *SpeI*, treated with DNA Polymerase I (Klenow), then cut with *EcoRI*. This digest allowed for insertion of the fragment between the SCS/SCS' insulating elements present in pHF10. The 6.2 kb *arm-lacZ* + *Runt* 3' UTR + *Sxl* 5' fragment was ligated into pHF10 (section 2.15) and plasmid DNA isolated (section 2.6.2) from transformant colonies (section 2.16) digested with *EcoRI* and *HindIII* (section 2.10.2). If the insert was present bands appeared at 6.46 kb, 6.2 kb, 3.22 kb and 0.54 kb (Figure 6D). The orientation of the insert was checked finally with an *Asp718* and *HpaI* digest (See section 3.2.2.2) (Figure 6E). These resulting plasmids were called pRH31 with the insert in the poly T orientation (binding) or pRH32 with the insert in the poly A orientation (non-binding).

3.2.2.4 β-galactosidase Assays of Autosomal Insertions of 5' UTR Sxl Binding Site Containing Construct– pRH31

One line carrying an autosomal insertion of pRH31 was obtained via P-element mediated transformation (section 2.20). β-galactosidase assays (section 2.22) were performed on one dose (heterozygous) males and females from the one line. Reporter gene expression was decreased in females carrying pRH31. Results of the assays are summarised in Table 9.

For flies with one copy of pRH31 the male:female ratios were 1.46 (se 0.06) (standardised with total protein) and 1.49 (se 0.05) (standardised with total fly weight).

These results indicate that the insertion of a Sxl site into the 5' UTR, of the reporter gene construct already carrying Sxl sites in the 3' UTR, does not led to the decrease in reporter gene expression in females that would give a 2:1 ratio. The addition of the 5' Sxl site did cause a decrease equivalent to *Small Forked* 3' UTR inserted into *arm-lacZ*, but no more. These results will be discussed later.

Figure 6. Restriction Enzyme Digestions of Sxl 5' Insert Containing Constructs to confirm Insertion and Orientation

A. *EcoRI and PstI* digestions of pRH33 to confirm the insertion of *arm-lacZ* + *Runt* into pUC118.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *EcoRI/PstI* digestion of plasmid pRH33.

B. *XbaI* and *NheI* digestion of pRH29 and pRH30 to confirm the insertion of the Sxl 5' linker into pRH33.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *XbaI/NheI* digestion of plasmid pRH29 or pRH30.

C. *Asp718* and *HpaI* digestion of pRH29 and pRH30 to confirm the orientation of the Sxl 5' linker in pRH33.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *Asp718/HapI* digestion of plasmid pRH30.
Lane 3. *Asp718/HpaI* digestion of plasmid pRH29.

D. *EcoRI* and *HindIII* digestions of pRH31 or pRH32 to confirm the insertion of *arm-lacZ* + *Runt* + Sxl 5' linker into pHF10.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *EcoRI/HindIII* digestion of plasmid pRH31 or pRH32.

E. *Asp718* and *HpaI* digestion of pRH31 or pRH32 to confirm the orientation of the Sxl 5' linker inserted into pHF10.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *Asp718/HpaI* digestion of plasmid pRH31 or pRH32.

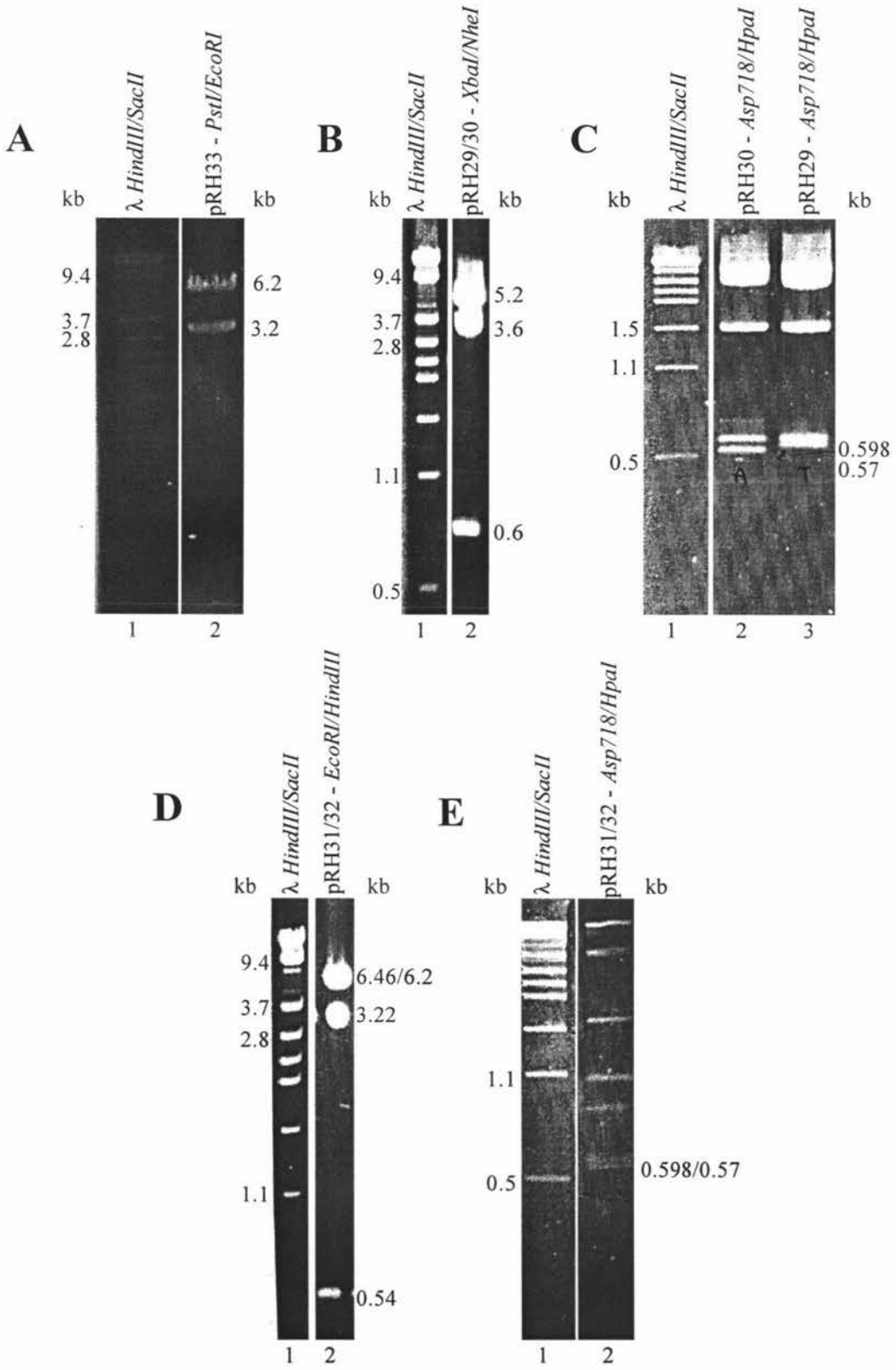

A
λ HindIII/SacII
pRH33 - PstI/EcoRI
kb
9.4
3.7
2.8
6.2
3.2
1
2
B
λ HindIII/SacII
pRH29/30 - XbaI/NheI
kb
9.4
3.7
2.8
1.1
0.5
5.2
3.6
0.6
1
2
C
λ HindIII/SacII
pRH30 - Asp718/HpaI
pRH29 - Asp718/HpaI
kb
1.5
1.1
0.5
0.598
0.57
A
T
1
2
3
D
λ HindIII/SacII
pRH31/32 - EcoRI/HindIII
kb
9.4
3.7
2.8
1.1
6.46/6.2
3.22
0.54
1
2
E
λ HindIII/SacII
pRH31/32 - Asp718/HpaI
kb
1.1
0.5
0.598/0.57
1
2

Table 9. β-galactosidase Assays of Transgenic Flies carrying Autosomal Insertions of pRH31

A single transgenic line was assayed from this construct. The expression of one dose males and females was compared. The assays were performed a minimum of five times and standardised using total protein measurements and total wet weight. The standard error represents the variability between the experiments.

Line	Chromosome	M:F Ratio		Standard Error
pRH31 #1	2	Protein	1.46	0.06
		Wet weight	1.49	0.05

3.3 INSERTION OF X-LINKED DNA INTO pHF11

A cosmid contig spanning approximately 84 % of the X chromosome has been constructed as part of the European *Drosophila* Genome project (Madueno *et al.*, 1995). Ten cosmids from the 1C region of the X chromosome were obtained for this project (Table 1). The 1C region was focussed upon because of recent findings that the MSL complex has a high affinity for this region (Lyman *et al.*, 1997). Cosmid DNA was digested with *EcoRI*, which cleaves genomic DNA on average once every 4.1 kb. Four fragments were derived from internal *EcoRI* sites within the vector - 1.7 kb, 1.6 kb, 1.2 kb and 0.05 kb.

An *EcoRI* digest (section 2.10.2) of all ten cosmids showed up to 13 fragments with very similar banding patterns for all the smaller bands (Figure 7A). A Southern Blot (section 2.11) using cosmid 34F3 DNA as a probe showed a strong signal with 34F3, 77G2 and 109H7, all of which are found predominantly in the 1C region. Cosmid 34F3 was chosen for cloning *EcoRI* digested fragments into pHF11.

The largest bands found from the *EcoRI* digest of cosmid 34F3 were chosen for cloning. Larger fragments would have a higher chance of containing a binding site for the MSL complex. These fragments were approximately 17.0 kb, 8.0 kb, and 5.0 kb (Figure 7B). The *EcoRI* digest of 34F3 (section 2.10.3) was fractionated on a seaplaque agarose gel (section 2.9), the three largest bands excised (section 2.9.1), and column purified. pHF11 was digested with *EcoRI* and treated with Calf Intestinal Phosphatase (CIP) (section 2.14.3) in preparation for ligation (section 2.15) of the X-linked DNA fragments. The ligation reactions were transformed into *E. coli* (section 2.16) and insertion of the fragments confirmed with an *EcoRI* digest. Because pHF11 has a unique *EcoRI* site, the *EcoRI* digest resulted in fragment sizes corresponding to 16.36 kb for the vector and the appropriate size of the insert (Figure 7C). The two smaller fragments were easily confirmed with this digest. These plasmids were named p34F3 5.0 and p34F3 8.0.

Due to the size of the largest fragment (approximately 17.0 kb), being about the same as pHF11, separate digestions with *BamHI* and *HindIII* were used to confirm the insertion. pHF11 alone resulted in fragment sizes of 9.8 kb and 6.6 kb for the *BamHI* digest and

6.9 kb, 6.6 kb, and 2.9 kb for the *HindIII* digest. Possible transformants were digested with each enzyme, resulting in banding patterns of four of five fragments totalling approximately 33 kb (Figure 7D). This confirmed that the largest 34F3 X-linked DNA fragment had been inserted into pHF11. This plasmid was called p34F3 17.0.

However, when a large scale DNA preparation (section 2.6.1) was carried out on p34F3 17.0, and the DNA checked by digestion with *HindIII* and separated on an agarose gel, the DNA appeared in a discreet band anywhere between 1.5 kb and 3.7 kb. Uncut DNA would still run as a much smaller fragment than expected. Large scale preparations were carried out using Qiagen midi, Concert midi, Bio Rad mini and Caesium Chloride gradient methods, and the DNA consistently appeared smaller than the expected 33 kb.

It is possible the large plasmid was undergoing recombination during the growth of the large cell culture and a smaller plasmid was forming that was then out competing the larger plasmid.

Plasmid DNA containing direct or inverted sequences are relatively stable in *E.coli* SBTL2 cells (Gibco BRL) which were found to reduce recombination in unstable plasmids. STBL2 competent *E. coli* cells are grown at 30°C in TB (section 2.3.5) to increase transformation efficiency. Transformation was carried out using a small scale preparation of DNA known to have the 34F3 17.0 kb fragment inserted into pHF11 (p34F3 17.0). The cells were plated onto LB (section 2.3.1) amp plates (section 2.3.6.1) and incubated at 30°C. Transformant colonies were then grown in TB at 30°C for small scale DNA preparations (section 2.6.2). The small scale preparations were checked for insertion with a *HindIII* digest. The first noticeable difference was the concentration of DNA - where the original small scale preparations had five faint bands (Figure 7D), the new transformants had the five original bands and another five bands further down the gel (Figure 7E). This indicated the concentration was much higher. The final test was growing the correct construct in a large culture. The cells were grown in TB at 30°C and plasmid DNA isolated using a Concert midi kit (Gibco BRL). The resulting isolated plasmid DNA was digested with *HindIII* and the ten bands observed in the small scale preparation were present. This plasmid was called p34F3 17.0.

3.3.1 β-galactosidase Assays of Autosomal Insertions of X-linked DNA Containing Constructs– p34F3 5.0, p34F3 8.0, p34F3 17.0

One line carrying an autosomal insertion of p34F3 5.0 and one line carrying an autosomal insertion of p34F3 8.0 were obtained via P-element mediated transformation (section 2.20). β-galactosidase assays (section 2.22) were performed on one dose (heterozygous) males and females with from the two lines. There was no noticeable increase in reporter gene expression in males carrying the constructs. Results of the assays are summarised in Table 10.

For flies with one copy of p34F3 5.0 the male:female ratios were 1.14 (se 0.02) (standardised with total protein) and 1.22 (se 0.09) (standardised with total fly weight).

Males with one copy of p34F3 8.0 the male:female ratios were 1.11 (se 0.01) (standardised with total protein) and 1.14 (se 0.02) (standardised with total fly weight).

No lines have been obtained of p34F3 17.0. Due to time restraints and the low survival rate of larvae from microinjection no transformants were found.

The results from assays of p34F3 5.0 and p34F3 8.0 suggest that no DCREs are present in the X-linked DNA fragments. Possible reasons why will be discussed later.

Figure 7. Restriction Enzyme Digestions of X-linked DNA Containing Constructs to confirm Insertion and Orientation

A. *EcoRI* digestions of cosmid DNA

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *EcoRI* digestion of cosmid 115C2.
Lane 3. *EcoRI* digestion of cosmid 118B3.
Lane 4. *EcoRI* digestion of cosmid 165D1.
Lane 5. *EcoRI* digestion of cosmid 77G2.
Lane 6. *EcoRI* digestion of cosmid 109H7.
Lane 7. *EcoRI* digestion of cosmid 107G5.
Lane 8. *EcoRI* digestion of cosmid 34F3.
Lane 9. *EcoRI* digestion of cosmid 40F6.
Lane 10. *EcoRI* digestion of cosmid 155B1.

B. *EcoRI* digestion of cosmid DNA in preparation for excision of the three largest bands and insertion into pHF11.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *EcoRI* digestion of cosmid 34F3.

C. *EcoRI* digestion of p34F3 8.0 and p34F3 5.0 to confirm the insertion of the 8.0 kb and 5.0 kb cosmid DNA fragments into pHF11.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *EcoRI* digestion of plasmid p34F3 8.0.
Lane 3. *EcoRI* digestion of plasmid p34F3 5.0.

D. *BamHI and HindIII* digestions of p34F3 17.0 in DH5α *E. coli* cells to confirm the insertion of the 17 kb cosmid DNA fragment into pHF11.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *BamHI* digestion of plasmid p34F3 17.0.
Lane 3. *BamHI* digestion of plasmid pHF11.
Lane 4. *HindIII* digestion of plasmid p34F3 17.0.
Lane 5. *HindIII* digestion of plasmid pHF11.

E. *HindIII* digestion of p34F3 17.0 in STBL2 *E. coli* cells to confirm the insertion of the 17 kb cosmid DNA fragment into pHF11.

Lane 1. λ *HindIII/SacII* ladder.
Lane 2. *HindIII* digestion of plasmid p34F3 17.0.

The comparison between D and E illustrates the effectiveness of STBL2 cells to reduce recombination in large-scale DNA preparations.

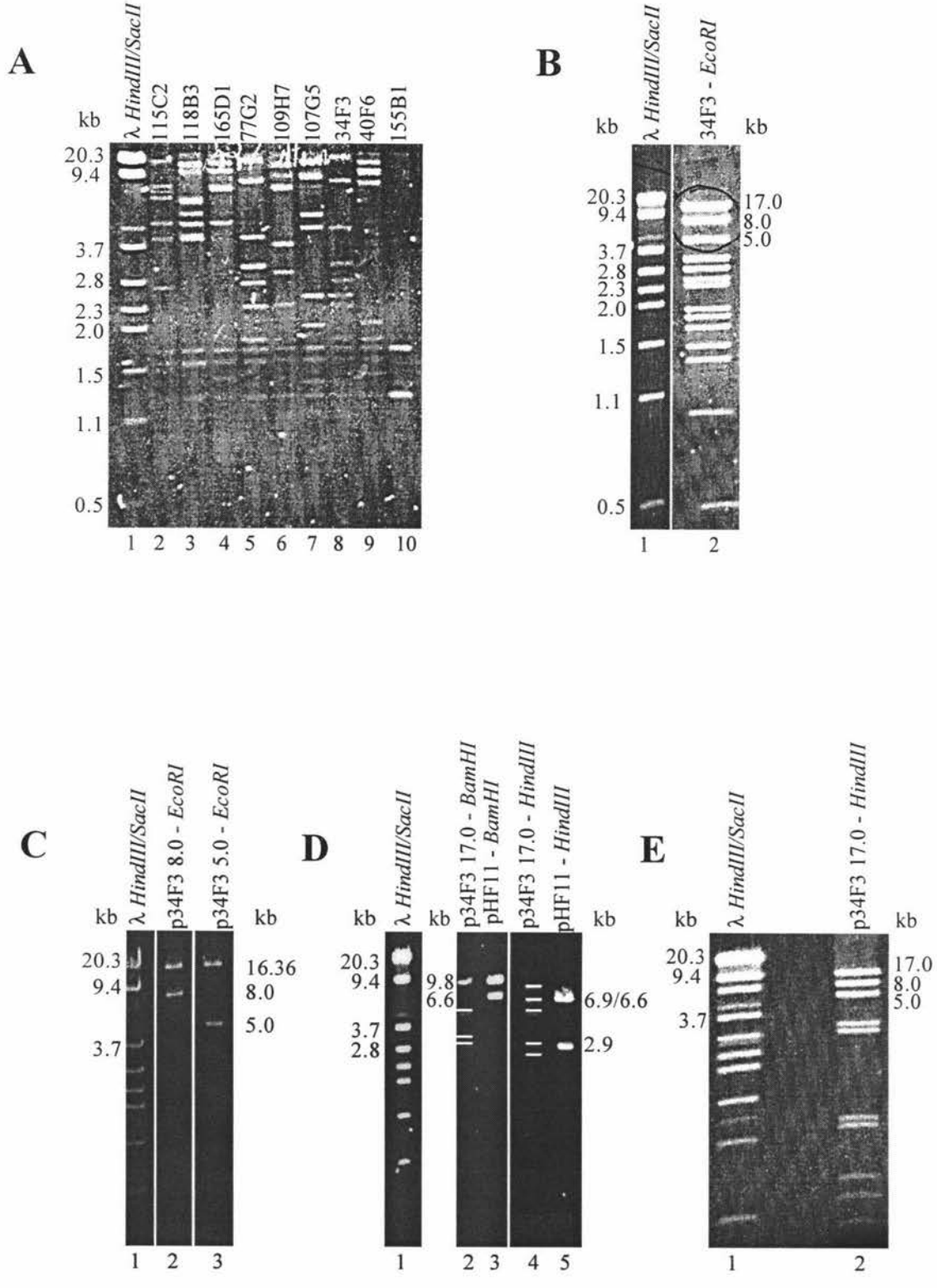

A
kb
λ HindIII/SacII
115C2
118B3
165D1
77G2
109H7
107G5
34F3
40F6
155B1
20.3
9.4
3.7
2.8
2.3
2.0
1.5
1.1
0.5
1 2 3 4 5 6 7 8 9 10
B
kb
λ HindIII/SacII
34F3 - EcoRI
kb
20.3
9.4
3.7
2.8
2.3
2.0
1.5
1.1
0.5
17.0
8.0
5.0
1 2
C
kb
λ HindIII/SacII
p34F3 8.0 - EcoRI
p34F3 5.0 - EcoRI
kb
20.3
9.4
3.7
16.36
8.0
5.0
1 2 3
D
kb
λ HindIII/SacII
kb
p34F3 17.0 - BamHI
pHF11 - BamHI
p34F3 17.0 - HindIII
pHF11 - HindIII
kb
20.3
9.4
3.7
2.8
9.8
6.6
6.9/6.6
2.9
1 2 3 4 5
E
kb
λ HindIII/SacII
p34F3 17.0 - HindIII
kb
20.3
9.4
3.7
17.0
8.0
5.0
1 2

Table 10. β-galactosidase Assays of Transgenic Flies carrying Autosomal Insertions of p34F3 5.0 and p34F3 8.0

Two transgenic lines were assayed from both constructs. For each line the expression of one dose males and females was compared. Each assay was performed a minimum of four times and standardised using total protein measurements and total wet weight. The standard error represents the variability between the experiments.

Line	Chromosome	M:F Ratio		Standard Error
p34F3 8.0 - Insertion of 8.0 kb *EcoRI* fragment into pHF11				
p34F3 8.0 #1	3	Protein	1.08	0.02
		Wet weight	1.15	0.03
p34F3 5.0 - Insertion of 5.0 kb *EcoRI* fragment into pHF11				
p34F3 5.0 #2	3	Protein	1.14	0.02
		Wet weight	1.22	0.09

4.0 DISCUSSION

4.1 EVALUATION OF THE ROLE OF *ROX* GENES DURING DOSAGE COMPENSATION

Both *roX1* and *roX2* RNAs are non-coding and male specific. Their accumulation in males is dependent upon the MSL proteins (Amrein and Axel, 1997; Meller *et al.*, 1997). Both *roX1* and *roX2* localise in a similar pattern to the MSL proteins along the male X chromosome (Franke and Baker, 1999; Kelley *et al.*, 1999). Both *roX1* and *roX2* have been shown to be non-essential for dosage compensation through mutational analysis using loss of function mutations. However, the loss of both *roX* genes prevents dosage compensation (Franke and Baker, 1999). Meller *et al.*, (1997) found that *roX1* has the ability to bind the X chromosome in *trans*, and Smith *et al.*, (2000) showed that *roX2* is present in the MSL complex, possibly in an auxiliary role to help maintain the association of MLE with the other MSLs.

4.1.1 Roles of *roX* Genes as MSL Entry Sites

Early studies into the *cis*-acting regulatory elements involved in dosage compensation focused upon translocation of hundreds of kilobases of X-linked DNA to autosomes. Lyman *et al.*, (1997) suggest that DCREs act over long distances rather than only on immediately adjacent genes. While the multi subunit MSL complex has been shown to bind hundreds of sites along the male X chromosome (Kuroda *et al.*, 1991; Palmer *et al.*, 1993), Lyman *et al.*, (1997) found that MSL-1 and MSL-2 co-localise to 30 – 40 'high affinity' sites along the male X chromosome, independently of MSL-3, MOF and MLE. Because no MSL protein can bind in the absence of MSL-1 and MSL-2, Lyman *et al.*, (1997) argue that this MSL-1/MSL-2 subunit is central to the MSL dosage compensation complex assembly. They also suggest that the 'high affinity' sites may be nucleation centres at which the MSL complex assembles and spreads from, predominantly in *cis*. Two of the 'high affinity' sites map to regions 3F and 10B/C of the X chromosome in males. These bands have been shown to contain the *roX1* (3F) and *roX2* (10B/C) genes (Amrein and Axel, 1997).

Tobler *et al.*, (1971) described the full dosage compensation of the X-linked *v+* (*vermilion*) gene and its associated enzyme, tryptophan pyrrolase. This gene was carried on the T(1;)ras translocation (regions 9F to 10C 1-2 of the X chromosome translocated to the third). The discovery that *roX2* is situated in the 10B/C region of the translocated fragment suggests that perhaps either *roX1* or *roX2* is required in *cis* for full dosage compensation to occur.

The *arm-lacZ* system allows for the insertion of gene fragments into a reporter gene construct and β-galactosidase assays to be performed independent of promoter sequences. Also this system detects the expression of a reporter gene rather than a gene product. This allows for the investigation of the effects of DNA sequences that may not produce a detectable product. This especially relates to the *roX* genes that produce a non-coding RNA. This assay system can show what effect the *roX* cDNA sequence has upon the expression of the *lacZ* reporter gene without the expression of the *roX* RNA.

Inserting the *roX* genes, both individually and together, into the insulated *arm-lacZ* reporter gene construct tested this hypothesis (section 3.1). An increase in *lacZ* expression in males was observed when either the RNA was expressed (*hsp83-roX2* [pRH02], [pRH03] and 4.9 kb *roX1* [pRH04]) or the cDNA sequence was present (3.7 kb *roX1* [pRH08], 1.1 kb *roX2* [pRH09] and 3.7 kb *roX1* and 1.1 kb *roX2* [pRH13]). In heterozygous males for these lines the increase was not fully two-fold. In homozygous males for both lines carrying the 3.7 kb *roX1* cDNA the increase in reporter gene expression was very close to two-fold. The increase in homozygous pRH08 #1 males was noticeably higher than homozygous males carrying either the *hsp83-roX2* fragment or the 4.9 kb *roX1* genomic DNA fragment. This raised the question of what roles the DNA sequence and the RNA play during dosage compensation.

4.1.2 *roX1* and *roX2* RNA Expression Partially Hypertranscribes *LacZ* in Males

β-galactosidase assays of flies carrying autosomal insertions of pRH02, pRH03 (*roX2* RNA expression) and pRH04 (*roX1* RNA expression) revealed that the β-galactosidase activity in one dose males was significantly increased over one dose females (Tables 4 and 5, sections 3.1.1.4 and 3.1.2.1). Lines carrying autosomal insertions of pRH02 and pRH03 showed β-galactosidase activity in one dose males to be 1.34 – 1.66 (pRH02)

and 1.51 – 1.86 (pRH03) times that of one dose females. Lines carrying autosomal insertions of pRH04 showed β-galactosidase activity in one dose males to be 1.41 – 1.67 times that of one dose females. The expression of *roX2* RNA from within the SCS/SCS' insulated region (pRH03) in one dose males increases the β-galactosidase activity to higher levels than the expression of *roX1* RNA (pRH04). However the increase in all one dose lines for these constructs is not fully two-fold.

β-galactosidase assays of homozygous flies carrying autosomal insertions of pRH02, pRH03 (*roX2* RNA expression) and pRH04 (*roX1* RNA expression) revealed that the β-galactosidase activity in males was increased over females (Tables 4 and 5, sections 3.1.1.4 and 3.1.2.1), but not to levels any higher than that seen in heterozygous flies. In fact three of the lines (pRH03 #3, pRH04 #1 and pRH04 #2), when homozygous, show β-galactosidase activity in two dose males to be only 1.21 – 1.39 times higher than two dose females. This is a noticeable lowering of the increase in males over females. These smaller increases may be due to the over expression of autosomal genes not normally dosage compensated.

The discovery that the *roX* genes reside within two of the 'high affinity' sites led Kelley *et al.*, (1999) to propose that one of the functions of the *roX* genes is provision of entry sites for the recognition of the X chromosome by the MSL complex. When these complexes form at the *roX1* and *roX2* entry sites the complexes incorporate *roX* RNA. Kelley *et al.*, (1999) observed that the MSL complex binds to autosomal insertions of the *roX1* and *roX2* transgenes. RNA *in situ* hybridisation to polytene chromosomes showed that the MSL complex is attracted to an autosome by the presence of a *roX1* transgene and the complex could spread in *cis* along the autosome. This presumably results in over expression in males of autosomal genes surrounding the insertion site of the transgene (Kelley *et al.*, 1999). This may explain why *lacZ* expression is lower in some homozygous lines when *roX* transgenes are present at autosomal sites – ie pRH02, pRH03, pRH04, pRH09 and pRH13. The spreading of the MSL complex and subsequent hypertranscription of autosomal genes may result in a lethal imbalance of autosomal gene products. This would result in cells, not experiencing high hypertranscription, surviving and giving lower M:F ratio values. This may also explain why in heterozygous lines the male:female ratio of β-galactosidase activity was less

than the expected two fold. Alternatively, recruitment of the MSL complex to the *roX* transgene may behave in an all-or-none mosaic, with some nuclei having the MSLs binding the autosomal *roX*, and some not. Another alternative is that full compensation requires a certain number or threshold of copies of the MSL complex. Perhaps the number of MSL complexes bound to an autosomal *roX* is less than the number required for full dosage compensation. Another possibility is that the *roX* RNA is acting as a negative factor within the MSL complex and plays a part in keeping the increase in transcription to the twofold level. Excess expression of *roX1* RNA maybe providing a level of RNA higher than is required by the complex resulting in a lowering of transcription.

4.1.3 *roX1* and *roX2* cDNA Sequences Partially Hypertranscribe *LacZ* in Males

β-galactosidase assays of flies carrying autosomal insertions of pRH08 (*roX1* cDNA), pRH09 (*roX2* cDNA), and pRH13 (*roX1* and *roX2* cDNAs) revealed that the β-galactosidase activity in one dose males was significantly increased over one dose females (Table 6, section 3.1.3.5). The β-galactosidase activity from lines carrying pRH08 and pRH09 showed heterozygous males to be 1.54 – 1.58 (pRH08) and 1.23 – 1.93 (pRH09) times that of heterozygous females. Assays for two lines carrying autosomal insertions of pRH13 showed β-galactosidase activity in one dose males to be 1.39 – 1.54 times that of one dose females. The third pRH13 line showed β-galactosidase activity in heterozygous males to be 2.02 – 2.30 times that of heterozygous females. This line carries an X-linked insertion of the construct and acts as a control to show that the construct can be fully compensated. Homozygous flies confirm the X-linked insertion by showing β-galactosidase activity in males to be only 1.09 – 1.17 times that of females.

β-galactosidase assays of homozygous males and females from pRH08 and pRH09 show enzyme activity levels close to a two-fold increase in males carrying the *roX1* cDNA, but not in males carrying the *roX2* cDNA. This is not consistent with the heterozygous lines carrying the *roX2* cDNA, which has increases higher than the *roX1* cDNA lines. A higher male:female ratio for the *roX1* cDNA homozygous line is consistent with the model that full compensation requires a threshold level of the MSL complex. *roX1* cDNA may contain more sites, than *roX2* cDNA, within its sequence

that the MSL complex recognises. However, if this were the case pRH13 lines would be expected to fully compensate due to the presence of both *roX* cDNAs.

The results from this study show that the *roX1* cDNA lines are generating significantly higher β-galactosidase activity ratios, between homozygous males and females, than the lines expressing the r*oX1* RNA. The role of the DNA sequence may involve targeting the MSL complex to the X chromosome in males, by possessing an MSL 'high affinity' site or sites within the sequence (Lyman *et al.*, 1997; Kelley *et al.*, 1999).

4.1.4 *roX* RNA Expression *vs* DNA Sequence

The ability of the MSL complex to bind and spread in *cis* along autosomal chromosomes suggests that the complex may be attracted to sequences or proteins that are common to active genes rather than specifically to X-linked genes. But because normally the 30 – 40 'high affinity' entry sites are only present on the male X chromosome the MSL complex is limited to compensating male X-linked genes.

This study investigated whether it was necessary to have a *roX* gene expressed in *cis* for compensation, or if the cDNA sequence alone is sufficient. The results obtained in this study show that the lines in which *roX1* or *roX2* RNA is expressed have β-galactosidase activity ratios that are predominantly lower than lines carrying *roX1* or *roX2* cDNA sequences. Kelley *et al.*, (1999) observed that both *roX1* and *roX2* transgenes not producing RNA had the MSL complex binding, which is consistent with the results from this study. However the possibility that the cDNAs are transcribed due to insertion near an autosomal promoter can not be ruled out.

Smith *et al.*, (2000) suggests that a function of the *roX2* RNA may be to help maintain the association of MLE with the other MSLs. It may be that the *roX1* gene sequence is required for targeting the MSL complex to the male X chromosome through the binding of MSL-1 and MSL-2 initially and the *roX* RNAs function redundantly within the MSL complex. Two lines of evidence suggest that *roX* RNAs are incorporated into the MSL complex. The first being that, ectopically expressed *roX* RNAs are highly unstable in the absence of the MSL complex. The second being that both *roX* RNAs and MSL proteins co-localise to the same sites along the male X chromosome (Franke and Baker,

1999; Kelley *et al.*, 1999), and *roX2* RNA co-immunoprecipitates with the MSL complex (Smith *et al.*, 2000).

4.1.5 Regions of the *roX1* cDNA may be Important of Dosage Compensation

Franke and Baker, (1999) discuss several possibilities for the function of the *roX* genes during dosage compensation. Double mutants of *roX1* and *roX2* generated by this group abolished MSL association with the X chromosome. Mutations involving the loss of one of the *roX* genes have no obvious phenotype and are not lethal. These results argue that the *roX* RNAs are functionally redundant, but that they are essential components of the dosage compensation machinery. The fact that loss of both *roX1* and *roX2* result in loss of MSL binding suggests that these two genes are the only members of the *roX* family. If there were other members, the *roX1* and *roX2* double mutants would still display MSL binding of the X chromosome.

Franke and Baker, (1999) also suggest that due to their redundant nature the *roX* RNAs might exhibit similarities in their primary and secondary structures. They identified a 30 base pair region of homology with no biological significance to date. This sequence was amplified using PCR in this study and the 250 bp fragment was inserted into the *arm-lacZ* reporter gene. β-galactosidase assays carried out on this construct showed no increase in *lacZ* expression in males (Table 7, section 3.1.4.1). The male to female ratios remained 1:1. This could mean that the sequence carries no significance in relation to dosage compensation or that it needs other sequences or protein binding sites in conjunction to function.

4.1.6 Regions of the *roX1* cDNA Sequence Partially Compensate *arm-lacZ*

pRH12, containing the 410 bp fragment with the possible GAGA binding sites showed a slight increase in one dose males in comparison to females. This fragment also contains a binding site for the MSL complex (R. Kelley, personal communication). The β-galactosidase assays on pRH12 heterozygous lines showed β-galactosidase activity in males to be 1.16 – 1.33 times higher than females (Table 7, section 3.1.4.1). One homozygous line was assayed for β-galactosidase activity and males found to have activity 1.25 – 1.34 times higher than females. This not noticeably higher than heterozygous lines. This increase in both heterozygous and homozygous males is lower

than the full length *roX1* cDNA. This may indicate that there are additional MSL binding sites in *roX1* and the number of MSL complexes recruited to the autosomal site is limiting.

4.1.7 GAGA Protein Binding Involvement in Dosage Compensation

GAGA protein is a sequence-specific DNA binding protein, which participates in the regulation of developmentally regulated genes as well as other housekeeping genes. Studies by Espinas *et al.*, (1999) have shown that GAGA oligomers bind DNA with high affinity and specificity and are capable of binding adjacent sites spaced by as many as 20 base pairs. They also show that GAGA dependent transcription activation depends strongly on the number of GAGA binding sites present - the more sites the higher the affinity. GAGA has been implicated in maintenance of chromatin structure at transcriptionally active loci (Wilkins and Lis, 1999). GAGA factor appears to relieve the repressive effects of histones without affecting the transcriptional machinery. This suggests that perhaps the GAGA sites in *roX1* play a role in opening up the chromatin to allow entry to the MSL proteins.

Insertion of a 410 bp fragment containing possible GAGA binding sites from *roX1* (found approximately 100 bp upstream of the P-element insertion site in *roX1*) into *arm-lacZ* resulted in an increase in *lacZ* expression in males. The 410 bp PCR fragment was from a region of *roX1* to which a male-specific DNAseI hypersensitivity site has been mapped (P. Becker, personal communication). The increase in males was 1.16 – 1.33 times that in females. The increase in males carrying the DNAseI *roX1* fragment is less than in lines carrying *roX* cDNAs or *roX* RNA expressing constructs, but is significant. There is also a possibility that it is not the GAGA sites that are facilitating the increase in transcription. There may be additional MSL binding sites present within the 410 bp fragment. The observation of only partial dosage compensation could be because: not all the MSL binding sites in *roX1* are found in the 410 bp fragment, the MSL complex is not binding the transgene in all nuclei, or the number of complete MSL complexes is too low for full levels of transcription to occur.

4.1.8 Role of *roX* RNA in RNA – Protein Interactions

Franke and Baker, (1999) draw parallels between the roles of *roX* RNAs and other RNA-protein complexes, ie. ribosomes, spliceosomes, and telomerase (reviewed in

Blackburn, 1998; Nilsen, 1998; Noller, 1998). They suggest that as the RNA components of these complexes perform major functions, rather than just acting as structural components, the *roX* RNAs may have additional roles in eliciting hypertranscription of the male X chromosome.

This study shows that the *roX* genes can mediate hypertransactivation in males. Lines carrying either a *roX* cDNA sequence or expressing a *roX* RNA facilitate an increase in transcription of the *arm-lacZ* reporter gene. Despite an increase being observed, it is still less than two-fold. What is surprising is that the homozygous *roX1* cDNA lines (pRH08) display a higher increase in males than homozygous lines expressing the *roX1* RNA. The *roX1* DNA sequence may be important for contact between the X chromosome and the MSL dosage compensation complex by acting as the initial binding site in the chromatin. The *roX1* RNA may be act as a negative factor within the MSL complex. A hypothesis has been suggested that *roX* RNAs may act to limit the transcription increase of MOF acetylase to two-fold. MOF acetylase strongly stimulates transcription in *Drosophila* nuclear extracts and in yeast (Akhtar and Becker, 2000). This suggests a component in the MSL complex acts to dampen the transcriptional stimulation due to MOF. This hypothesis is supported by the results of this study. Both constructs carrying the 3.7 kb *roX1* cDNA sequence (pRH08) and the 4.9 kb *roX1* genomic sequence (pRH04) contain the *roX1* DNA sequence. The difference between the two is the expression of *roX1* RNA. Lines homozygous for both the constructs show a larger increase in males carrying the *roX1* cDNA. Excessive expression (homozygous) of *roX1* RNA may act to dampen the MSL mediated hypertransactivation rather than maintain the level. This dampening of the activation links *Drosophila roX* RNAs and mammalian *Xist* RNA closer than before as *Xist* RNA acts to inactivate a mammalian X chromosome.

4.1.9 Parallels Between *roX* and *Xist*

Both flies and mammals must equalise gene expression between two X chromosomes in females and one in males. In both, dosage compensation involves opposite changes in the pattern of histone H4 acetylation. Both mechanisms involve non-coding, nuclear RNAs as integral components of the dosage compensation machinery and result inactivation in mammals, or hypertransactivation in *Drosophila.*

Kelley *et al.*, (1999) strengthen the parallels between *roX* and *Xist* RNAs with experimental observations of the *roX* RNAs. Firstly, *roX* RNAs and the MSL proteins can spread long distances (100 - 1000 kb) in *cis* from a *roX1* transgene at an autosomal site into previously unrecognised autosomal chromatin. They propose that this autosomal spreading reflects what normally occurs along the male X chromosome. *Xist* RNA spreads from a single origin on the inactive X chromosome in females, to cover almost 155 Mb of X chromosomal DNA (reviewed in Lee and Jaenisch, 1997). *Xist* RNA also does this in X to autosome translocations, or if expressed from an autosomal transgene. The 30 - 40 chromatin entry sites distributed along the 25 Mb X chromosome in *Drosophila* are utilised to allow *roX* RNA and the MSLs to spread, on average, less than 1 Mb from each entry site to cover the entire X chromosome (Kelley *et al.*, 1999; Meller *et al.*, 1997). As well as acting in *cis*, *roX* RNA has been shown to bind the X chromosome in *trans* (Meller *et al.*, 1997), while the *Xist* RNA only functions in *cis* (Lee *et al.*, 1996).

4.1.10 Conclusions and Future Work

The main conclusion reached in this study is that *roX1* and *roX2* cDNAs act as MSL assembly points, which leads to an increase in transcription in males. It has been hypothesised that the *roX* RNAs are members of a larger family in which the RNAs act redundantly within the MSL protein complex. Mutational analysis of these RNAs has proven that if a double mutant is generated, the MSLs fail to bind the X chromosome (Franke and Baker, 1999). This suggests that *roX1* and *roX2* are the only X-linked non-coding RNAs that are part of the MSL complex.

The findings from this study, that the lines in which the *roX1* RNAs are expressed show smaller increases in *lacZ* expression than the lines carrying the *roX1* cDNA, suggest that the role of the *roX1* RNA maybe as an inhibiting factor for the increase in transcription elicited by MOF acetylase. The *roX* cDNA sequence on the other hand appears to play a role in the targeting of the MSL complex to the male X chromosome. Results from lines carrying either the *roX1* or *roX2* cDNA sequence strengthen the argument that the 30 - 40 'entry sites' found along the X chromosome are the initial site for the binding of the MSL protein complex. Lines carrying autosomal insertions of the *roX* cDNA sequences displayed predominantly higher β-galactosidase male to female ratios than lines expressing the *roX* RNAs.

In an initial attempt to identify the MSL binding site within *roX1*, two regions thought to be important in the targeting of the MSL complex to the X chromosome were tested in the *arm-lacZ* system. A fragment containing the 30 bp homologous sequence suggested by Franke and Baker, (1999) in the 3' end of *roX1* did not show an increase in heterozygous males over heterozygous females. But the fragment carrying the possible GAGA binding sites, found approximately 100 bp upstream of the P-element insertion site, did show an increase in males over females. It has been suggested that the GAGA binding sites act in conjunction with other protein binding sites elsewhere in the DNA sequence. These additional sites may be additional GAGA binding sites or perhaps the sequence present that targets the MSL complex to the *roX* 'entry site'. For our study to achieve full dosage compensation we may have needed these other sites present in the reporter gene construct. All lines, containing the *roX* genes in one form or another, never reach a full 2:1 ratio of males to females. This partial compensation could be a result of the increase in *lacZ* expression not occurring in all nuclei, or that the MSL complex is incomplete.

To clarify what is causing the partial compensation further experiments are required. Distribution of the MSLs in nuclei can be checked using antibody staining. Antibody staining will also show if mosaicism is occurring ie. if some nuclei are not binding the MSL complex. Partial compensation will occur if the MSL complex is incomplete. Therefore the observation of less than two-fold increases in males obtained in this study may be due to a missing component within the complex. This missing component may be MOF, which is expected to increase transcription (Akhtar and Becker, 2000), however it may be limiting the levels of compensation by not being present in all nuclei. As discussed previously there may also be a component of the complex that dampens the increase in transcription. An excess of this component at an autosomal site would explain the observation that homozygous males carrying the 4.9 kb *roX1* genomic fragment do not have an increase as large as homozygous males carrying the 3.7 kb *roX1* cDNA. These results suggest that it may be the RNA that is the dampening agent. Further lines from both constructs need to be generated by mobilising the construct to new autosomal sites. These additional lines would confirm if the trend is consistent. An additional construct that would help determine the role of the *roX2* gene is one carrying the *roX2* genomic DNA fragment. This would act as a comparison to the *roX2*

cDNA construct to see if the RNA expression results in a transcriptional increase lower than in males carrying the *roX2* cDNA.

roX1 and *roX2* have both been found to lie within two of the 30 – 40 MSL 'entry sites' and the presence of the *roX* cDNA sequence produces larger increases in β-galactosidase activity in males over females. The next step is to use the *roX* DNA sequence to determine which regions of sequence are important for dosage compensation. By cutting the sequence into four of five overlapping fragments and inserting these smaller fragments into the *arm-lacZ* assay system, the β-galactosidase activity levels in males and females, from subsequent lines, would narrow down the sequence requirements for MSL binding to the male X chromosome.

roX1 and *roX2* reside in only two of the 30 - 40 suggested MSL 'entry sites' along the X chromosome. *roX1* and *roX2* may be the only members of the *roX* family of non-coding RNAs involved in dosage compensation, but the fact that there are 30 - 40 sites found to bind the MSL-1/MSL-2 initial complex suggests that there may be sequence similarities between other members of this group. Work in the future may also centre upon identifying similarities between all these 'high affinity entry sites'.

4.2 EXAMINATION OF DOSAGE COMPENSATION INVOLVING SEX-LETHAL

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 (Kelley *et al.*, 1995). Sxl is best known as a regulator of alternative RNA splicing during *Drosophila* sex determination. A second function for the Sxl protein in translational regulation has been identified through studies of the control of *Msl-2* translation during sex determination (Bashaw and Baker, 1997; Kelley *et al.*, 1997). Sxl protein is thought to prevent translation in females through binding to poly (U) stretches in the 3' and 5' UTRs of female mRNA. The *arm-lacZ* system also allows for the insertion of 3' and 5' UTRs from unrelated genes into the reporter gene construct without the rest of the sequence.

The aim of this study was to further investigate the function of Sxl binding sites in both 3' and 5' UTRs of genes within the subset identified to contain three or more Sxl binding sites (Kelley *et al.*, 1995). Fitzsimons *et al.*, (1999) began the investigation into these sites using the insulated *arm-lacZ* reporter system by inserting a 170 bp fragment from the *Runt* 3' UTR (containing three Sxl binding sites) into the *arm-lacZ* 3' UTR. Lines carrying autosomal insertions of this construct displayed partial dosage compensation.

4.2.1 Dosage Compensation Involving Sex-lethal

β-galactosidase assays of flies carrying autosomal insertions of pRH23 (*Cut* 3' UTR), pRH24 (*Sxl* 3' UTR), and pRH25 (*Small Forked* 3' UTR) revealed that the β-galactosidase activity in one dose males varied considerably in relation to one dose females (Table 8, section 3.2.1.3). Lines carrying the *Forked* 3' UTR fragment and the *Cut* 3' UTR fragment, in the poly T orientation, displayed β-galactosidase activity in heterozygous males to be 1.26 - 1.34 *(Forked)* or 1.31 - 1.50 (*Cut*) times higher than that of heterozygous females. In *Small Forked* this corresponds to a 21 - 25 % decrease in females or 42 – 50 % of the decrease required for the 2:1 ratio. In *Cut* this corresponds to a 24 - 33 % down-regulation of *lacZ* expression in females, which is the

equivalent to 48 – 66 % of the required decrease. This decrease in reporter gene expression shows that the presence of Sxl binding sites in the 3' UTR can promote down regulation by the Sxl protein. This decrease in females was similar to lines carrying autosomal insertions of the *Runt* 3' UTR (Fitzsimons *et al.*, 1999), which showed a 24 - 32 % down regulation of *lacZ* in females.

4.2.2 Differences Between Length, Position and Number of Binding Sites

The genes chosen for this study contained either longer poly (U) Sxl binding sites than that of *Runt* (ie *Small Forked*), more Sxl binding sites (ie *Sxl*), or larger spacing between sites (ie *Cut*). It was hypothesised that combinations of these variables could be controlling the levels of gene expression via Sxl. In this study the decrease in females carrying any of these additional 3' UTRs was not any more significant than the decrease observed in females carrying autosomal insertions of the *Runt* 3' UTR (Fitzsimons *et al.*, 1999). It seems possible then that these variables are not alone in controlling the Sxl mediated dosage compensation pathway in females. Surprisingly the lines carrying the *Sxl* 3' UTR fragment, which contains nine Sxl poly (U) binding sites, showed β-galactosidase activity in one dose males to be 1.07 - 1.31 times higher than that of one dose females. This corresponds to only a 7 - 24 % down-regulation of *lacZ* in females. Moreover, two of the *Sxl* 3' UTR fragment lines (pRH24 #1 and pRH24 #3) did not show a significant decrease in females. These results suggest that it is not simply the number of predicted Sxl binding sites that determine the level of translational repression in females.

4.2.3 Sxl Binding Sites Required in Both 3' UTR and 5' UTRs?

One suggestion to explain the results obtained in this study, was that the genes undergoing Sxl mediated dosage compensation (MSL independent) in females required Sxl binding sites to be present in both the 3' UTR and the 5' UTR. This may explain why complete compensation of the *arm-lacZ* reporter system is not observed. Studies into the efficient repression of *Msl-2* expression in females requires Sxl binding sites in both the 3' and 5' UTRs (Bashaw and Baker, 1997; Kelley *et al.*, 1997). Of the 20 genes identified to contain three or more Sxl binding sites (Kelley *et al.*, 1995), only eight, including *Sxl* and *Cut*, were shown to contain Sxl binding sites in the 5' UTR.

4.2.4 Sxl Protein Binding of mRNA

Critical to the diverse regulatory activities of the Sxl protein is the recognition of substrate RNAs. The Sxl protein consists of 354 amino acids and contains two tandem RNA-recognition motif (RRM) domains, separated by a short flexible linker segment. These domains are responsible for the sequence-specific interactions with poly (U) runs in mRNAs (Reviewed in Hughson and Schedl, 1999). Sxl protein blocks *Msl-2* mRNA translation by binding to poly (U) tracts in both the 3' and 5' UTRs (Bashaw and Baker, 1997; Kelley *et al.*, 1997; Zhou *et al.*, 1995). Substrate recognition by the Sxl protein requires the co-ordinate activity of both RRM domains. Studies into the crystal structure of the Sxl protein have shown that the two RRM domains in the Sxl molecule do not touch one another (Crowder *et al.*, 1999). The linking region between the two RRM domains shows flexibility, suggesting that the RRM domains are flexibly tethered in solution and are not pre-organised to bind RNA (Crowder *et al.*, 1999). The finding that Sxl proteins interact with each other and subsequently stabilise binding of the target RNA may help explain why genes known to contain three or more Sxl binding sites in their mRNA show no similarities to each other in relation to the number of sites, length of the poly (U) stretches and distance between the sites. The interactive nature of the Sxl protein would mean that the protein-protein associations could help adjust between sites close together and further apart.

4.2.5 Other Protein Binding Sites in 5' UTR for Interaction with Sxl

Additional protein-protein interactions may also be important for Sxl regulatory functions. Two Sxl proteins associate with the closely spaced Sxl binding sites in the *tra* pre-mRNA (Reviewed by Hughson and Schedl, 1999) and it has been suggested that translational regulation of *Msl-2* might also involve interactions between Sxl proteins associated with the 3' and 5' UTRs (Bashaw and Baker, 1997; Kelley *et al.*, 1999). The Sxl protein is associated in a large complex containing Snf and other components of the splicing apparatus *in vivo* and can interact directly with Snf protein *in vitro* (Reviewed by Hughson and Schedl, 1999). An important aim for the future will be to identify the proteins that interact with Sxl and contribute to Sxl's wide range of regulatory activities.

This study generated a construct carrying an oligonucleotide linker, containing an 11(T) Sxl binding site, in the 5' UTR of *arm-lacZ* called pRH31. To generate pRH31 the oligonucleotide linker was inserted into pHF13 to obtain an *arm-lacZ* construct with Sxl

binding sites present in both the 3' and 5' UTRs. pHF13 has the 170 bp *Runt* 3' UTR fragment inserted into the 3' UTR of *arm-lacZ* (Fitzsimons *et al.*, 1999). β-galactosidase assays of flies carrying autosomal insertions of pRH31 showed enzyme activity levels in one dose males to be 1.46 - 1.49 times higher than one dose females (Table 9, section 3.2.2.4). This 32 - 33 % down-regulation of *lacZ* in one dose females (corresponding to 64 – 66 % of the decrease needed for a 2:1 ratio) was not significantly larger than that of lines carrying only the 3' UTR of *Runt*. pRH31 may not display a larger decrease in females because the Sxl protein binding the *Runt* 3' UTR does not recognise the Sxl binding site in the 5' UTR. Both *Sxl* and *Cut* normally have an additional Sxl binding site present in the 5' UTR, but the constructs generated in this study do not. *Small Forked* on the other hand is like *Runt*, it does not normally have a Sxl site in the 5' UTR, so perhaps it needs other protein binding sites to be present for Sxl mediated dosage compensation to occur at an optimal rate.

4.2.6 Conclusions and Future Work

Kelley *et al.*, (1995) proposed that some X-linked genes with three or more Sxl binding sites could be compensated by an MSL independent mechanism. This study extends previous work, that fragments containing Sxl binding sites can lead to a decrease of expression in females. It is important to remember that it is only a hypothesis that some genes are compensated by an MSL-independent mechanism. The only evidence is studies of *Runt* using non-quantitative assays. It may be that the Sxl mediated compensation of *Runt* is less than two-fold.

The results from this study show that Sxl can partially dosage compensate *arm-lacZ* in adult females when Sxl binding sites are present in the 3' UTR. Although compensation of *arm-lacZ* was observed in females, only 42 – 66 % (*Cut* and *Small Forked*) and 7 – 24 % (*Sxl*) of the hypothesised down-regulation occurred when 3' UTR fragments were inserted into the 3' UTR of *arm-lacZ*. Only 64 – 66 % of the hypothesised two-fold down regulation was observed in females when Sxl binding sites are present in both the 3' UTR and 5' UTRs.

It seemed logical to assume that the Sxl binding sites were the controlling factor in female down regulation, and that the number, length and distance between the sites were dictating the extent to which genes were being compensated. But, of the 20 genes

identified to contain three or more Sxl binding sites in their 3' UTRs, only eight also carry sites in their 5' UTR. We have shown that the presence of Sxl sites in the 3' and 5' UTRs does not increase the decrease in *lacZ* expression in females. This study used pHF13 (Fitzsimons *et al.*, 1999) which contains the 3' UTR of *Runt*. *Runt* is one of the genes found to not carry a Sxl binding site in the 5' UTR. When the Sxl protein binds the *Runt* 3' UTR in pRH31 it may be that the Sxl protein binds in such a way that it is not able to interact with a Sxl protein binding the 5' UTR. Because *Runt* does not normally carry Sxl binding sites in it's 5' UTR, the Sxl protein binding the 3' UTR sites may bind the mRNA in such a way that it is ready to interact with another type of protein. Because we inserted a synthetic oligonucleotide into the 5' UTR of pRH31 and not *Runt's* own 5' UTR, these alternative proteins have no site to bind and therefore no additional down-regulation was observed in females.

Future studies may need to look in further detail at the untranslated regions of the genes thought to undergo Sxl mediated dosage compensation (MSL independent) in females. Constructs may need to be generated that contain both the 3' and 5' UTRs of the genes. Constructs containing both the 3' and 5' UTRs of *Sxl, Small Forked, Cut* and *Runt* would provide an interesting comparison to results found in this study. If indeed the 5' UTR is required these constructs would show a more significant decrease in β-galactosidase activity in females. It would then be interesting to identify the proteins that bind the 5' UTR and study their interaction with Sxl.

4.3 ISOLATION OF X-LINKED DOSAGE COMPENSATION REGULATORY ELEMENTS

4.3.1 Are Genes Present within the X-linked Regions Studied?

Three constructs containing X-linked DNA fragments of sizes 5 kb, 8 kb and 17 kb cloned into pHF11 were generated and transformants from two of the constructs were obtained via P-element mediated transformation. Results from β-galactosidase assays performed on one dose males and females, from both transgenic lines, showed a 1:1 male to female ratio. This result indicates that the fragments used in this study do not contain DCREs.

There are several hypotheses as to why no DCREs were isolated. The first is that the regions of X-linked DNA that were chosen for this study either do not contain any genes or the genes within the fragments do not contain any DCREs. This study focussed upon the 1C region of the X chromosome, which has been shown recently to contain one of the 30 - 40 'high affinity' sites for MSL protein binding (Lyman *et al.*, 1997). It is highly possible that the fragments taken from this region (totalling ~30 kb) do not contain the DCRE or MSL 'high affinity' site as the 1C region of the X chromosome is approximately 100 kb.

The MSL 'high affinity' sites are thought to be the initial point of contact for the MSL protein complex through the binding of MSL-1and MSL-2 as an independent complex. Once the MSL-1/MSL-2 core complex has bound to the 30 - 40 entry sites, MSL-3, MLE, MOF and perhaps one *roX* RNA interact together to complete the MSL complex. This full complex then spreads along the male X chromosome and binds other sequences which may correspond to the hundreds of bands found during immunoprecipitation experiments (Kelley *et al.*, 1999). Kelley *et al.*, (1999) found that the *roX1* gene provides a nucleation site for extensive spreading of the MSL complex into flanking chromatin, even when inserted into an autosome.

4.3.2 What is the Nature of DCREs?

It is highly probable that the DCREs correspond to the 30 - 40 'high affinity' sites. The presence of these DCREs as the MSL entry sites does not explain why X-linked genes such as *White* and *6-Pgd* show partial compensation when translocated to an autosome.

This partial compensation may be due to regions within the gene facilitating compensation. There may be sequences present that allow for the spreading of the MSL complex. The fact that these sequences have not been identified to date suggests that they are short oligonucleotide sequences that are not easily identifiable. These oligonucleotide sequences may be long stretches of mono- and dinucleotide repeats, ie $(dC\text{-}dA)_n.(dT\text{-}dG)_n$ (Lowenhaupt *et al.*, 1989; Pardue *et al.*, 1987). Pardue *et al.*, (1987) reported that the long CA/TG repeats are distinctively distributed throughout the euchromatic chromatin of both the X chromosome and the 2nd and 3rd chromosomes, but not the heterochromatin. One striking feature of the hybridisation pattern was the high concentration of the CA/TG sequences on the X chromosome. In fact the X chromosome appeared the have at least twice as many long CA/TG repeats as any autosome. They comment that this distribution correlates with the overall activity of the chromosomal regions. The repeats are found throughout the euchromatin, and are twice as prevalent in chromosomes that are dosage compensated.

Translocation of X-linked transgenes or fragments to autosomal sites can lead to the spreading of the MSL complex along the autosomal chromatin. This presents the idea that the MSL complex may be attracted to sequences that are common to active genes rather than specifically to X-linked genes. The finding that both the X chromosome and autosomes carry long dinucleotide repeats (Lowenhaupt *et al.*, 1989; Pardue *et al.*, 1987) supports the possibility of there being a common sequence for active genes. Following this idea, the X-linked genes are dosage compensated because the X chromosome carries the MSL 'high affinity' sites identified by Lyman *et al.*, (1997).

4.3.3 Conclusions and Future Work

Three constructs containing X-linked DNA, inserted into the insulated *arm-lacZ* reporter system, were generated and two produced transformant flies after micro-injection into *D. melanogaster* embryos. β-galactosidase assays of the transgenic lines showed no increase in *lacZ* expression in males (Table 10, section 3.3.1). This suggests that no DCREs were isolated that could confer dosage compensation onto *arm-lacZ*. This study focussed on the 1C region of the X chromosome, identified as one of the 'high affinity' sites for MSL protein complex binding (Lyman *et al.*, 1997). Unfortunately only two transgenic lines were generated, spanning approximately 13 kb from a very small area of the overall region. This region has high affinity for the MSL

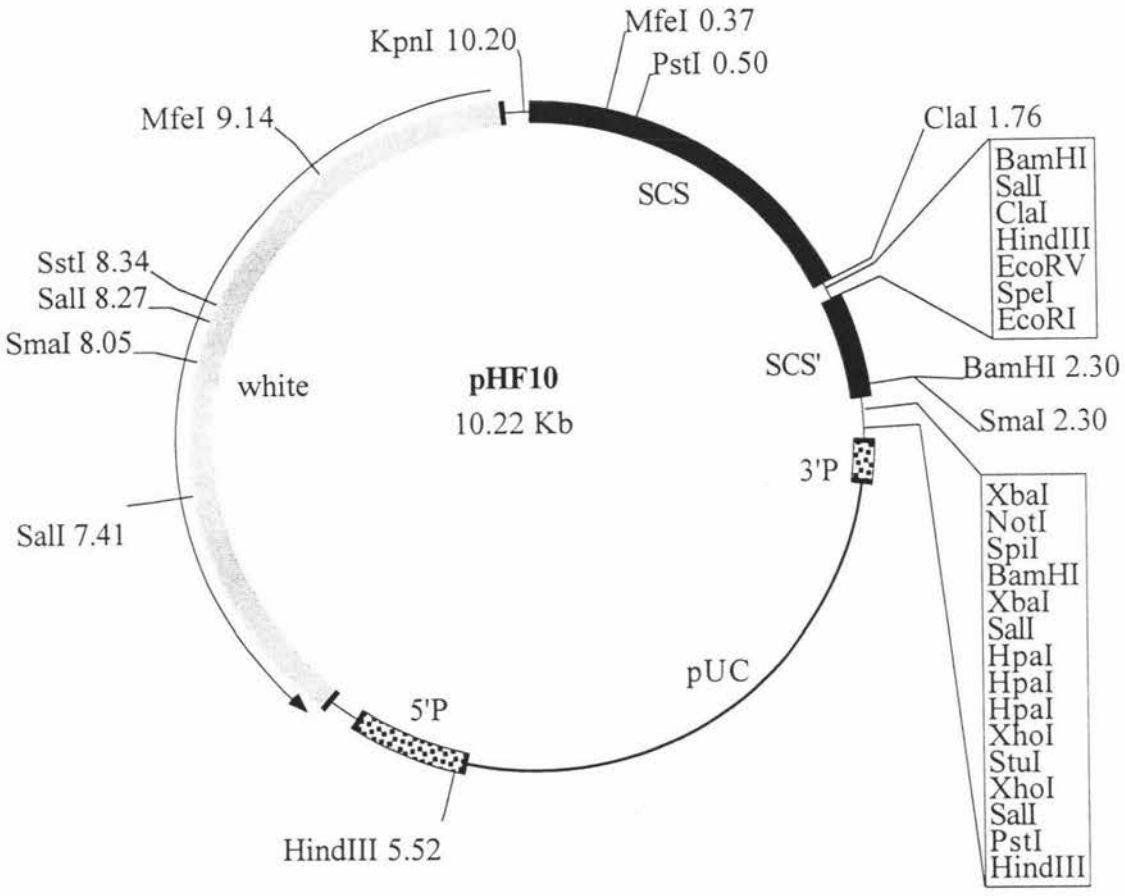
complex (Lyman *et al.*, 1997) so future work would benefit from generating more transgenic lines from other areas within this region.

The identification of DCRE sequences will continue to prove difficult, due to the probability that the sequences may vary considerably. But if studies focus upon the regions known to display high affinity, the search will eventually be narrowed down. The *arm-lacZ* reporter assay system works well for the identification of genes involved in dosage compensation that do not generate an RNA or protein gene product (ie *roX1* and *roX2* cDNA sequences, *roX1* 3' and DNAseI fragments). This system would allow for the fragmentation of X chromosomal regions without the requirement for detection of gene products, and thereby facilitating the narrowing down of sequences of importance for dosage compensation. Identification of sequences within the X specific high affinity sites (ie *roX1* and *roX2* sequences) would only require the insertion of fragments into the reporter system. However the identification of the specific sequences thought to be bound by the spreading MSL complex (Kelley *et al.*, 1999) may require the presence of a *roX* DNA sequence in conjunction with the X-linked DNA being investigated.

5.0 APPENDICES

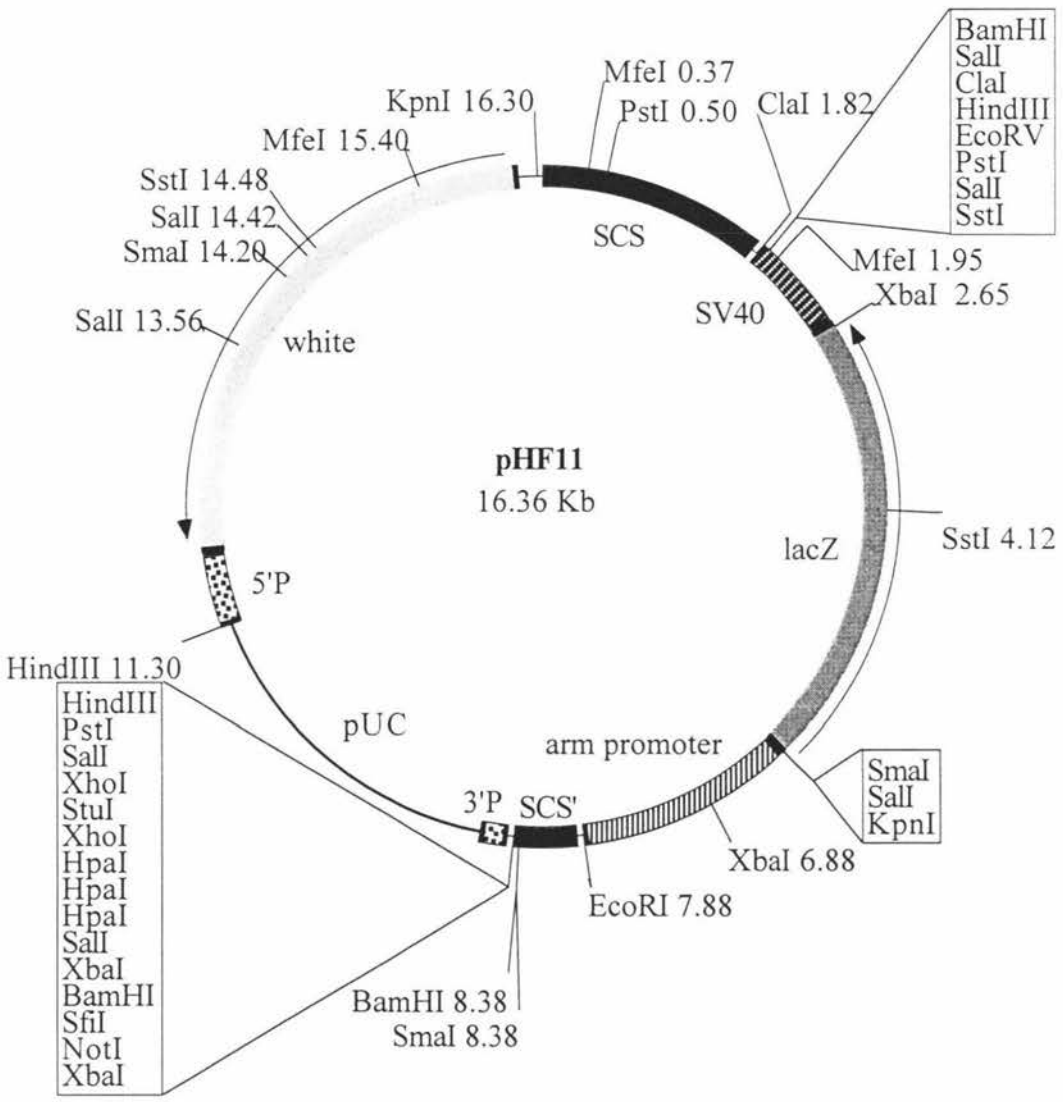
APPENDIX 1. Physical Maps of Plasmids

Plasmids included in this appendix include the most important cloning vectors and the plasmids microinjected during the course of this study.



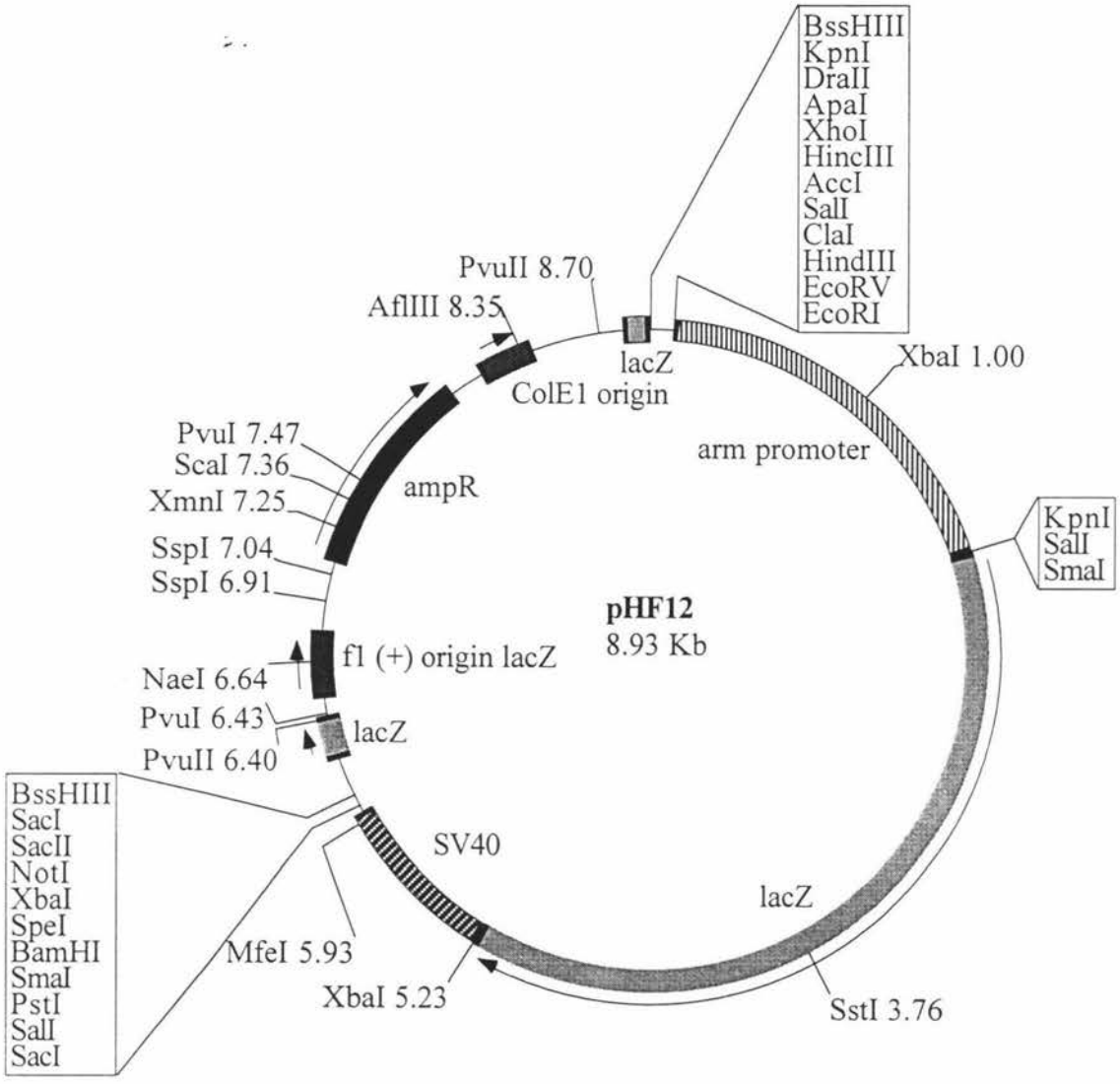
Physical Map of pHF10

10.32 kb, pCaSpeR4 with *EcoRI* and *SpeI* sites removed, containing a 2.4 kb *KpnI/NotI* SCS-SCS' fragment from Elba6 and a *EcoRI-SpeI* linker. pHF10 was used to generate pRH23, pRH24, pRH25, pRH26, pRH27, pRH28, pRH31, and pRH32.



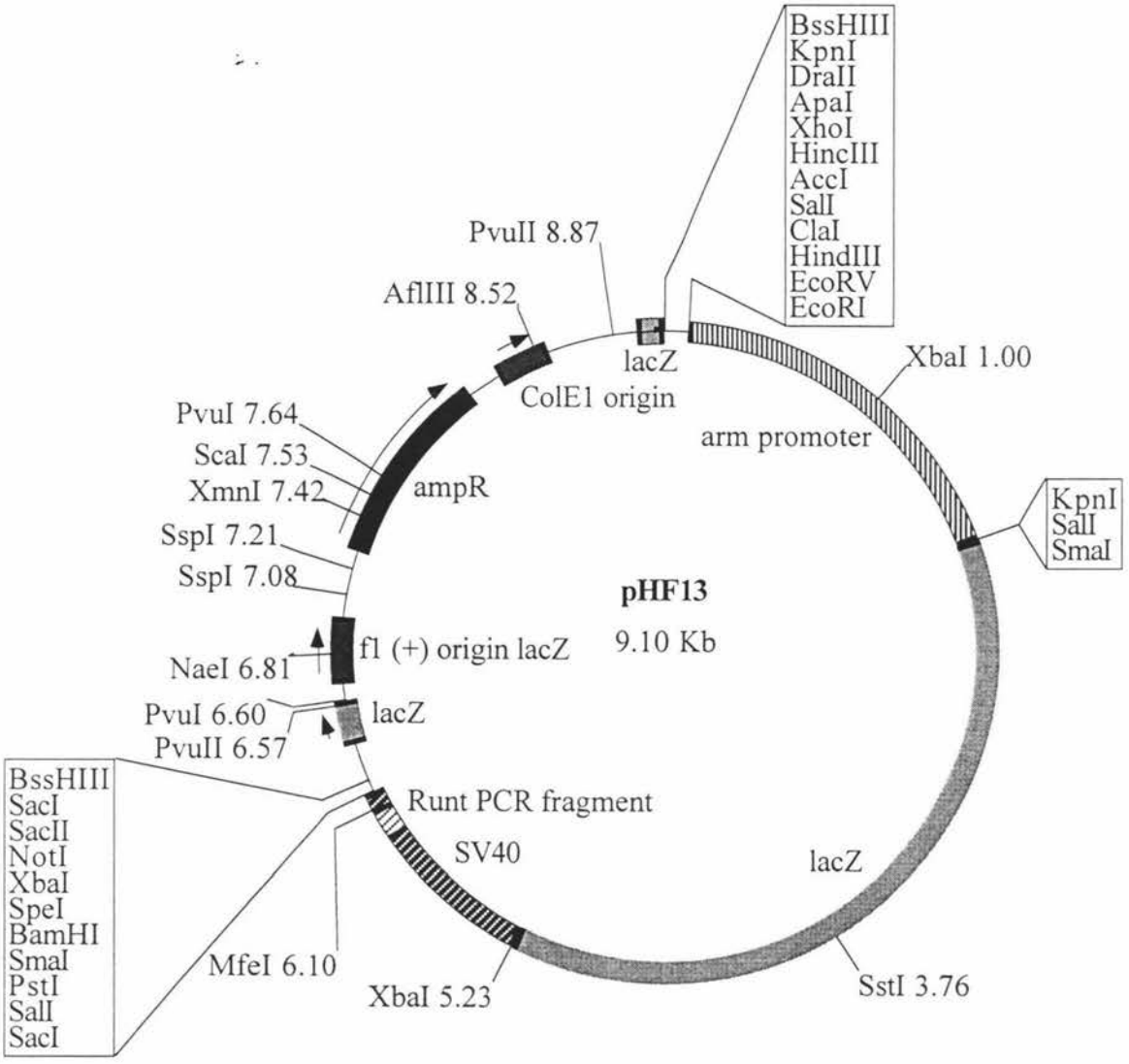
Physical Map of pHF11

16.36 kb, pHF10 containing a 6.2 kb *EcoRI/HindIII arm-lacZ* fragment in *EcoRI/SpeI* sites. pHF11 was used to generate pRH02, pRH03, pRH04, pRH06, pRH11, pRH12, p34F3 5.0, p34F3 8.0, and p34F3 17.0.



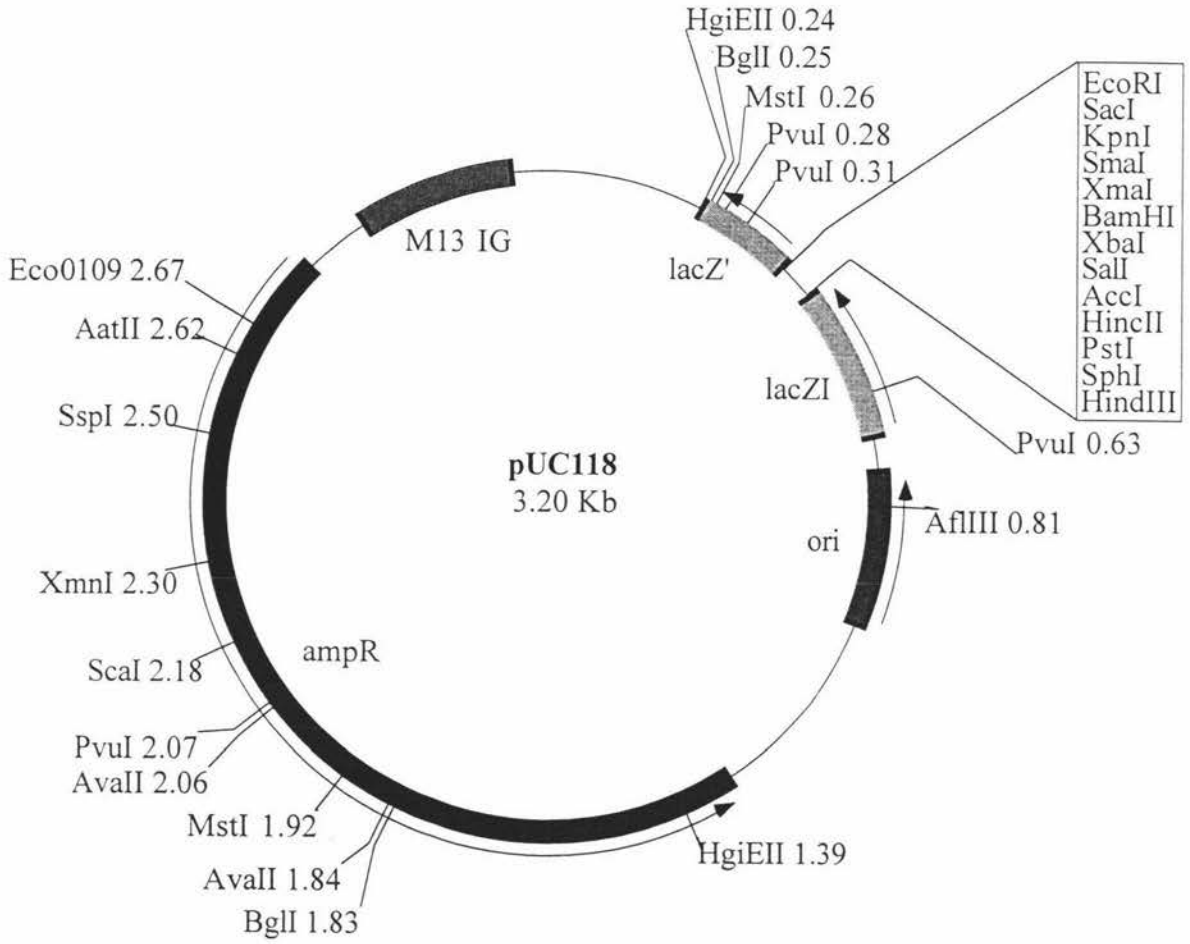
Physical Map of pHF12

8.93 kb, pBSII KS- containing a 6.2 kb *PstI/EcoRI arm-lacZ* fragment. pHF12 was used to generate pRH17, pRH18, pRH19, pRH20, pRH21, and pRH22.



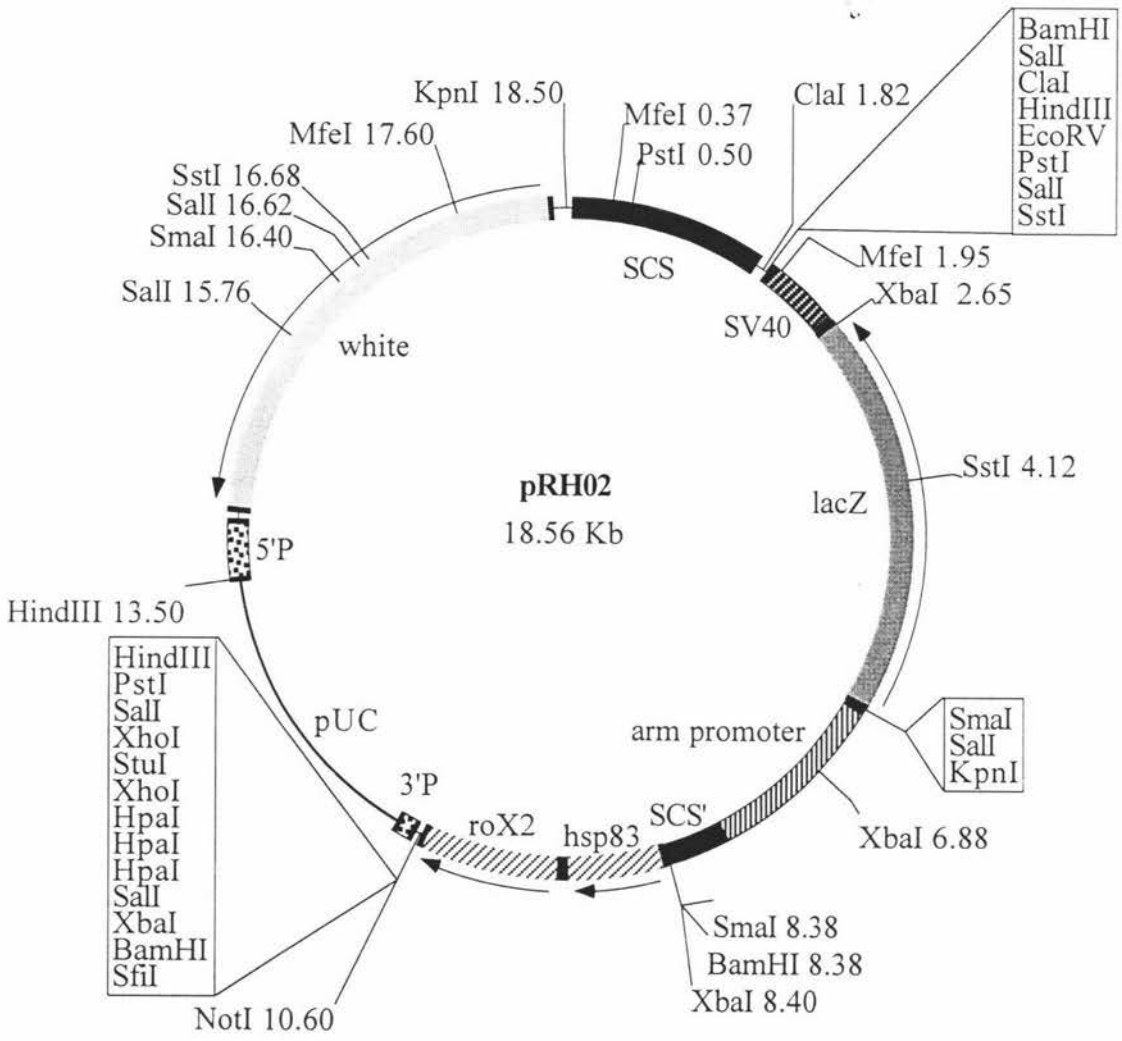
Physical Map of pHF13

9.10 kb, pBSII KS- containing a 6.2 kb *PstI/EcoRI arm-lacZ* fragment with a 170 bp *runt* PCR product (Sxl orientation) in the *MfeI* site of *arm-lacZ*. pHF13 was used to generate pRH29, pRH30, pRH31, pRH32, and pRH33.



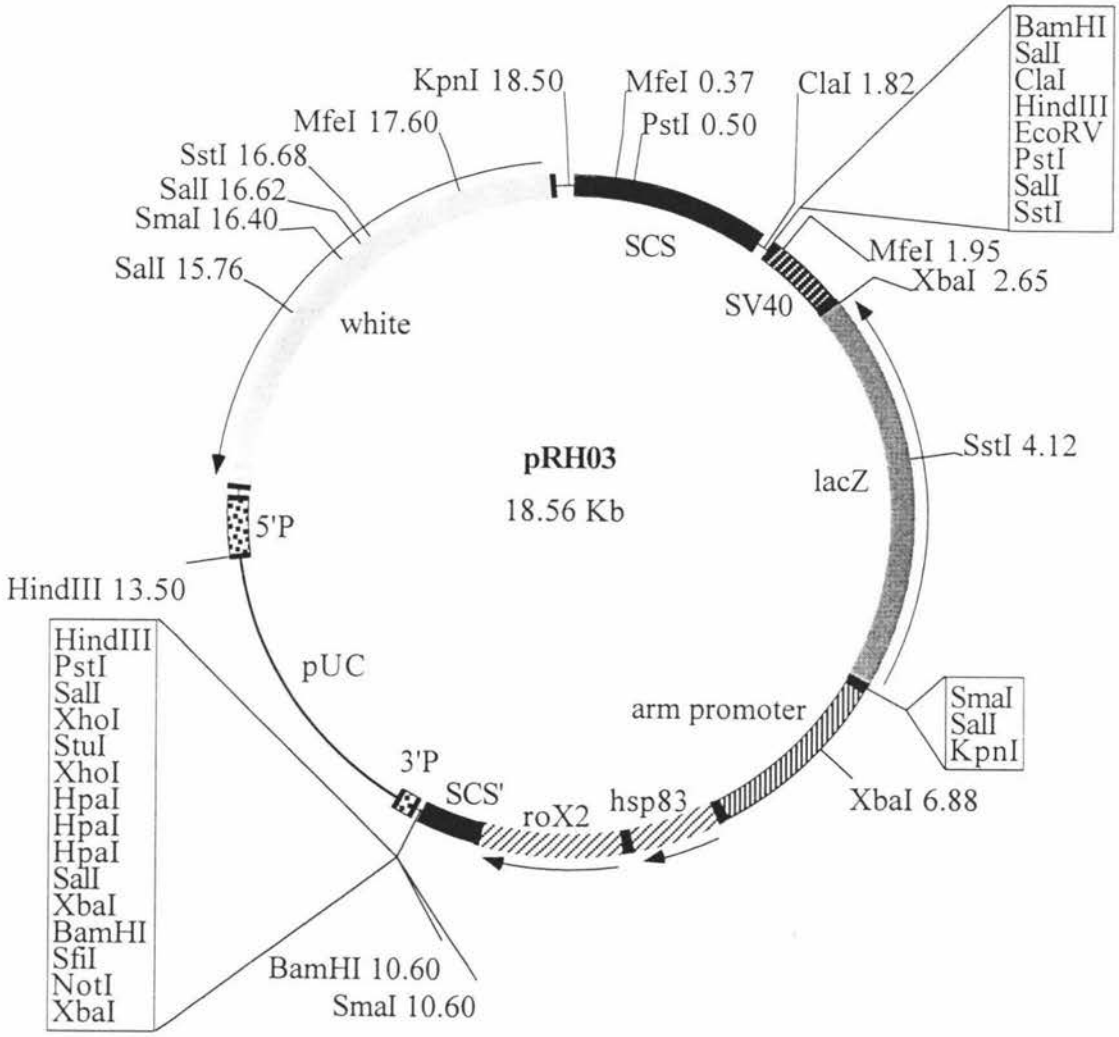
Physical Map of pUC118

3.2 kb, cloning vector with an *EcoRI* site downstream of P_{lac}. pUC118 was used to generate pRH29, pRH30, and pRH33.



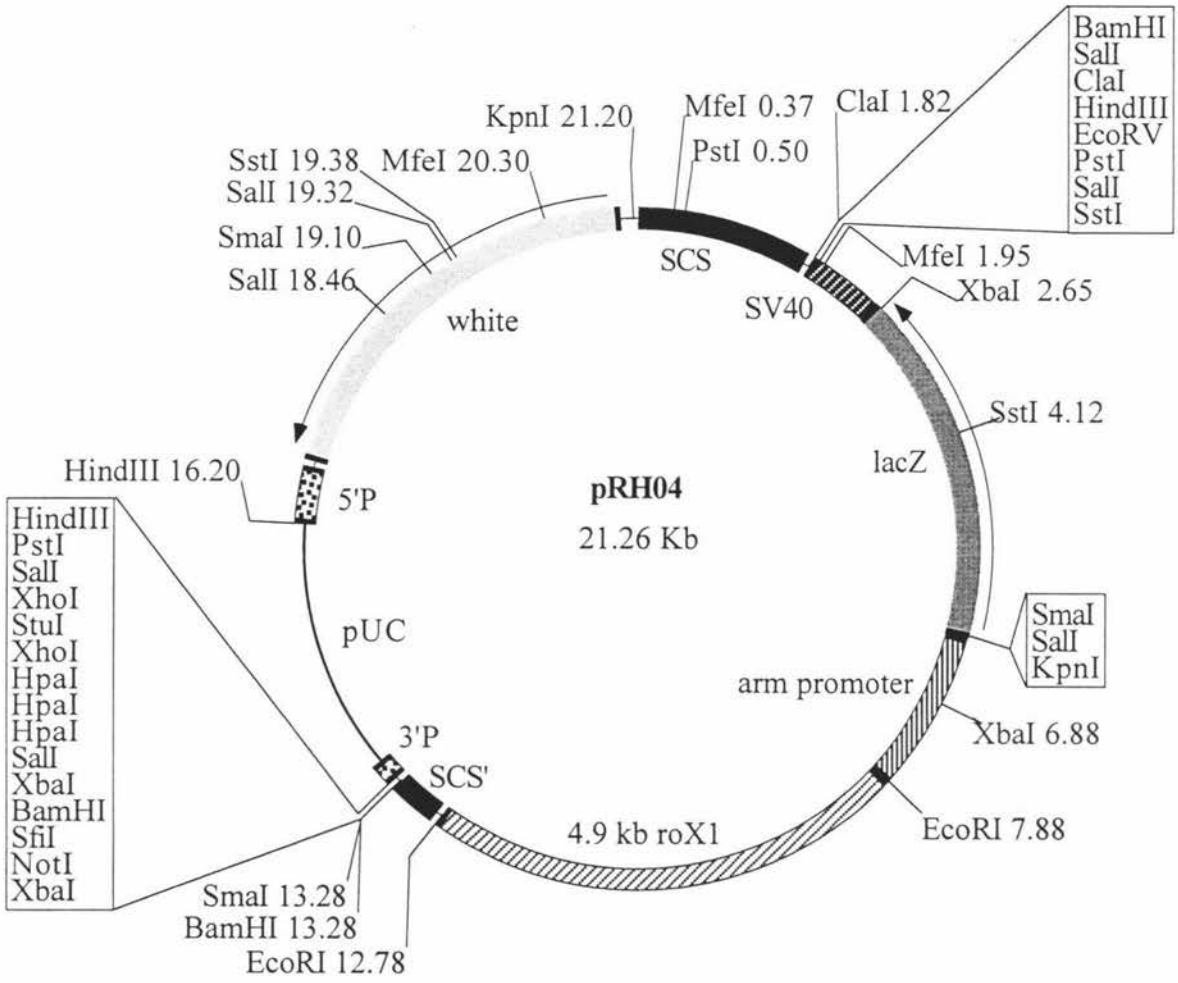
Physical Map of pRH02

18.56 kb, pHF11 containing the 2.0 kb *NotI/PspOMI* (*ApaI*) *hsp83-roX2* fragment, from pRH01, inserted into the *NotI* site present in the polylinker found upstream of the SCS' insulator element.



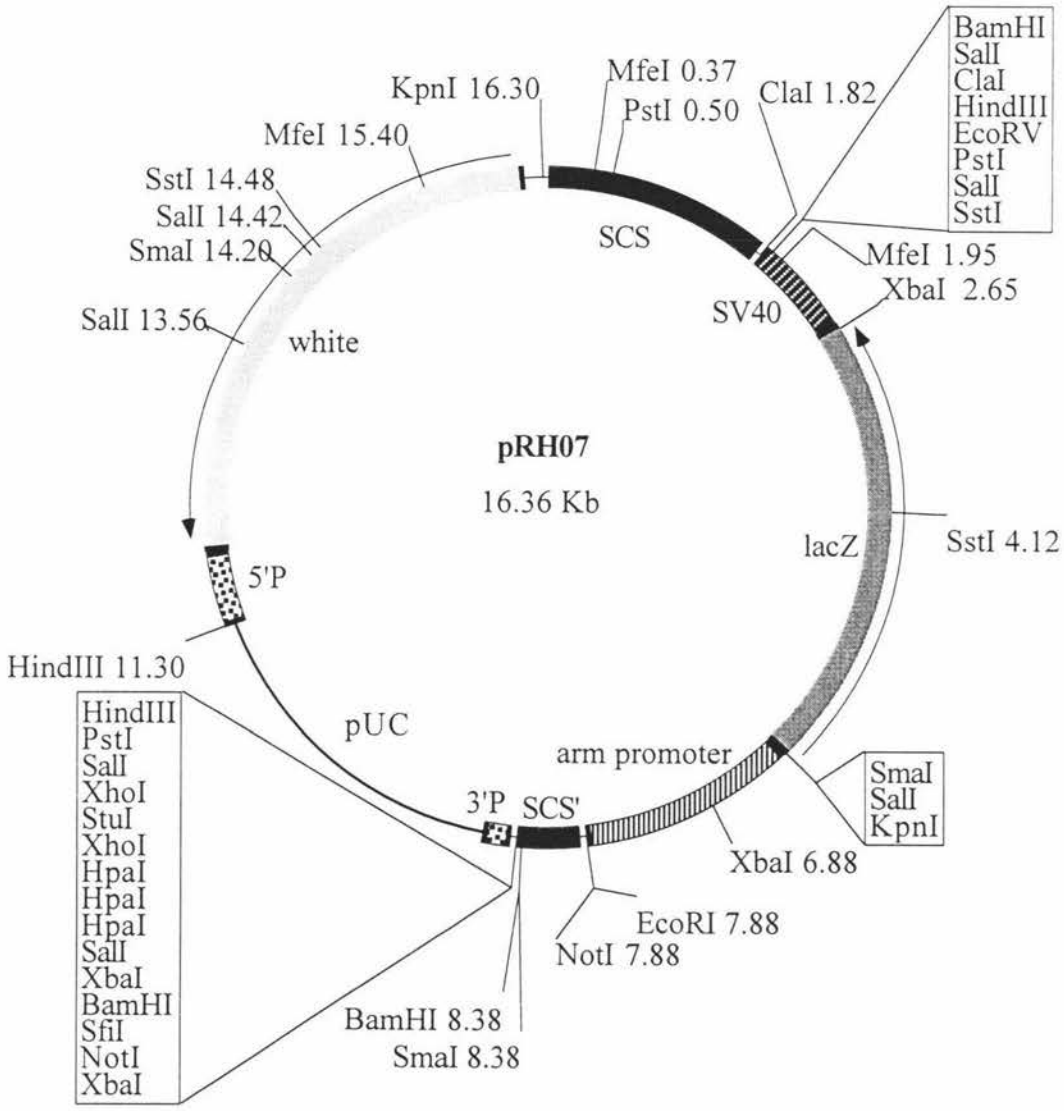
Physical Map of pRH03

18.56 kb, pHF11 containing the 2.0 kb *NotI/PspOMI (ApaI) hsp83-roX2* fragment, from pRH01, inserted into the *EcoRI* site of *arm-lacZ,* within the SCS/SCS' element insulated region.



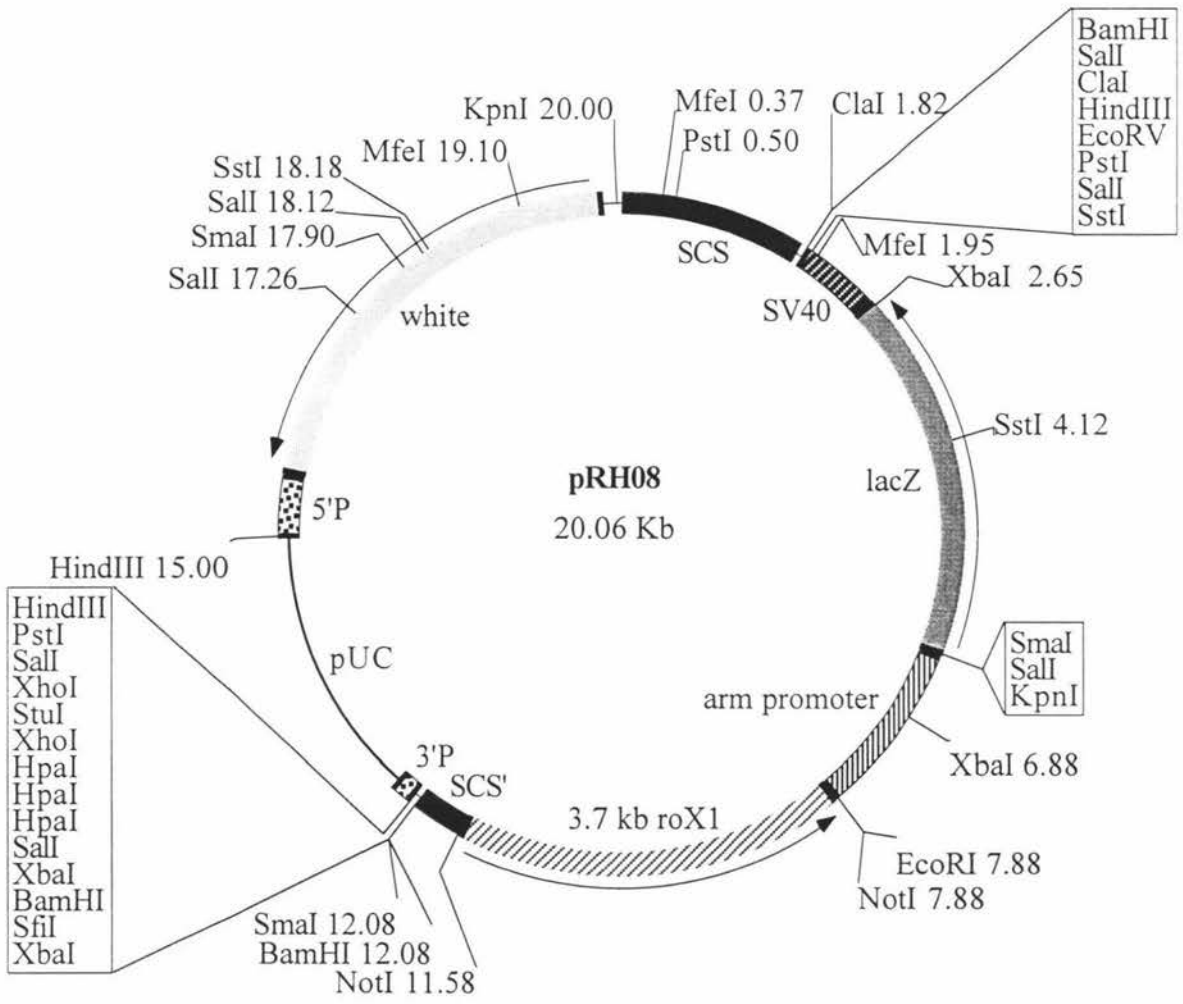
Physical Map of pRH04

21.26 kb, pHF11 containing the 4.9 kb genomic DNA *roX1 EcoRI* fragment, from pGMroX5, inserted into the *EcoRI* site of *arm-lacZ*.



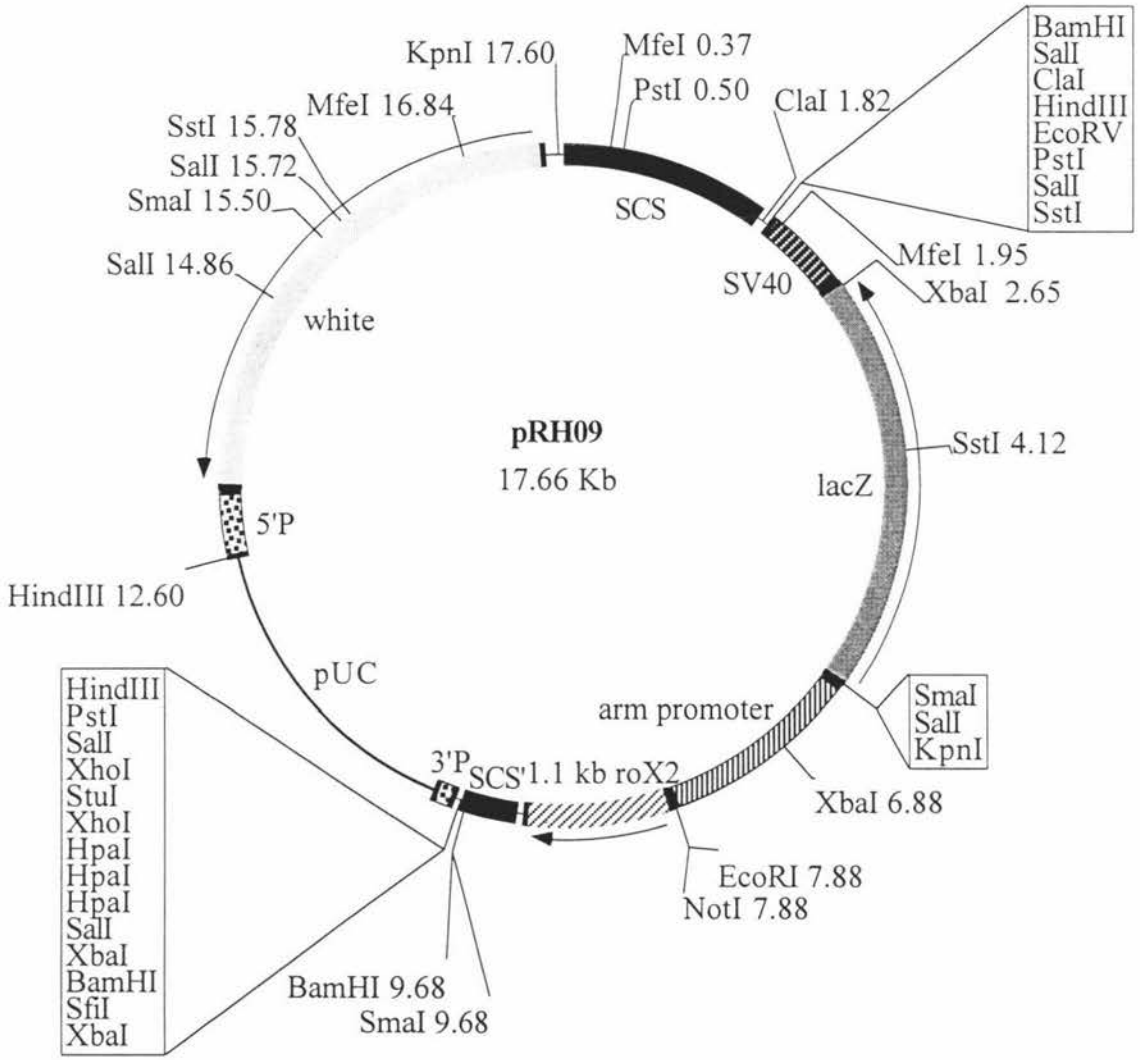
Physical Map of pRH07

16.36 kb, pRH06 with the *NotI* linker inserted into the *EcoRI* site of *arm-lacZ*, within the SCS/SCS' insulated region



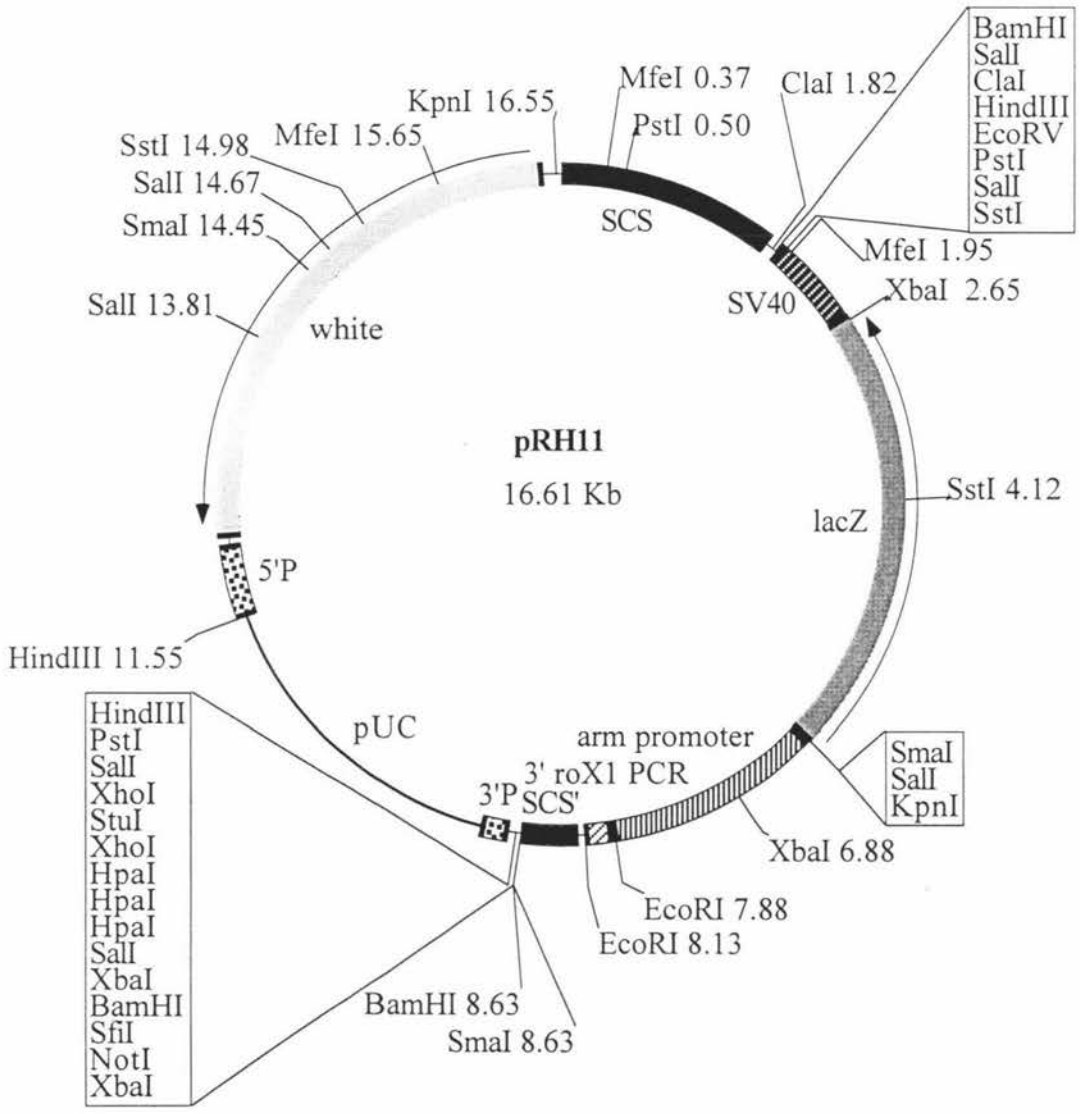
Physical Map of pRH08

20.06 kb, pRH07 with the 3.7 kb *NotI* (10.A2) roX1 cDNA fragment, from pRh05, inserted into the *NotI* site in *arm-lacZ*.



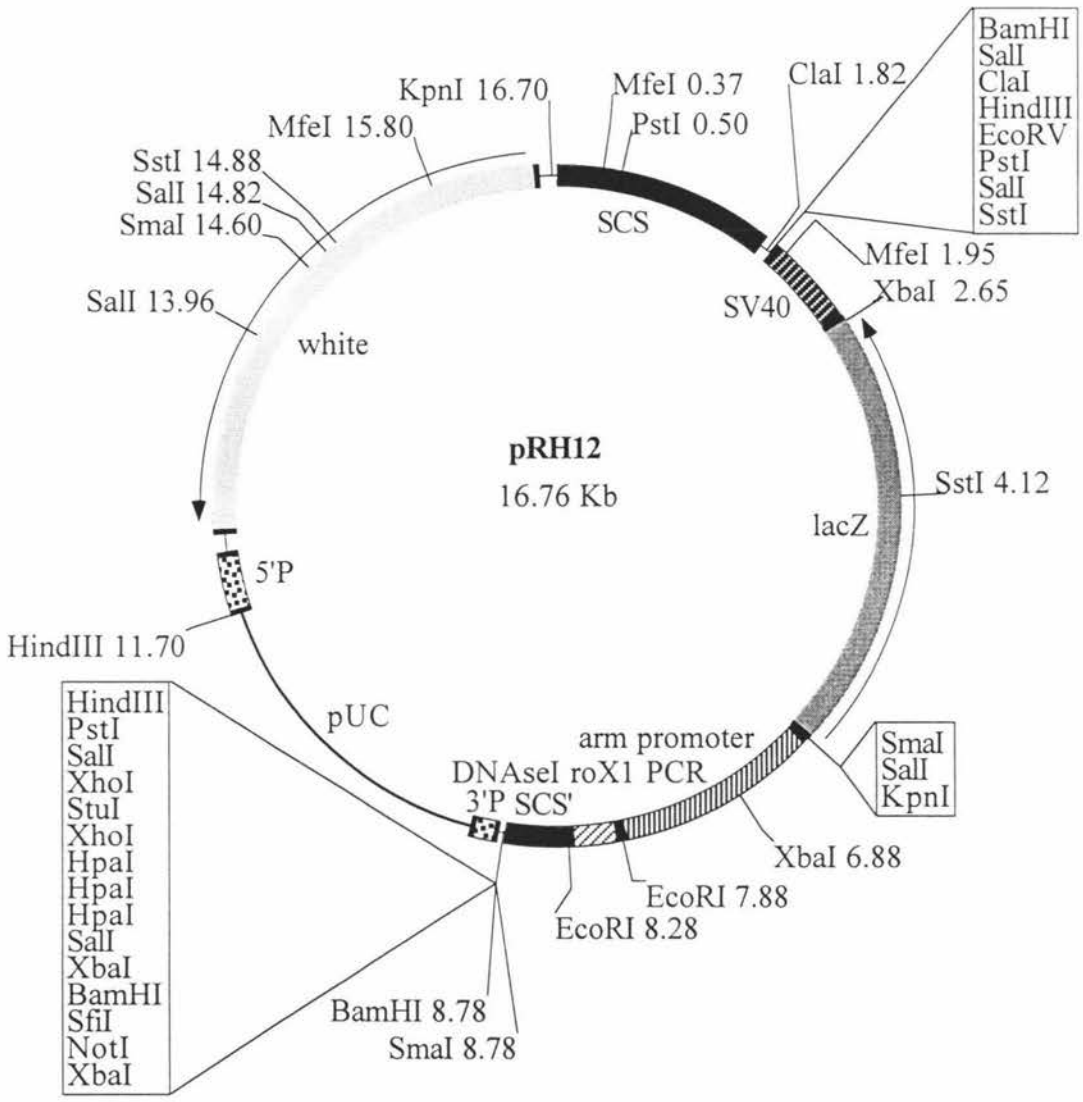
Physical Map of pRH09

17.66 kb, pRH07 with the 1.1 kb *NotI/PspOMI* (73.13) *roX2* cDNA fragment, from *roX2* in pBS SK-, inserted into the *NotI* linker site in *arm-lacZ.*



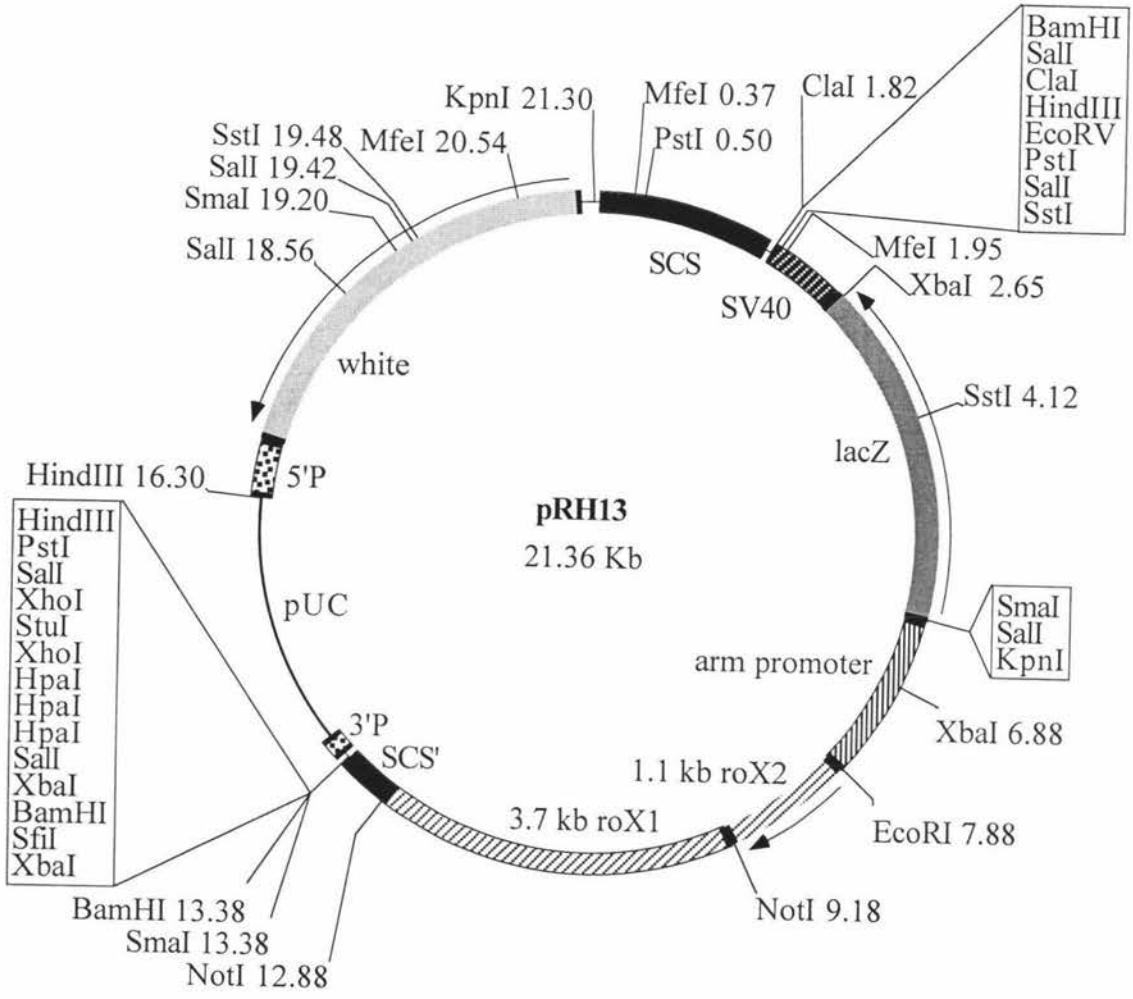
Physical Map of pRH11

16.61 kb, pHF11 with the 250 bp 3' *roX1* PCR fragment (similar sequence to *roX2*), from the *roX1* cDNA, inserted into the *EcoRI* site of *arm-lacZ*.



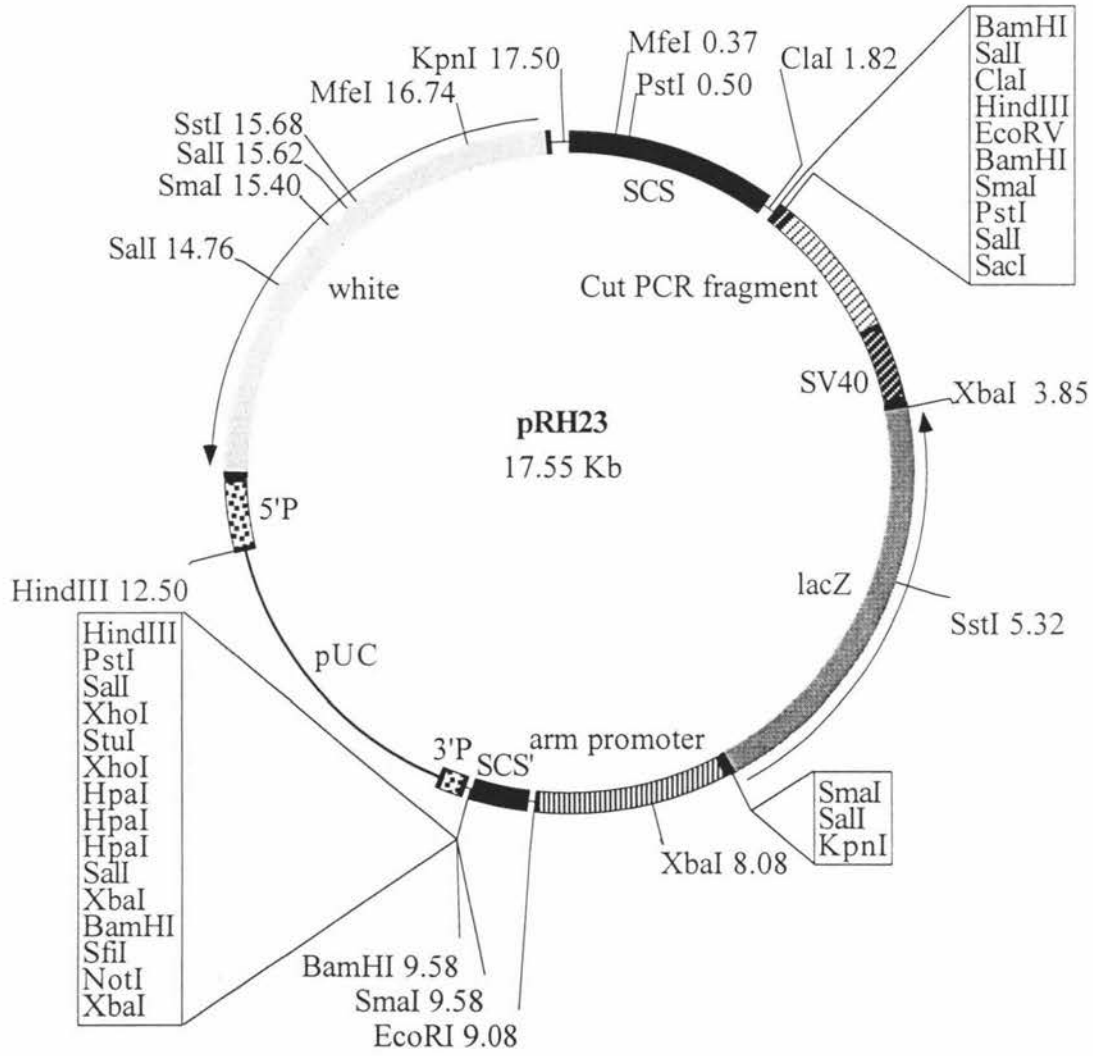
Physical Map of pRH12

16.76 kb, pHF11 with the 410 bp DNAseI roX1 PCR fragment (DNAseI hypersensitivity site), from the *roX1* cDNA, inserted into the *EcoRI* site of *arm-lacZ.*



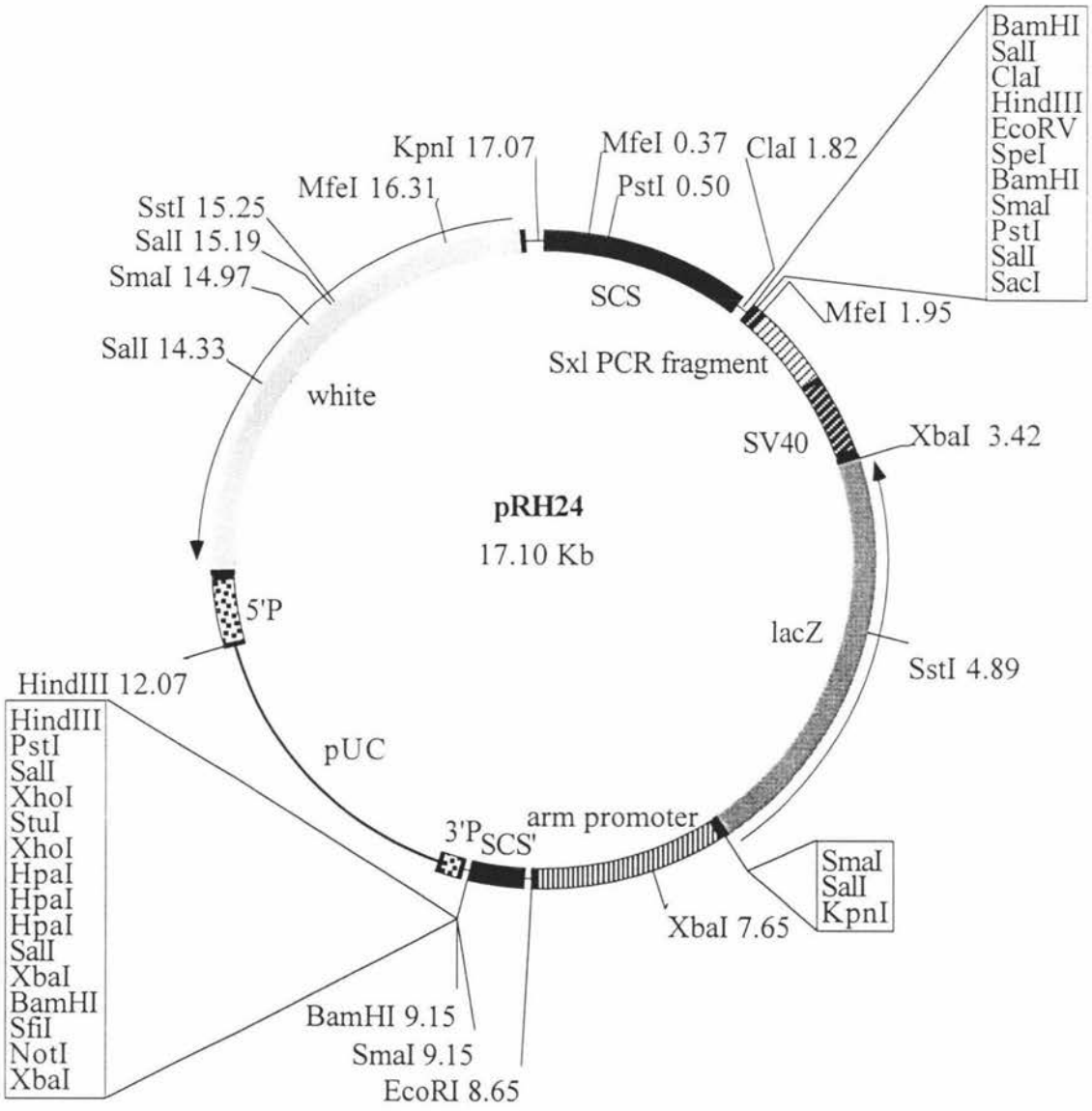
Physical Map of pRH13

21.36 kb, pHF09 with the 3.7 kb *roX1 NotI* cDNA fragment, from pRH05, inserted into the *NotI* site of *arm-lacZ* (reconstituted by *roX2* insertion into the *NotI* linker in pRH07).



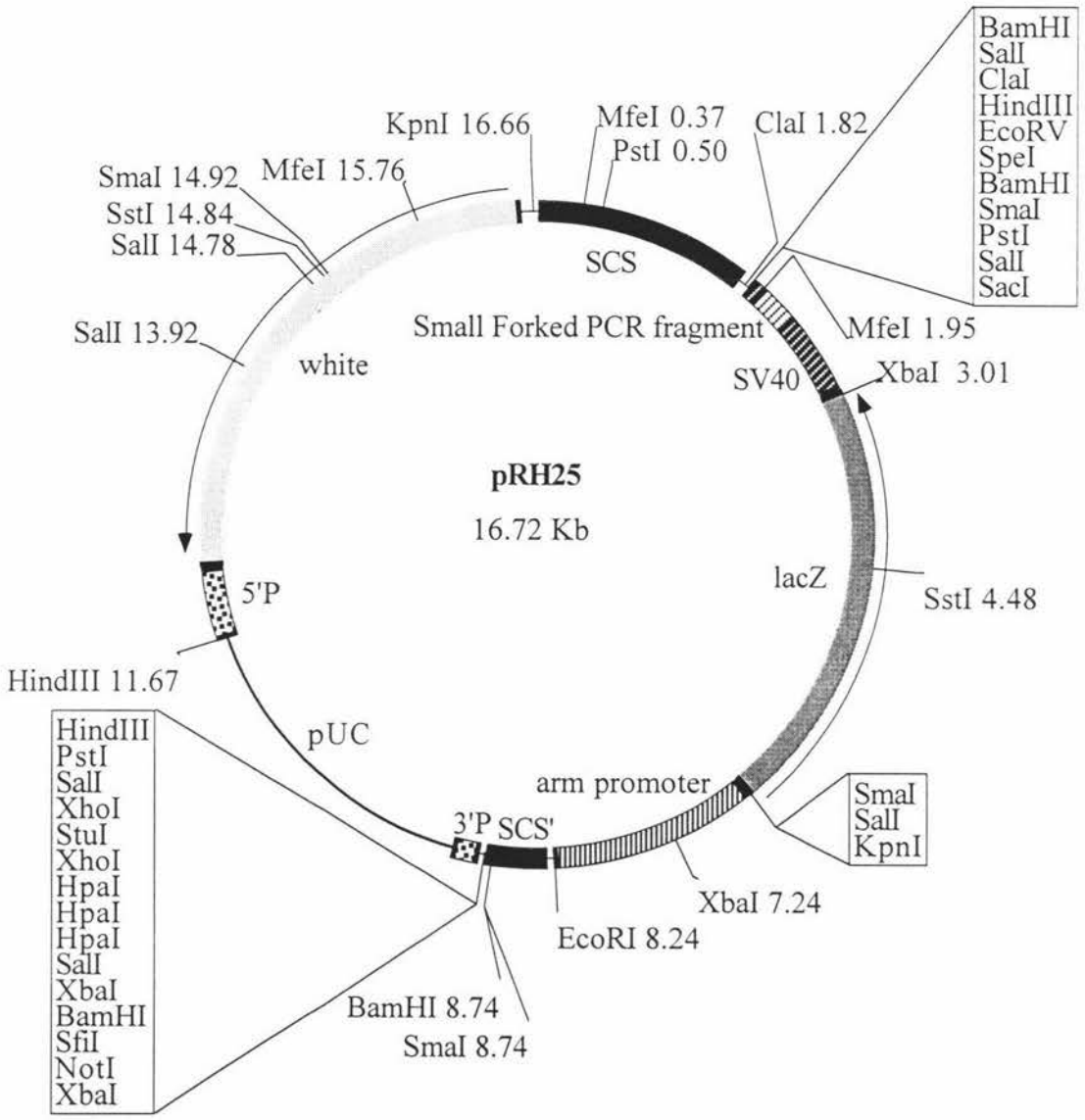
Physical Map of pRH23/pRH26

17.55 kb, pHF10 containing the 7.33 kb *SpeI/EcoRI* (*arm-lacZ* + *Cut* PCR), from pRH17 (poly T orientation) to generate **pRH23**, and pRH20 (poly A orientation) to generate **pRH26**.



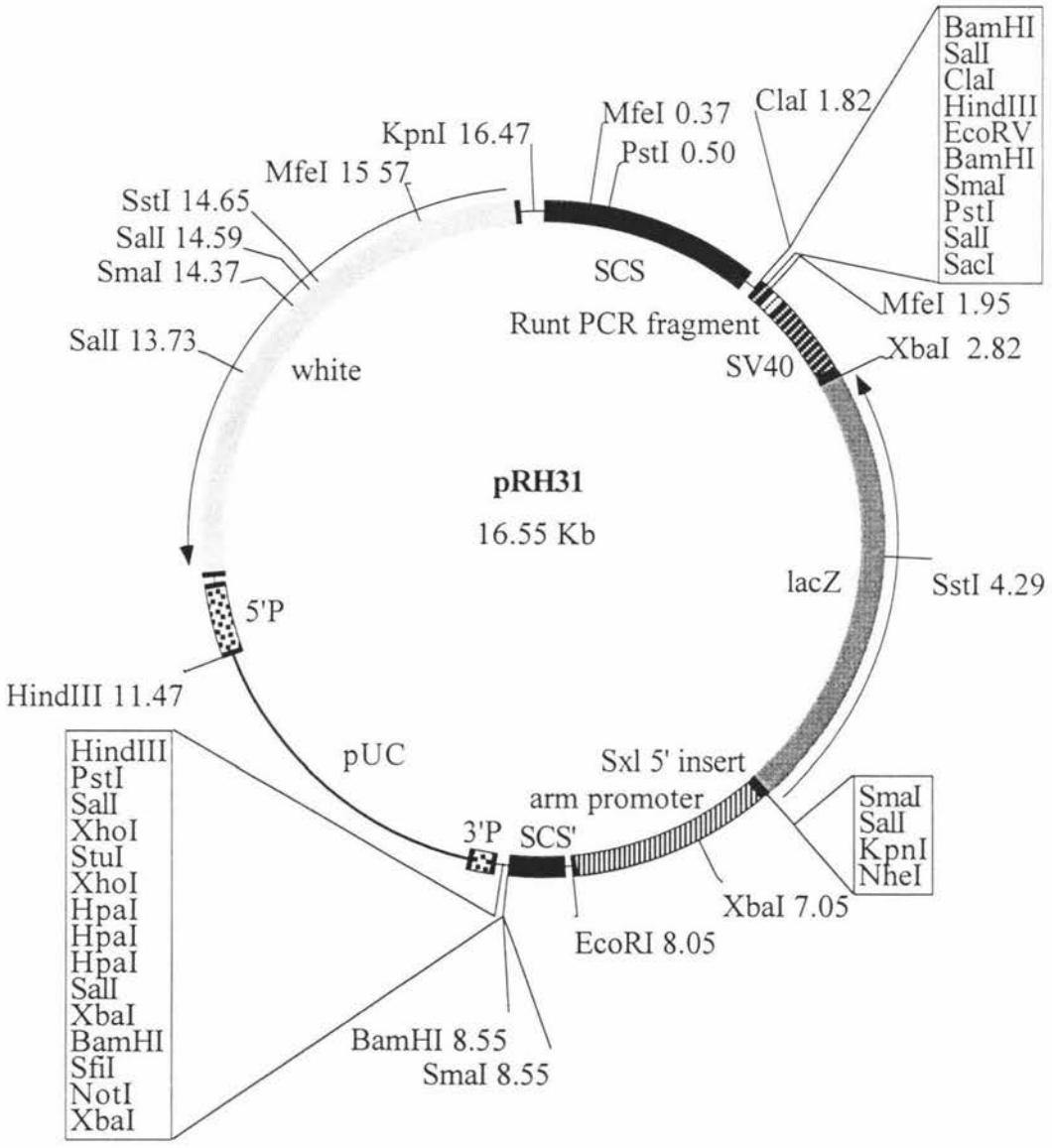
Physical Map of pRH24/pRH27

17.10 kb, pHF10 containing the 7.33 kb *SpeI/EcoRI* (*arm-lacZ* + *Cut* PCR), from pRH17 (poly T orientation) to generate **pRH24**, and pRH20 (poly A orientation) to generate **pRH27**.



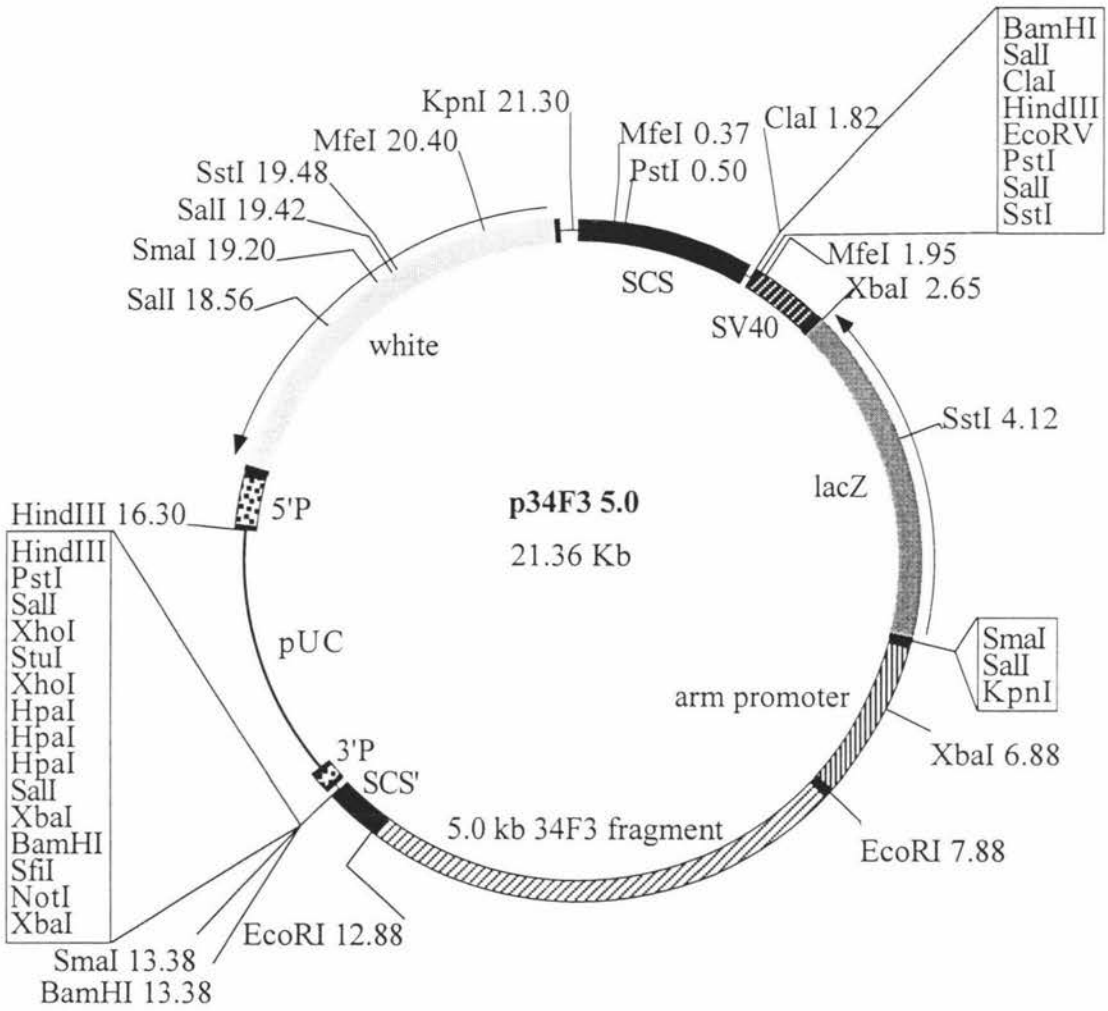
Physical Map of pRH25/pRH28

16.72 kb, pHF10 containing the 6.47 kb *SpeI/EcoRI* (*arm-lacZ* + *Small Forked* PCR), from pRH19 (poly T orientation) to generate **pRH25**, and pRH22 (poly A orientation) to generate **pRH28**.



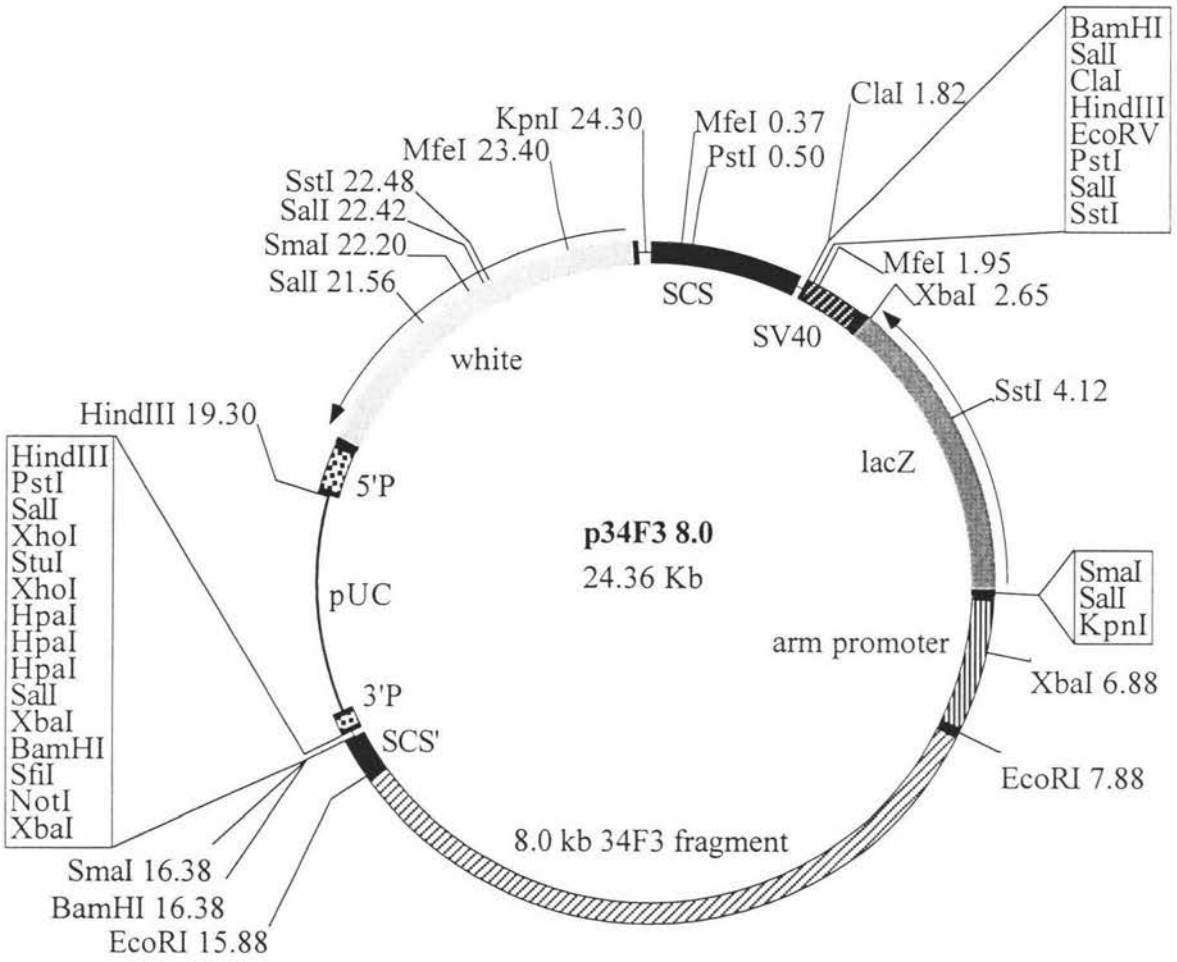
Physical Map of pRH31/pRH32

16.55 kb, pHF10 containing 6.3 kb *arm-lacZ* fragment, from pRH29 (poly T orientation) to generate **pRH31**, and pRH30 (poly A orientation) to generate **pRH32**, inserted into the *SpeI/EcoRI* sites within the SCS/SCS' insulated region.



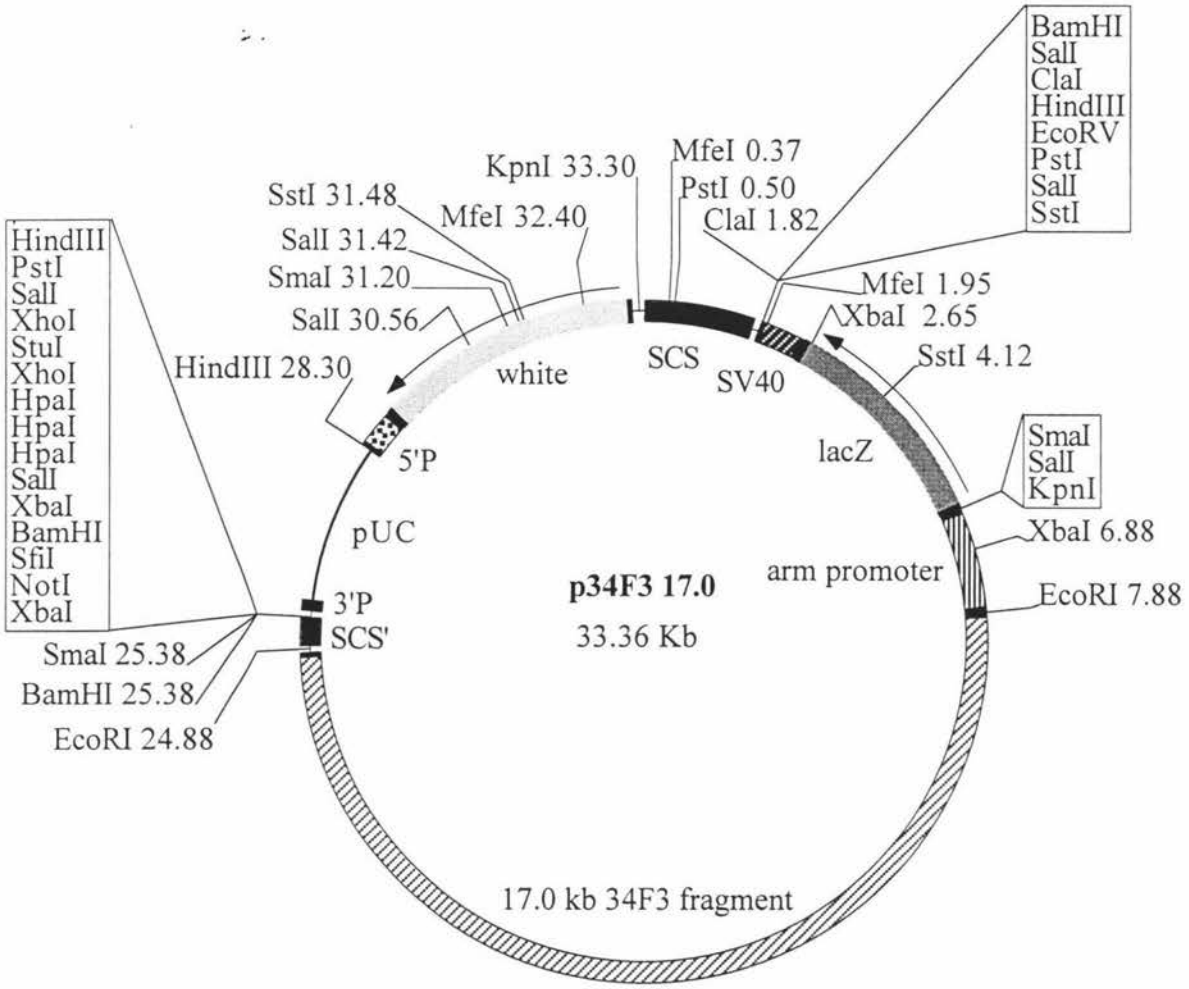
Physical Map of p34F3 5.0

21.36 kb, pHF11 containing the 5.0 kb *EcoRI* fragment from cosmid 34F3 inserted into the *EcoRI* site of *arm-lacZ*.



Physical Map of p34F3 8.0

24.36 kb, pHF11 containing the 8.0 kb *EcoRI* fragment from cosmid 34F3 inserted into the *EcoRI* site of *arm-lacZ*.



Physical Map of p34F3 17.0

33.36 kb, pHF11 containing the 17.0 kb *EcoRI* fragment from cosmid 34F3 inserted into the *EcoRI* site of *arm-lacZ*.

APPENDIX 2. β-Galactosidase Activities of Transgenic Flies

β–Galactosidase activity was expressed as Δabsorbance(abs)/min/μg of protein x 10^{-4} or Δabs/min/mg wet weight x 10^{-2}. The male to female ratio (M/F) and the average β-galactosidase activity was calculated for each line.

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH02 #1	1	M	7.011	1.29	3.567	1.37
		F	5.417		2.606	
		M	6.296	1.32	3.183	1.42
		F	4.752		2.245	
		M	7.965	1.46	4.776	1.73
		F	5.471		2.759	
		M	7.471	1.28	3.968	1.52
		F	5.84		2.608	
Average Activity		M	7.186		3.874	
		F	5.438		2.555	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH02 #2	1	M	5.000	1.63	3.906	1.76
		F	3.070		2.216	
		M	4.729	1.52	3.431	1.75
		F	3.118		1.960	
		M	3.274	1.59	2.576	1.47
		F	2.057		1.756	
		M	4.538	1.65	3.551	1.79
		F	2.756		1.987	
Average Activity		M	5.570		3.366	
		F	2.750		1.980	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH02 #2	2	M	7.350	1.51	6.178	1.76
		F	4.872		3.520	
		M	6.475	1.35	5.784	1.60
		F	4.803		3.614	
		M	7.132	1.52	6.032	1.63
		F	4.682		3.712	
		M	6.586	1.44	6.178	1.66
		F	4.558		3.732	
		M	6.929	1.36	6.075	1.59
		F	5.104		3.816	
Average Activity		M	6.894		6.049	
		F	4.504		3.679	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH03 #1	1	M	3.174	1.60	2.507	2.09
		F	1.980		1.198	
		M	3.926	1.67	2.310	1.65
		F	2.344		1.404	
		M	2.790	1.72	2.449	2.02
		F	1.622		1.211	
		M	2.763	1.76	2.058	1.66
		F	1.574		1.239	
		M	2.657	1.49	2.216	1.69
		F	1.781		1.311	
Average Activity		M	3.061		2.308	
		F	1.860		1.273	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH03 #1	2	M	4.530	1.48	3.664	1.52
		F	3.063		2.418	
		M	4.684	1.54	3.648	1.52
		F	3.048		2.399	
		M	4.724	1.70	4.252	1.86
		F	2.784		2.288	
		M	5.008	1.62	4.210	1.74
		F	3.082		2.414	
		M	4.829	1.68	4.057	1.76
		F	2.882		2.299	
Average Activity		M	4.755		3.966	
		F	2.972		2.364	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH03 #2	1	M	4.184	1.20	3.340	1.28
		F	3.476		2.602	
		M	5.240	1.76	3.535	1.68
		F	2.980		2.110	
		M	3.794	1.52	3.928	1.52
		F	2.488		2.592	
		M	3.526	1.48	3.589	1.54
		F	2.379		2.325	
		M	4.852	1.41	3.429	1.68
		F	3.450		2.039	
		M	4.125	1.64	3.222	1.51
		F	2.509		2.132	
Average Activity		M	4.287		3.507	
		F	2.880		2.300	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH03 #3	1	M	4.641	1.33	3.535	1.66
		F	3.490		2.131	
		M	4.484	1.49	3.449	1.51
		F	3.001		2.278	
		M	4.273	1.78	3.793	1.88
		F	2.394		2.019	
		M	4.271	1.54	4.100	1.62
		F	2.778		2.525	
		M	5.211	1.66	3.782	1.80
		F	3.131		2.100	
Average Activity		M	4.576		3.732	
		F	2.959		2.211	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH03 #3	2	M	8.510	1.26	9.201	1.26
		F	6.760		7.309	
		M	8.016	1.32	9.352	1.55
		F	6.074		6.047	
		M	7.778	1.26	8.023	1.26
		F	6.180		6.350	
		M	7.829	1.23	12.02	1.66
		F	6.350		7.258	
		M	9.238	1.04	11.07	1.22
		F	8.889		9.053	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH04 #1	1	M	4.685	1.41	3.823	1.68
		F	3.330		2.278	
		M	4.539	1.38	3.823	1.64
		F	3.288		2.330	
		M	3.868	1.37	4.307	1.79
		F	2.829		2.411	
		M	3.796	1.49	3.755	1.56
		F	2.553		2.400	
Average Activity		M	4.222		3.927	
		F	3.000		2.355	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH04 #1	2	M	7.430	1.18	8.981	1.41
		F	6.298		6.389	
		M	7.263	1.22	9.291	1.52
		F	5.969		6.111	
		M	8.291	1.40	10.36	1.53
		F	5.935		6.769	
		M	7.319	1.11	10.02	1.36
		F	6.581		7.375	
		M	7.704	1.16	9.630	1.13
		F	6.667		8.547	
Average Activity		M	7.601		9.656	
		F	6.290		7.038	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH04 #2	1	M	3.423	1.30	3.293	1.41
		F	2.633		2.329	
		M	4.708	1.41	3.375	1.59
		F	3.333		2.122	
		M	4.430	1.56	3.502	1.33
		F	2.837		2.625	
Average Activity		M	4.187		3.390	
		F	2.934		2.359	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH04 #2	2	M	7.181	1.26	8.206	1.21
		F	5.712		6.771	
		M	6.598	1.20	8.170	1.29
		F	5.492		6.313	
		M	6.524	1.25	8.908	1.39
		F	5.227		6.389	
		M	7.103	1.26	8.165	1.31
		F	5.644		6.250	
		M	7.038	1.27	7.680	1.35
		F	5.541		5.710	
Average Activity		M	6.889		8.226	
		F	5.523		6.287	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH08 #1	1	M	3.331	1.52	4.027	1.46
		F	2.191		2.752	
		M	3.194	1.63	4.027	1.67
		F	1.964		2.416	
		M	3.056	1.51	3.849	1.67
		F	2.021		2.311	
		M	3.005	1.59	3.874	1.55
		F	1.885		2.493	
		M	3.091	1.53	3.743	1.44
		F	2.025		2.596	
		M	2.897	1.44	3.957	1.63
		F	2.006		2.425	
Average Activity		M	3.096		3.128	
		F	2.015		2.499	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH08 #1	2	M	13.89	2.10	13.35	2.05
		F	6.601		6.497	
		M	15.38	1.63	14.89	1.97
		F	9.447		7.540	
		M	16.71	1.73	14.66	2.40
		F	9.678		6.111	
		M	14.78	1.57	13.19	1.80
		F	9.405		7.315	
		M	20.57	2.01	15.77	1.91
		F	10.22		8.244	
Average Activity		M	16.27		14.37	
		F	9.070		7.141	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH08 #2	1	M	6.016	1.51	4.933	1.55
		F	3.984		3.190	
		M	5.801	1.40	5.672	1.63
		F	4.146		3.484	
		M	5.775	1.46	4.567	1.46
		F	3.947		3.125	
		M	5.524	1.49	4.700	1.60
		F	3.709		2.933	
		M	3.937	1.81	3.724	1.56
		F	2.177		2.380	
		M	3.659	1.76	3.724	1.66
		F	2.077		2.248	
Average Activity		M	5.119		4.553	
		F	3.340		2.575	

Line	Dose	Sex	β–galactosidase activity Δabs/min/μg protein x 10-4	M/F Ratio	β–galactosidase activity Δabs/min/mg wet weight x 10-2	M/F Ratio
pRH08 #2	2	M	12.10	1.96	11.71	2.11
		F	6.180		5.556	
		M	11.41	1.69	11.21	1.70
		F	6.765		6.609	
		M	11.24	1.79	11.30	1.91
		F	6.286		5.914	
		M	10.91	1.57	11.63	1.84
		F	6.952		6.337	
		M	11.92	1.63	12.14	1.93
		F	7.333		6.291	
Average Activity		M	11.52		11.60	
		F	5.703		6.141	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH09 #1	1	M	3.833	1.15	3.434	1.37
		F	3.330		2.511	
		M	4.120	1.36	3.584	1.44
		F	3.021		2.491	
		M	5.235	1.30	3.720	1.49
		F	4.020		2.503	
		M	4.276	1.08	3.467	1.29
		F	3.947		2.689	
		M	4.607	1.26	3.367	1.30
		F	3.658		2.600	
Average Activity		M	4.414		3.514	
		F	3.595		2.559	

Line	Dose	Sex	β–galactosidase activity Δabs/min/μg protein x 10-4	M/F Ratio	β–galactosidase activity Δabs/min/mg wet weight x 10-2	M/F Ratio
pRH09 #1	2	M	11.18	1.55	8.871	1.57
		F	7.222		5.642	
		M	9.745	1.29	8.249	1.51
		F	7.531		5.466	
		M	10.15	1.39	8.333	1.53
		F	7.308		5.460	
		M	10.39	1.35	8.244	1.42
		F	7.701		5.816	
		M	10.39	1.33	7.986	1.48
		F	7.816		5.397	
Average Activity		M	10.37		8.337	
		F	7.516		5.556	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH09 #2	1	M	5.317	1.30	4.467	1.47
		F	4.102		3.040	
		M	6.071	1.57	5.100	1.68
		F	3.877		3.040	
		M	5.947	1.63	4.994	1.87
		F	3.659		2.664	
		M	3.582	1.67	4.251	1.76
		F	2.150		2.416	
		M	4.152	1.98	4.407	1.64
		F	2.101		2.685	
		M	3.345	1.48	4.015	1.64
		F	2.262		2.454	
Average Activity		M	4.736		4.539	
		F	3.025		2.717	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH09 #3	1	M	1.979	1.89	1.855	2.08
		F	1.048		0.891	
		M	1.716	1.69	1.622	1.78
		F	1.014		0.912	
		M	1.946	1.82	1.654	1.86
		F	1.072		0.891	
		M	1.920	1.48	1.899	1.84
		F	1.293		1.030	
		M	1.902	1.71	1.435	1.81
		F	1.110		0.792	
		M	1.923	2.00	1.589	2.22
		F	0.960		0.716	
		M	1.768	1.59	1.620	1.94
		F	1.110		0.837	
Average Activity		M	1.879		1.668	
		F	1.087		0.867	

Line	Dose	Sex	β–galactosidase activity Δabs/min/μg protein x 10-4	M/F Ratio	β–galactosidase activity Δabs/min/mg wet weight x 10-2	M/F Ratio
pRH09 #3	2	M	7.425	1.75	6.306	2.16
		F	4.234		2.923	
		M	7.153	1.70	5.293	1.74
		F	4.198		3.034	
		M	6.984	1.63	6.011	2.01
		F	4.290		2.990	
		M	7.154	1.87	6.011	1.98
		F	3.833		3.034	
Average Activity		M	7.179		5.905	
		F	4.138		2.995	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH13 #1	1	M	4.047	1.39	3.587	1.52
		F	2.917		2.357	
		M	4.088	1.42	3.722	1.59
		F	2.875		2.348	
		M	4.109	1.40	3.860	1.58
		F	2.937		2.449	
		M	4.167	1.52	3.722	1.50
		F	2.737		2.479	
		M	3.704	1.32	3.509	1.49
		F	2.816		2.348	
Average Activity		M	4.023		3.680	
		F	2.856		2.396	

Line	Dose	Sex	β–galactosidase activity Δabs/min/μg protein x 10-4	M/F Ratio	β–galactosidase activity Δabs/min/mg wet weight x 10-2	M/F Ratio
pRH13 #1	2	M	12.05	1.34	9.331	1.41
		F	8.967		6.630	
		M	11.21	1.38	9.415	1.45
		F	8.130		6.510	
		M	11.23	1.21	10.00	1.39
		F	9.268		7.202	
		M	11.21	1.37	9.635	1.43
		F	8.188		6.726	
		M	10.96	1.34	9.204	1.36
		F	8.161		6.779	
		M	10.98	1.35	9.224	1.40
		F	8.156		6.609	
Average Activity		M	11.27		9.468	
		F	8.478		6.744	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH13 #2	1	M	4.150	1.35	3.563	1.32
		F	3.067		2.690	
		M	4.117	1.54	3.626	1.46
		F	2.678		2.481	
		M	4.188	1.50	3.964	1.51
		F	2.789		2.622	
		M	3.626	1.27	3.870	1.45
		F	2.855		2.664	
		M	3.877	1.33	3.340	1.38
		F	2.910		2.427	
		M	3.819	1.32	3.499	1.42
		F	2.891		2.466	
Average Activity		M	3.963		3.644	
		F	2.865		2.558	

Line	Dose	Sex	β–galactosidase activity Δabs/min/μg protein x 10-4	M/F Ratio	β–galactosidase activity Δabs/min/mg wet weight x 10-2	M/F Ratio
pRH13 #2	2	M	11.16	1.51	8.759	1.44
		F	7.407		6.083	
		M	8.616	1.21	8.743	1.48
		F	7.113		5.908	
		M	11.80	1.47	9.367	1.25
		F	8.025		7.467	
Average Activity		M	10.53		8.956	
		F	7.515		6.486	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH13 #3	1	M	6.889	1.89	5.287	2.09
		F	3.649		2.528	
		M	6.386	2.13	4.781	2.07
		F	3.000		2.313	
		M	6.751	1.94	5.581	2.40
		F	3.476		2.322	
		M	7.402	2.32	5.287	2.48
		F	3.190		2.131	
		M	6.476	1.84	5.423	2.40
		F	3.528		2.258	
Average Activity		M	6.781		5.272	
		F	3.369		2.310	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH13 #3	2	M	7.179	1.14	6.965	1.02
		F	6.296		6.800	
		M	7.067	1.24	6.448	1.04
		F	5.683		6.190	
		M	7.179	1.21	7.650	1.24
		F	5.917		6.185	
		M	7.694	1.26	7.467	1.09
		F	6.090		6.825	
		M	6.988	1.11	7.200	1.07
		F	6.275		6.723	
		M	6.923	1.06	7.377	1.09
		F	6.532		6.762	
Average Activity		M	7.172		7.185	
		F	6.132		6.581	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH11 #1	1	M	3.021	1.08	2.599	1.03
		F	2.803		2.527	
		M	2.813	1.06	2.832	1.15
		F	2.650		2.467	
		M	2.415	1.00	2.425	1.10
		F	2.415		2.200	
		M	2.639	1.02	2.570	1.18
		F	2.598		2.182	
Average Activity			2.669		2.475	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH11 #2	1	M	1.550	1.03	1.725	1.19
		F	1.512		1.450	
		M	2.114	1.15	1.776	1.16
		F	1.841		1.530	
		M	1.830	1.06	1.628	1.19
		F	1.725		1.368	
		M	1.806	0.96	1.587	1.08
		F	1.886		1.467	
		M	1.720	1.02	1.467	1.04
		F	1.684		1.404	
Average Activity			1.578		1.540	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH11 #3	1	M	5.955	1.01	5.630	0.99
		F	5.885		5.680	
		M	6.478	1.02	6.045	0.97
		F	6.367		6.217	
		M	6.967	1.14	5.680	1.07
		F	6.088		5.306	
		M	5.785	0.91	5.635	1.09
		F	6.372		5.167	
		M	6.511	1.17	6.388	1.19
		F	5.567		5.362	
		M	6.923	1.08	5.884	1.07
		F	6.395		5.500	
Average Activity			6.274		5.708	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH12 #1	1	M	2.896	1.14	2.627	1.32
		F	2.545		1.991	
		M	2.889	1.17	2.600	1.37
		F	2.463		1.891	
		M	2.483	1.16	2.433	1.27
		F	2.139		1.912	
		M	2.644	1.29	2.620	1.26
		F	2.057		2.074	
		M	2.430	1.14	2.289	1.18
		F	2.127		1.934	
Average Activity		M	2.668		2.514	
		F	2.266		1.960	

Line	Dose	Sex	β–galactosidase activity Δabs/min/μg protein x 10-4	M/F Ratio	β–galactosidase activity Δabs/min/mg wet weight x 10-2	M/F Ratio
pRH12 #1	2	M	7.469	1.25	6.489	1.39
		F	5.952		4.664	
		M	7.756	1.28	7.600	1.46
		F	6.061		5.208	
		M	7.997	1.24	7.787	1.22
		F	6.462		6.400	
		M	6.667	1.23	6.489	1.28
		F	5.442		5.089	
Average Activity		M	7.472		7.091	
		F	5.979		5.340	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH12 #2	1	M	2.233	1.18	1.832	1.13
		F	1.889		1.622	
		M	2.039	1.10	2.070	1.30
		F	1.856		1.595	
		M	2.908	1.26	2.061	1.26
		F	2.309		1.630	
		M	2.481	1.25	2.131	1.28
		F	1.978		1.667	
		M	2.265	1.03	2.055	1.25
		F	2.189		1.639	
Average Activity		M	2.385		2.030	
		F	2.044		1.631	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH12 #3	1	M	3.611	1.22	3.215	1.30
		F	2.955		2.481	
		M	3.404	1.22	2.996	1.24
		F	2.801		2.411	
		M	4.207	1.35	3.509	1.35
		F	3.120		2.602	
		M	4.028	1.19	3.776	1.39
		F	3.390		2.715	
		M	4.379	1.26	3.907	1.35
		F	3.483		2.894	
Average Activity		M	3.926		3.481	
		F	3.150		2.621	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH24 #1	1	M	2.374	1.19	2.105	1.30
		F	1.991		1.623	
		M	2.125	1.10	1.491	1.15
		F	1.934		1.294	
		M	2.065	1.03	1.558	1.12
		F	2.007		1.386	
		M	2.130	0.97	1.532	1.00
		F	2.207		1.529	
Average Activity		M	2.174		1.672	
		F	2.035		1.458	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH24 #2	1	M	1.048	1.30	0.874	1.33
		F	0.804		0.655	
		M	0.972	1.15	0.802	1.25
		F	0.843		0.641	
		M	0.744	1.23	0.067	1.34
		F	0.603		0.050	
		M	0.748	1.21	0.716	1.29
		F	0.616		0.553	
Average Activity		M	0.878		0.615	
		F	0.717		0.475	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH24 #3	1	M	2.447	1.04	1.822	1.12
		F	2.358		1.627	
		M	2.698	1.12	2.036	1.34
		F	2.417		1.518	
		M	1.986	1.03	2.029	1.19
		F	1.930		1.711	
		M	2.714	1.06	1.900	1.08
		F	2.557		1.767	
		M	2.765	1.14	2.269	1.29
		F	2.436		1.759	
Average Activity		M	2.522		2.011	
		F	2.340		1.676	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH25 #1	1	M	2.403	1.25	1.893	1.29
		F	1.920		1.464	
		M	2.245	1.30	1.623	1.25
		F	1.730		1.302	
		M	2.083	1.16	1.832	1.38
		F	1.789		1.332	
		M	2.118	1.32	1.822	1.42
		F	1.608		1.282	
Average Activity		M	2.212		1.793	
		F	1.762		1.345	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH23 #1	1	M	1.991	1.27	1.489	1.33
		F	1.565		1.117	
		M	1.765	1.32	1.800	1.43
		F	1.333		1.261	
		M	1.960	1.42	1.973	1.59
		F	1.381		1.239	
		M	1.680	1.18	1.713	1.41
		F	1.422		1.209	
Average Activity		M	1.849		1.744	
		F	1.425		1.207	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH23 #2	1	M	2.037	1.37	1.553	1.40
		F	1.485		1.113	
		M	1.836	1.31	1.891	1.38
		F	1.400		1.367	
		M	1.906	1.38	1.927	1.66
		F	1.377		1.160	
		M	1.863	1.37	1.856	1.43
		F	1.361		1.298	
Average Activity		M	1.911		1.807	
		F	1.406		1.235	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH23 #3	1	M	1.604	1.55	1.742	1.63
		F	1.035		1.072	
		M	1.425	1.23	1.615	1.41
		F	1.154		1.145	
		M	1.533	1.34	1.787	1.51
		F	1.143		1.180	
		M	2.095	1.45	1.961	1.58
		F	1.444		1.243	
		M	2.171	1.44	1.913	1.40
		F	1.512		1.366	
Average Activity		M	1.766		1.804	
		F	1.258		1.201	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
pRH31 #1	1	M	2.017	1.37	2.318	1.56
		F	1.470		1.483	
		M	2.083	1.43	2.155	1.32
		F	1.460		1.636	
		M	2.009	1.33	2.251	1.50
		F	1.511		1.499	
		M	2.500	1.50	2.664	1.48
		F	1.667		1.805	
		M	3.167	1.67	2.474	1.60
		F	1.902		1.545	
Average Activity		M	2.355		2.372	
		F	1.602		1.594	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
p34F3 5.0 #1	1	M	3.207	1.12	2.481	1.25
		F	2.867		1.988	
		M	2.994	1.14	2.544	1.44
		F	2.636		1.771	
		M	2.515	1.18	1.838	1.16
		F	2.139		1.578	
		M	2.297	1.11	1.873	1.03
		F	2.063		1.824	
Average Activity			2.590		2.028	

Line	Dose	Sex	β-galactosidase activity Δabs/min/μg protein x 10^{-4}	M/F Ratio	β-galactosidase activity Δabs/min/mg wet weight x 10^{-2}	M/F Ratio
p34F3 8.0 #1	1	M	3.841	1.02	1.856	1.18
		F	3.752		1.576	
		M	3.632	1.09	2.704	1.19
		F	3.333		2.279	
		M	1.333	1.11	2.396	1.13
		F	1.206		2.122	
		M	1.394	1.08	2.853	1.08
		F	1.288		2.636	
Average Activity			2.426		2.303	

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