Progress Towards Development of a Genetically Modified Strain of the Australia Sheep Blowfly *Lucilia cuprina* Suitable for a Sterile Release Program

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Abstract

The sterile insect technique (SIT) is concerned with the mass-rearing and release of sterilized insects which mate with "wild-type" females in the field, producing no viable offspring. The aim of this study was to use genetic engineering methods to make a strain of the Australia sheep blowfly *Lucilia cuprina* which is suitable for an area-wide sterile-male release program. The main objectives were: development of an efficient germline transformation system for introducing a target gene into *Lucilia* and development of an inducible female killing system to produce a male only population.

The *piggyBac* and *Minos* transposons were evaluated as transformation vectors for *L. cuprina*. Firstly, *Drosophila melanogaster* was used as a model system to determine if the frequency of both inter-plasmid transposition and germ-line transformation increases with the level of expression of the *piggyBac* transposase. Expression of the *piggyBac* transposase gene was controlled with either the *α1-tubulin*, *hsp83* or *hsp70* promoter, which have strong, intermediate and low constitutive activity respectively. The results show that the frequency of *piggyBac*-mediated germ-line transformation does increase with the level of expression of the transposase. In contrast, there does not appear to be a simple correlation between the level of expression the transposase and the frequency of transposition measured using an inter-plasmid transposition assay. This suggests that this widely used assay may not necessarily predict which is the best "helper" plasmid for germ-line transformation. Secondly, inter-plasmid transposition assays have shown that both *piggyBac* and *Minos* transposases are active in blowfly embryos. Thirdly, *Drosophila* eye color genes and the enhanced green fluorescent protein (*EGFP*) gene were tested as potential markers for identifying transgenic flies. The most promising marker based on transient expression appears to be *EGFP* driven by the Drosophila *polyubiquitin* gene promoter (*pUb-EGFP*). Fourthly, blowfly embryos were coinjected with the *piggyBac* helper driven by the *D. melanogaster*

hsp70 promoter and the *PUbnlsEGFP* marker gene. Two transgenic *L. cuprina* lines were isolated and characterised by Southern DNA hybridisation analysis and inverse PCR. The transformation frequency was 1.4 to 1.9%. Of the two transformant lines obtained, one had a single copy of the transgene and the other most likely has four copies. This is the first report of germ line transformation of *L. cuprina*.

A tetracycline regulated inducible expression system was adopted to develop a controllable female-killing genetic system based on the *D. melanogaster msl2* gene. One component of the system is the tetracycline dependent transactivator (*tTA*) gene controlled by a constitutive promoter. The other (*tetO-msl2*) is the *msl2* coding region controlled with a promoter bearing seven copies of the tetracycline operator (*tetO*) sequence. Female *D. melanogaster* carrying both a *promoter-tTA* and *tetO-msl2* gene constructs would be predicted to die in the absence of tetracycline due to expression of *msl2*.

In this study several *promoter-tTA* constructs were developed including *WH-arm* which uses the constitutive *armadillo* promoter. Drosophila carring both *WH-arm* and *tetO-lacZ* transgenes were shown by spectrophotometric and histochemical staining assays to express β–galactosidase but only if raised on media that lacked tetracycline. There was a significant decrease in viability of females carrying both *WH-arm* and *tetO-msl2* gene constructs raised on media lacking tetracycline. However lethality was not 100%. Assembly of the MSL complex on female X chromosomes (due to expression of *msl2*) was confirmed by immuno staining of polytene chromosomes with anti-MSL3 antibody. Thus it appears that induction of 100% female lethality will require higher levels of *msl2* expression than obtained with the *WH-arm/tetO-msl2* system for controlling female viability in transgenic *Lucilia*.

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ABBREVIATIONS

arm	*armadillo*
β	beta
Δ	delta
λ	lambda
bp	base pairs
BSA	bovine serum albumin
BHI	brain heart infusion
cn	*cinnabar* gene
°C	degrees Celsius
cDNA	complementary DNA
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
D. melanogaster	*Drosophila melanogaster*
dNTPs	dinucleotide triphosphates
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
EGFP	enhanced green fluorescent protein
F	female
g	gram
GFP	green fluorescent protein
hsp	heat shock protein
kb	kilobase pairs
mRNA	messenger ribonucleic acid
L	liter
L. cuprina	*Lucilia cuprina*
μ	micro
M	male
M	molar

Mb	million base pairs
min	minute
mle	*maleless*
msl	*male specific lethal*
mof	*males absent on the first*
nm	nanometer
OD	optical density
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNase	ribonuclease
RNA	ribonucleic acid
rpm	revolutions per minute
TE	tris-EDTA
tetO	*tetracycline operator*
tetR	tetracycline repressor
tTA	tetracycline dependent transactivator
tub	tubulin
UV	ultra violet
U	units
UTR	untranslated region
V	volts
v/v	volume per volume
w/v	weight per volume
yp	yolk protein

LIST OF PUBLICATIONS ARISING FROM THIS THESIS

X. Li, J.C. Heinrich and M. J. Scott (2001) *piggyBac*-mediated transposition in *Drosophila melanogaster*: an evaluation of the use of constitutive promoters to control transposase gene expression. *Insect Molecular Biology* **10(5)**: 447-455.

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*These authors contributed equally to this study.

CHAPTER 1

GENERAL INTRODUCTION

1.1 STERILE INSECT TECHNIQUE (SIT)

1.1.1 Overview

The sterile insect technique (SIT) is concerned with the mass-rearing and release of sterilized insects which mate with "wild-type" females in the field, producing no viable offspring. SIT can result in suppression or eradication of the target insect (Gilmore, 1989; Knipling, 1960). Since it was developed by Knipling (1955) for use against the *screwworm* fly, *Cochliomyia hominivorax* (Coquerel), it has become an efficient target-specific and environmentally nonpolluting method available for controlling insect pests.

The success of screwworm eradication programs in the United States during the 1950s and 1960s (Baumhover *et al.,* 1955; Knipling 1979) stimulated wider use of the SIT against other insects, including at least 13 species of tephritid fruit flies (Steiner, 1969; Klassen *et al.,* 1994). Successful past SIT programs include the eradication of screwworm from North America (Knipling, 1960), Queensland fruit fly from Western Australia (Fisher, 1994), melonfly from the Okinawa islands (Kakinohana, 1997) and tsetse fly from Zanzibar (Vreysen *et al.,* 2000). SIT has also been used for eradication or suppression of the Mediterranean fruit fly (medfly) in various parts of the world (Gilmore, 1989; Hendrichs, 1994).

There are several factors to consider in appraising the feasibility of SIT (Knipling *et al.,* 1955):

- An economical method of rearing millions of insects must be known, or capable of development;
- The insect must be of a type that can be readily dispersed so that released sterile males will be about as accessible to the virgin females in nature as are the competing fertile males;
- Females must normally mate only once;

- The insect to be controlled must have a low inherent population, or the species must be under natural or induced circumstances, reach a population sufficiently low to make it economically feasible to rear and release enough sterile males to effect a further downward trend in the population of subsequent generations.

Mass-rearing facilities initially produce equal numbers of the two sexes, but females are generally separated and discarded before release. From an economic perspective, single-sex releases would reduce the costs associated with production, transportation, and avoid the problem of sterile stings which may render fruit unmarketable (Proverb, 1974). From a biological viewpoint male-only releases may be more effective at reducing wild populations than standard bisexual releases, because sterile males would direct mating efforts at wild females exclusively. Male competitiveness has been estimated to be 3-5 times greater in unisexual releases than bisexual ones (Robinson *et al.,* 1986; McInnis *et al.,* 1994). In addition the females of some species require a much higher irradiation dose than the males to be sterilized (as in the screwworm) and the higher dose impairs the males competitiveness in the field (Whitten and Taylor, 1970; Bushland, 1971). For medfly, SIT has been shown to be most effective when only sterile males are released in the field (McInnis *et al.,* 1994).

1.1.2 Methods of Development of Male-Only Strains

The development of male-only strains represents a potentially important step toward improving SIT (Hendrichs *et al.,* 1995). Various female-killing and sex-sorting genetic systems have been developed, known generically as genetic sexing (GS) strains.

1.1.2.1 Chromosome translocation (aberration) based GS strains

Genetic sexing systems have been constructed in a number of dipteran species, either for use with the SIT or with other genetic methods of population control (Whitten 1969, 1979; Curtis *et al.,* 1976; Whitten *et al.,* 1977; Foster *et al.,* 1978;

Kaiser *et al.,* 1978; Baker *et al.,* 1979, 1980; Rossler, 1979; Robinson and Van Heemert, 1982; Saul, 1984; 1985Kerremans and Franz, 1995). So far, most GS strains in factory production have used radiation-induced translocations to the Y chromosome as dominant selectable markers, complementing an X-linked or autosomal recessive trait such as pupal color, temperature-sensitive lethality, blindness, or insecticide resistance (Seawright *et al.,* 1978; Foster *et al.,* 1988; Robinson *et al.,* 2000).

The first type of GS strains was based on pupal colour mutations (Whitten, 1969; Rossler, 1979, 1980; Robinson and van Heemert, 1982) with males emerging from wild-type pupae and females emerging from mutant pupae. These GS systems require the use of expensive pupal separation machines that can separate pupae on the basis of colour. However, machines are too slow for an operational mass-rearing factory, and the extra handling lowers the quality of the released males.

The second type of GS strains was developed by Kerremans and Franz (1995). These GS strains were based on a temperature-sensitive lethal (*tsl*) mutation (Franz *et al.,* 1994) so that female zygotes can be killed following a high temperature treatment.

Cladera (1995) developed the 3rd type of GS strain, by using a gene causing slow development during the larval and pupal stages. This gene is the eye color mutation *sw-z* in medfly (*Ceratitis capitata*), in which the traits of slow development and mutant eye phenotype behave as pleiotropic effects of a single gene. The delaying effect of the gene can be used to separate males at the larva stage. The separation of sexes was improved by lowering the rearing temperature. Another mutant strain *sw-y*, which is allelic to *sw-z*, was developed by Pizarro *et al.* (1997). This strain has a slower developmental rate than *sw-z* with respect to wild type, which could make it possible to separate males from females at an early stage of development.

A type of field-female killing strains, a modification to the SIT system, was also developed based on GS method (Whitten *et al.,* 1977, 1979; Foster *et al.,* 1985). This system uses recessive eye-color mutations as the selective marker. Females are homozygous for the mutations and unable to survive to reproductive maturity in the field, because they are functionally blind. Genetic death in field populations is caused by semisterility of the translocation and by homozygosis of the mutations in females and non-translocation males of field origin (Foster *et al.,* 1988). Foster (1991) showed that a field female-killing system using a Y-linked translocation can achieve higher genetic death rates than the sterile insect technique in density-influenced populations.

The translocation-based GS strains all have potential genetic instability, either due to recombination in the chromosomal region between the translocation breakpoint and the selectable marker or due to the survival of adjacent-1 segregants (Franz *et al.,* 1994; Robinson, 2000). To reduce the frequency of recombination, crossover-suppressing inversions are included in some genetic sexing systems, to ensure tight linkage of the deleterious genes to sex (Curtis *et al.,* 1976; Kaiser *et al.,* 1978; Baker *et al.,* 1979, 1980; Foster, 1991). These chromosome aberration-based systems also tend to reduce the fitness of the insects, making them less effective agents for SIT (Franz *et al.,* 1994; Kerremans & Franz, 1995; Robinson *et al.,* 2000).

1.1.2.2 The transgene based GS strains

An alternative approach of making a GS strain is to use a transgene system to induce repressible female-specific lethality. This could be used simply as a GS strain.

Heinrich and Scott (2000) reported that they had developed a tetracycline-repressible female-specific lethal genetic system in the vinegar fly *Drosophila melanogaster* (see section 1.5). One component of the system is the tetracycline-controlled transactivator gene under the control of the fat body and female-specific transcription enhancer from the *yolk protein 1* (*yp1*) gene. The other

component consists of the proapoptotic gene *hid* under the control of a tetracycline-responsive element. Males and females of a strain carrying both components are viable on medium supplemented with tetracycline, but only males survive on normal medium.

A similar system has also been developed by Thomas *et al.* (2000). They also showed a simple model of the release of insects carrying this trait into a target population. They demonstrated two methods that have the required genetic characteristics in an otherwise wild-type genetic background. The first system uses a sex-specific promoter or enhancer to drive the expression of a repressible transcription factor, which in turn controls the expression of a toxic gene product. The second system uses non-sex-specific expression of the repressible transcription factor to regulate a selectively lethal gene product. Both methods work efficiently in *Drosophila melanogaster.*

1.2 THE SHEEP BLOWFLY, *LUCILIA CUPRINA* (DIPTERA)

The current world-wide distribution of the sheep blowfly, *Lucilia cuprina*, has been given as Asia, Africa, Australia, Egypt, Fiji, Indonesia, New Caledonia, Pakistan (Waterhouse & Paramov, 1950; Norris, 1990) and more recently New Zealand (Heath, 1990). *L. cuprina*, is the most economically important species involved in flystrike, or myiasis of sheep in Australia (Watts *et al.,* 1976), and New Zealand (Heath, 1990). The annual costs of flystrike in lost production and control measures have been estimated to be A$149 million in Australia (Beck *et al.,* 1985) and NZ$37 million in New Zealand (Heath and Bishop, 1995). *L. cuprina* is believed to have been present in New Zealand since the late 1970s (Heath, 1990; Heath *et al.,* 1991; Bishop, 1993) but was firstly reported only in 1988 (Holloway, 1991) and is now found to have spread throughout the North Island and through much of the South Island and is the species most commonly associated with flystrike (Heath and Bishop, 1995). *L. cuprina* is also the principal fly involved in myiasis of sheep in South Africa and has been known to cause myiasis in other African countries and India.

Several control methods have been developed against *L. cuprina*, including insecticides (Gleeson *et al.,* 1994; Wilson and Heath, 1994), GS systems (Whitten 1969; Rossler, 1979; Robinson and van Heemert, 1982) and FK (field-female killing) systems (Whitten *et al.,* 1977; Whitten, 1979; Foste *et al.,* 1985, 1988; Foster, 1989). To date, none of these methods have proved effective at eradicating *L. cuprina.*

In New Zealand, *L. cuprina* is primarily controlled through the application of insecticides. However, increasing resistance to some insecticides coupled with demand for wool with low insecticide residue has resulted in an increased interest in biological methods of control.

1.3 DOSAGE COMPENSATION IN *D. MELANOGASTER*

1.3.1 Overview

Dosage compensation is the process by which males (XY) express a level of X-linked gene products equivalent to that of females (XX).

In *D. melanogaster*, males are the heterogametic sex; they carry one X and one Y chromosome, whereas females have two X chromosomes. In this species dosage compensation is achieved by transcriptionally up regulating genes on the male X chromosome to equal expression from the two female X chromosomes (Offermann 1936; Mukherjee and Beerman, 1965). Failure to compensate for the difference in X chromosome dosage results in a lethal genetic imbalance of X-linked and autosomal gene products in males.

Dosage compensation in *Drosophila* is mediated by a multiprotein, RNA-containing complex that associates with the X chromosome at multiple sites. The complex is preferentially associated with numerous sites on the X chromosome in somatic cells of males but not of females.

1.3.2 Genes Involved in Dosage Compensation

The products of at least five genes, *maleless* (*mle*), *male-specific lethal 1* (*msl1*), *male-specific lethal2* (*msl2*), *male-specific lethal 3* (*msl3*) and *males absent on the first* (*mof*), collectively called the msls and 2 non-coding RNAs, *RNA on X1* (*roX1*) and *roX2*, are necessary for dosage compensation (Fulunaga *et al.,* 1975; Belote and Lucchesi, 1980; Uchida *et al.,* 1981; Lucchesi *et al.,* 1982; Hilfiker *et al.,* 1997; Lucchesi, 1998; Kelley and Kuroda, 2000; Gu *et al.,* 2000; Meller *et al.,* 2000; Smith *et al.,* 2000). The *msl* genes were identified by genetic means for example as *msl1*-mutant males die in the third instar larval stage due to a failure to dosage compensate (Lucchesi, 1987). Each gene has been cloned and the encoded proteins have been characterized to varying degrees.

1.3.2.1 *mle* encodes for an RNA-DNA helicase

The *mle* gene encodes for an RNA-DNA helicase of the DExH subfamily of ATPase/helicases (Kuroda *et al.,* 1991; Lee *et al.,* 1997). A predicted MLE protein contains seven short segments that define a superfamily of known and putative RNA and DNA helicases. MLE, while present in the nuclei of both male and female cells, differs in its association with polytene X chromosomes in the two sexes. MLE is associated with hundreds of discrete sites along the length of the X chromosome in males but not in females (Kuroda *et al.,* 1991). MLE has short sequences that identify it as a member of one of two superfamilies of nucleic acid helicases (Kuroda *et al.,* 1991). MLE is highly homologous to human RNA helicase A (Lee *et al.,* 1993) and bovine nuclear DNA helicase II (Zhang *et al.,* 1994). These features of the MLE protein imply that it interacts with RNA (Kuroda *et al.,* 1991; Gibson *et al.,* 1994). MLE remains associated with the male X throughout mitosis (Lavender *et al.,* 1994). It seems that the association of MLE with the MSL complex is mediated through interaction with a stable RNA (Richter *et al.,* 1996). MLE is weakly associated with the MSL complex (Copps *et al.,* 1998). Immunofluorescence studies have shown that *mle+* function is required for localization of MOF and MSL3 to the X chromosome (Gu *et al.,* 1998). MLE does not interact with either MSL3 or MOF (Scott *et al.,* 2000).

1.3.2.2 *msl1* encodes a protein with a highly acidic N terminus

msl1 encodes a protein with a highly acidic N terminus that includes short stretches composed almost entirely of aspartate or aspartate and glutamate residues as well as numerous interspersed glutamate doublets. These characteristics are common to a large group of proteins thought to be involved in transcription regulation. In the central region of the protein, there are many proline, serine and threonine residues some of which could be sites of phosphorylation by protein kinases of the cdc2 type as means of regulating the activity of the protein (Palmer *et al.,* 1993).

Two regions of MSL1, one near the N-terminus and the other at the C-terminus, are important for assembly of the MSL complex *in vivo* as overexpression of either region caused male-specific lethality. MSL2 co-purified from crude fly extracts with the amino terminal domain of MSL1. Both MOF and MSL3 co-purified with the C-terminal domain of MSL1. It appears that the main role of MSL1 is to serve as the backbone for assembly of the MSL complex (Scott *et al.,* 2000).

The amount of MSL1 protein in females is reduced compared to males (Chang and Kuroda, 1998). Ectopic expression of MSL2 protein in females is not sufficient to induce an insurmountable level of dosage compensation, suggesting that an additional component is limiting in females. A candidate for this limiting factor is MSL1, because the amount of MSL1 protein in females is reduced compared to males. There are two levels of negative regulation of *msl1* in females. The predominant regulation is at the level of protein stability, while a second regulatory mechanism function is at the level of protein synthesis. Overcoming these control mechanisms by overexpressing both MSL1 and MSL2 in females results in 100% female-specific lethality (Chang and Kuroda, 1998).

1.3.2.3 *msl2*-the primary target of negative regulation in females-encodes a RING finger protein

msl2 encodes a key male-limited regulator of dosage compensation in *Drosophila*. It consists of 769 amino acid residues and has a RING finger (C3HC4 zinc finger) and a metallothionein-like domain with eight conserved and two non-conserved cysteines. The RING finger is present in more than 60 proteins from plants, bacteria, animals and viruses, many of which are present in macromolecular complexes (reviewed in Freemont, 1993; Saurin *et al.,* 1996). In each case, two zinc atoms are coordinated by interleaved pairs of cysteines, or cysteine plus histidine, in a 1-3, 2-4 pattern. The RING finger may serve as scalffold for the evolution of different. A requirement for the MSL2 RING finger in male viability was demonstrated by site-directed mutation of conserved RING finger cysteines (Lyman *et al.,* 1997). Residues around the first zinc-binding site of the RING finger were essential for this interaction (Copps *et al.,* 1998). MSL2 has been shown to bind to MSL1 using a yeast 2-hybrid system (Copps *et al.,* 1998)

MSL2 protein is not produced in females, as *msl2* mRNA translation is repressed by the female-specific SXL protein (see section 1.3.3). Ectopic expression of *msl2* in females results in female-specific developmental delay and reduction in viability (Kelley *et al.,* 1995). This is probably because the MSL complex assembles onto both X chromosomes and upregulates the transcription of most X-linked genes.

1.3.2.4 *msl3* encodes a chromodomain protein

MSL3 was initially described as a novel protein with only a very weak similarity to some human and rice sequences of unknown function (Gorman *et al.,* 1995). Subsequently, Koonin *et al.* (1995) showed that MSL3 contains a chromodomain at the amino end and a chromoshadow domain at the carboxy end. The "chromodomain," has been found in many proteins involved in chromatin regulation, such as POLYCOMB and HP1 (Aasland and Stewart, 1995). Bertram *et al.* (1999) and Prakash *et al.* (1999) described several mammalian genes with

similarities to *msl3.* Gorman *et al.* (1995) found that there were two types of *msl3* RNAs in *D. melanogaster*, one of which would lack part of the chromodomain-like region, suggesting that there may be significant roles for the truncated version of the MSL3.

By comparison of the *D. melanogaster* and *D. virillis* sequences as well as the mammalian genes, Marin and Baker (2000) found that MSL3 is actually a member of a class of proteins found in many other eukaryotic organisms, including not only animals, but also yeast and plants. They concluded that only the more N-terminal chromodomain-containing regions hypothesized by Koonin *et al.* (1995) have a pattern of conserved residues in the members of this gene family that is compatible with being a chromodomain-related structure. Most data suggest that the chromodomain is a protein-protein interaction domain, MSL3 might be able to form dimers or interact using such a surface with MSL1 or MSL2. Recently it has been shown that the chromodomain is an RNA binding domain (Akhtar *et al.,* 2000). This suggests that MSL3 may bind to either *roX1* or *roX2* RNA.

1.3.2.5 *mof* gene encodes a putative acetyl transferase

The *mof* mRNA encodes an 827 amino acid protein that contains a 250 amino acid domain common to many acetyl transferases. 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). A null allele of mof, mof´, has a single nucleotide mutation that leads to a substitution of Gly691 (the most conserved residue in the 250 amino acid motif) with glutamic acid. More recently it has been shown that MOF is a histone acetylase preferentially acetylating histone H4 at lysine 16 (Smith *et al.,* 2000). Staining of polytene chromosomes with anti-MSL antibodies suggests that the association of MSL1 and MSL2 with the male X-chromosomes of mutant *mof* larvae is slightly reduced. However, the association of MLE is substantially reduced, and the H4 isoform, H4Ac16, appears to be absent (Gu *et al.,* 1998). By using loss-of-function mutations it has been demonstrated that MOF co-localises

with the MSL complex on the male X chromosome (Gu *et al.,* 1998). MOF activities are necessary for complexes to access the various X chromosome sites (Gu *et al.,* 2000).

1.3.2.6 *roX1* and *roX2 RNAs* encode non-coding RNAs

roX1 and *roX2 RNAs* were isolated using an enhancer detector screen for β–galactosidase activity in the mushroom bodies of the *Drosophila* brain. Both genes are X-linked and each encodes a RNA without a significant open reading frame. Their expression is confined to the nucleus of male flies, and paint the male X chromosome (Amrein and Axel, 1997; Meller *et al.,* 1997). The *roX* RNAs associate with the X chromosome with a distribution that mirrors that of the MSLs, and *roX2* is present in a purified MSL complex isolated from cultured cells (Smith *et al.,* 2000). *roX1* is not essential for male-viability or dosage compensation but may be functionally redundant with *roX2* (Franke and Baker, 1999). With the exception of a 30 bp region near the 3′ ends, there is no significant sequence similarity between *roX1* and *roX2*. The RNA components probably play an early role in MSL binding, assembly or spreading (Meller *et al.,* 2000; Kageyama *et al.,* 2001).

1.3.2.7 The regulation of dosage compensation

The MSL proteins are thought to form a complex that is preferentially associated with numerous sites on the X chromosome in somatic cells of males but not females (Kuroda *et al.,* 1991; Palmer *et al.,* 1993; Gorman *et al.,* 1995; Bashaw and Baker 1995; Kelley *et al.,* 1995; Zhou *et al.,* 1995; Hilfiker *et al.,* 1997; Gu *et al.,* 1998). The complex is needed to mediate hypertranscription of the hundreds of active genes along the male X. When any subunit is removed through mutation, the MSL complex is lost from most sites along the X, dosage compensation fails, and males die (Kelley and Kuroda, 2000).

Immunoprecipitation and yeast two-hybrid assays suggest that MSL1, MSL2, MSL3, and MOF are strongly interacting, while MLE is less tightly associated with the complex (Kelley *et al.,* 1995; Copps *et al.,* 1998; Smith *et al.,* 2000). MLE

activities are necessary for complexes to access the various X chromosome sites (Gu *et al.*, 2000).

MOF plays a direct role in the specific histone acetylation associated with dosage compensation (Turner *et al.*, 1992; Bone *et al.*, 1994; Hilfiker *et al.*, 1997; Akhtar and Becker 2000; Smith *et al.*, 2000). Association of MOF with the male X chromosome depends on its interaction with RNA (Akhtar and Becker, 2000). In vitro analyses of the MOF and MSL3 chromodomains indicate that these chromodomains may function as RNA interaction modules. Their interaction with non-coding RNA may target regulators to specific chromosomal sites (Akhtar *et al.*, 2000).

The binding of MSL1 and MSL2 to several "high affinity" or "chromotin-entry" sites on the X chromosome does not require MLE, MOF or MSL3 (Lyman *et al.*, 1997). The binding of MLE, MOF or MSL3 proteins to the male X chromosome is absolutely dependent on MSL1 and MSL2 (Gu *et al.*, 1998). The MSL complex does not associate with the female X chromosomes because MSL2 protein is absent from females (Bashaw and Baker, 1995; Kelley *et al.*, 1995; Zhou *et al.*, 1995).

The *roX1* and *roX2* gene each contains a chromotin-entry site for the MSL complex (Kelley *et al.*, 1999). MSL proteins bind a small DNA region near the middle of the *roX1* gene corresponds to a male-specific DNase I hypersensitive site in chromatin of intact nuclei (Kageyama *et al.*, 2001).

In order to become stably associated with the numerous other sites along the X chromosome where it is normally found, the MSL complex requires the histone acetyltranferase activity of MOF as well as the ATPase activity of MLE (Gu *et al.*, 2000). If either of these activities is impaired, complexes containing the known MSLs are formed but are unable to access X-chromosome chromatin beyond the entry sites. Over-expression of MOF leads to the acetylation of numerous autosomal association of the MSL complex.

Recently, a JIL-1 protein kinase has been found associated with the MSL complex, but its function in the dosage compensation system is still unknown (Jin *et al.,* 1999, 2000).

MSL-mediated dosage compensation begins early in embryogenesis. MSL binding to the male X chromosome is observed in all somatic tissues of embryos and larvae. The MSL proteins first associate with the male X chromosome as early as the blastoderm stage, slightly earlier than the histone H4 isoform, acetylated at lysine 16 is detected on the X chromosome (Franke *et al.,* 1996).

1.3.3 *Sex Lethal* (*Sxl*) Controls Dosage Compensation in *Drosophila* by a Non-splicing Mechanism

Sxl encodes an RNA binding protein that regulates sexual differentiation by influencing alternative splicing in the sex determination hierarchy (Bell *et al.,* 1988; Sosnowski *et al.,* 1989; Inoue *et al.,* 1990; Samuels *et al.,* 1994). Sxl regulates its own expression by regulating splicing (Bell *et al.,* 1991). In females, SXL protein-directed splicing of *Sxl* pre-mRNA generates an open reading frame. In males, default splicing results in a mRNA with premature stop codons. *Sxl* also prevents X chromosome hypertranscription in females (Lucchesi and Skripsky, 1981; Cline, 1984).

Activation of early transcription of the *Sxl* gene is affected by the assessment of the X:A ratio (Cline, 1993). Females (X:A=1.0) make SXL protein, which represses the *msl* pathway and results in basal transcription of both X chromosomes. Males (X:A=0.5) lack SXL protein, leaving the *msl* pathway active for hypertranscription of most X-linked genes.

One of the molecular targets of *Sxl* repression is the *msl2* mRNA. There are poly(U) tracts in both the 5′ and 3′ UTRs that resemble the SXL-binding sites in *Sxl* and *tra*. SXL protein associates with multiple sites in the 5′ and 3′

untranslated regions (UTR) of the *msl2* transcript and represses its translation in females (Kelley *et al.,* 1997; Bashow and Baker, 1997). The SXL-binding sites in the *msl2* 5′ UTR are within the intron, and thus they are only retained in the female message. Removal of the *Sxl* binding sites in either 3′ or 5′ UTRs results in expression of MSL2 protein in females. Expression of MSL2 also results in the X chromosome association of the other MSLs, suggesting that *msl2* is the primary sex-specific target of Sxl (Kelley *et al*., 1995; Bashaw *et al.,* 1995).

Sex-lethal acts synergistically through sequences in both the 5′ and 3′ untranslated regions of MSL2 to mediate repression. The repression of MSL2 is directly regulated by Sex-lethal at the level of translation. In contrast to numerous other developmentally regulated mRNAs, the regulation of *msl2* mRNA occurs by a poly(A) tail-independent mechanism (Gebauer *et al.,* 1999).

MSL1 is highly dependent on MSL2 for its protein stability. In addition, a subset of *msl1* transcripts also contain SXL-binding sites in their 3′ untranslated regions and these contribute to MSL1 repression internals (Chang *et al.,* 1998).

1.3.4 A Second Dosage Compensation Process

Dosage compensation of the *runt* gene during embryogenesis is dependent on *Sxl* but independent of the *msl* genes (Gergen, 1987; Bernstein *et al.,* 1994). This indicates that there are at least two kinds of dosage compensation and may explain the inability of mutations in the *msl* genes to rescue females which lack *Sxl* function. Colocalization of the MSLs and H4Ac16 on the male X chromosome in all somatic cells beginning in the late blastoderm stage also supports the idea that both kinds of dosage compensation function side by side throughout the life of *Drosophila* (Rastelli *et al.,* 1995; Bashaw *et al.,* 1996).

Insight into the identity of a second dosage compensation process that could be controlled by *Sxl* has come from the finding *Sxl* completely represses translation

of *msl2* RNA in females. The *msl2* RNA contains 2 *Sxl* polyU binding sites in the 5´ UTR and 4 sites in the 3´ UTR. Both 5´ and 3´ UTR binging sites are required for complete repression. However, either the 5´ or 3´ UTR sites all are sufficient for a modest (about 2 fold) decrease in MSL2 translation in female (Bashaw *et al.,* 1995). Computer searching for other genes that have polyU clusters in their 3´ UTRs indicated that genes which have three or more such U stretches, including *runt*, are all X-linked with the exception of *msl1* and *msl2*. This finding has led to the proposal that *Sxl* may directly mediate a second dosage compensation process in females (Kelley *et al.,* 1995).

1.3.5 MSL Proteins Occurs in Other Drosophilid Species

Ignacio *et al.* (1996) reported that the sex-and chromosome-specific binding of three of the MSL proteins (MSL-1, MSL-2, MSL-3) occurs in other drosophilid species, spanning four genera. By using antibodies directed against the MSL-1, MSL-2 and MSL-3 proteins for immunostaining in 14 drosophilid species, they observed male X chromosome-specific staining by at least one of these antibodies in 12 of these species. Staining in females is not observed in any of these 14 species. The male- and chromosome-specific binding of the MSL proteins provides strong evidence for the functional conservation of this system of dosage compensation in all of these species. Staining is seen in both *Chymomyza* and *Hirtodrosophila*, among the more distant relatives of *Drosophila* within the *Drosophilinae* subfamily (Ignacio *et al.* 1996). The *msl*-based system of dosage compensation is therefore much older than the *Drosophila* genus; the *Chymomyza-Drosophila* split is estimated to have occurred at least 55 million years ago (Russo *et al.,* 1995).

1.4 DEVELOPMENT OF INSECT GENETIC TRANSFORMATION SYSTEMS

1.4.1 Overview

1.4.1.1 Transformation and transgenic insects

Efforts to develop genetic-transformation technology for insects span 3 decades. Gene introduction or "genetic-transformation" technologies permit genes of any origin to be introduced into insects, either temporarily or permanently. These will play a critical role in gene function identification and testing. Gene transformation will also enable manipulation of insect genotypes and the potential to devise chemical-free methods for controlling pest insect populations (Atkinson *et al.,* 2001). There are two particular advantages of transgenic technology over classical genetics: (1) the potential to exploit genes and gene constructs across species barriers; and (2) the ability to introduce particular, defined sequences without the genome disruption of a conventional cross (Warren and Crampton, 1994).

The two key requirements for successful transformation are: i.) an efficient means for introducing the DNA into the germ line, and ii.) a sensitive marker gene that can distinguish the transgenic insect from the much more plentiful non-transgenic siblings.

1.4.1.2 Strategies for transforming insects

1.) Physical introduction of DNA

Genetic transformation technologies depend on viable physical and biological methods for delivering transgenes to cells and, ultimately, to the nuclei of the cells of interest. For stable insect transformation, the germ cells are the targets of all efforts at DNA introduction (Atkinson *et al.,* 2001). A number of methods for delivering DNA to developing insect germ cells have been used, including microinjection, biolistics, and electroporation (Rubin and Spradling, 1982; Baldarelli and Lengyel, 1990; Mialhe and Miller, 1994; Leopold *et al.,* 1996).

Microinjection remains the best option for penetrating insect chorions and delivering vector DNA to the germ cells and typically techniques used for handling and microinjecting insect embryos are based on those that have been developed for *D. melanogaster* (Rubin and Spradling, 1982). The characteristics of the insect species will determine how to introduce the DNA into their germline. The simplest method is injection of embryos that works well in *Drosophila* (Rubin *et al.,* 1982), Medfly (Loukeris *et al.,* 1995), blowfly (Atkinson *et al.,* 1992) and mosquito (Jasinskiene *et al.,* 1998; Coates *et al.,* 1998). The modifications required for each species are important for ensuring the survival of the embryos and generation of transgenic insects.

2.) Biological introduction of DNA

Except in *Drosophila*, several gene vectors have been developed that enable the movement of genes in non-drosophilid insects. These include transposable elements, viruses, and *in vivo* recombination systems such as the FLP/FRT system of *Saccharomayces cerevisiae* (Morris, 1997). Only six of these vectors have proven to be of sufficient robustness to be used as genetic tools in insects. These vectors include four transposable elements, the Sindbis alphavirus, and pseudotyped pantropoic retroviruses. The two viral systems are currently limited to the transient expression of genes in individuals that have been directly infected with engineered virus. Nonetheless, the Sindbis system is proving to be a very powerful tool for the analysis of gene expression in mosquitoes. The four transposable-element vectors that have been developed are also proving to be useful genetic tools for the generation of transgenic insects. Transposable elements are the only vectors discussed in this study.

1.4.2 Transposable Elements as Gene Vectors in Insects

1.4.2.1 Classification of transposable elements

Transposable elements include a diverse collection of genetic elements which have in common the ability to promote recombination reactions that result in the movement of the element from one location in the genome to another. The

transposable elements are divided into two classes depending upon the general features of the mechanisms of movement (Finnegan, 1989). Class I elements are those that transpose by reverse transcription of an RNA intermediate, similar to retroviral integration. Class II elements appear to transpose directly from DNA to DNA. They are usually of small to moderate length (less than 10kb).

1.4.2.2 Short inverted repeat-type elements involved in constructing gene vectors

A subclass of class II elements have in common the presence of terminal sequences that form inverted repeats. These terminal inverted repeats are usually less than 50 base pairs and can be as few as eight base pairs. Members of this subclass of elements are called short inverted repeat-type elements and have proven most useful in constructing gene vectors for *Drosophila* and are becoming increasingly important for plant genome manipulation (Walbot, 1992; Hehl, 1994).

Short inverted repeat-type elements have two major functional components, a transcription unit that encodes a protein (transposase) required for transpositional recombination and the terminal inverted repeats of the element including 100 bases or more of sub-terminal DNA. Although not well characterized in eukaryotes, transposases are probably recombinases. The role of the terminal repeats of this class of elements appear to serve as pointers, directing the recombinase and /or other factors to the proper position on the element to initiate recombination.

Short inverted-repeat type elements are particularly amenable to being converted to vectors because the two components of the element can function in *trans*. That is, transposase produced by one element can act on the terminal sequences of another element of the same type and promote its transposition. Consequently, attaching the terminal sequences to any other non-transposable element sequence results in the creation of a chimeric transposable element capable of transposing when the appropriate transposase is present.

Trans-mobilization is a useful feature of this type of transposable element because it permits the investigator to stabilize the movement of the element following integration by removing the source of transposase. These characteristics were first exploited in eukaryotes for the purposes of gene vector development using the *P*-element from *D. melanogaster* (Rubin and Spradling, 1982; Spradling and Rubin, 1982), and this is refereed to as the *P*-element paradigm. Extensions of this technology include transposon tagging (Searles *et al.,* 1982) and enhancer trapping (Bellen *et al.,* 1989) which are powerful methods for identifying and isolating genes.

1.4.2.3 The functional insect vector systems

1.) *P*-element family

P elements were the prototypical short inverted repeat-type element in eukaryotes; they are almost 3 kb in length, encode a single transposase and have terminal inverted repeat sequences of 31 nucleotides.

P-elements were identified in the hybrids of certain inter-strain crosses of *D. melanogaster* (Kidwell *et al.,* 1977). Movement of *P*-elements in the host is strain-dependent with certain strains repressing almost all P movement and others supporting high rates of *P*-element excision and transposition (Engels, 1989). Within *D. melanogaster*, *P*-elements are effective agents for genome manipulation because of their rates of movement (Rio, 1991).

The *D. melanogaster P*-element has been successfully used as a transformation vector in the closely related species, *D. simulans* (Scavarda and Hartl, 1984) and a more distantly related drosophilid, *D. hawaiiensis* (Brennan *et al.,* 1984). Neither of these species possess an endogenous *P*-element.

The *P*-element ultimately proved to be useless for those wishing to genetically transform non-drosophilid insects (Atkinson *et al.,* 2001). The successful creation of transgenic non-drosophilid insects using *P*-element vector-containing plasmids

has been reported but in no case has the integrated DNA consisted of only sequences precisely delimited by the termini of *P*-elements (Miller *et al.,* 1987; McCrane *et al.,* 1988; Morris *et al.,* 1989) suggesting integration was not catalysed by the *P* transposase.

2.) The *hobo* or *hat* element family

The *hobo* element, which was identified in *D. melanogaster* (Lim *et al.,* 1983), is a typical short inverted repeat-type element with a copy number of 0-50 per genome, depending on the strain. It is 3 kb in length with 12 bp terminal inverted repeats, creates 8 bp duplications at the points of integration and probably encodes a single protein (transposase) (Blackman and Gelbart, 1989).

Blackman *et al.* (1989) used *hobo* as a gene vector for germ-line transformation of *D. melanogaster*. Twenty percent or more of the fertile adults developing from embryos injected with the vector produced transgenic progeny. The *hobo* mobility is confined predominantly to the germline by virtue of tissue specific expression of the *hobo* transposase gene (Calvi and Gelbart, 1994). While *hobo* integration rates are comparable to that observed using *P*-elements, insertion site preferences of these two elements are distinct. The spatial distribution of *hobo* and *P*-element integration sites within the *Drosophila* genome overlapped, but there were chromosomal regions that were preferred by only one of the elements (Smith *et al.,* 1993).

The first direct demonstration of *hobo's* ability to function in a non-host insect was reported by Atkinson *et al.* (1993). They demonstrated that *hobo* could excise from plasmids introduced into and transiently maintained extrachromosomally in cells of developing *Musca domestica* (housefly) embryos. Similar results were reported in distantly related *Drosophila* species (Handler and Gomez, 1995) and currently the range of species in which *hobo* has been shown to excise includes the mosquitoes, *Aedes aegypti* and *Aedes australis* (Sarkar *et al*, 1997b) and the lepidopteran, *Helicoverpa armigera* (Pinkerton *et al.,* 1996). Interplasmid

transposition assays in a number of diverse non-drosophilid insect species including the housefly, *M. domestica*, the Queensland fruitfly, *Bactrocera tryoni* and the corn earworm, *H. armigera* demonstrated *hobo's* ability to transpose accurately in non-host insects (O'Brochta *et al.,* 1994; Pinkerton *et al.,* 1996). The frequency of *hobo* transposition in non-drosophilids was less than that observed in *D. melanogaster* under similar conditions. *hobo* has been successfully used as a germ-line transformation vector in *D. virilis* (Lozovskaya *et al.,* 1996).

Using *hobo* excision as a bioassay for *hobo* or *hobo*-like transposase activity, several *hAT* elements have been isolated from non-drosophilid insects:

Hermes, isolated from *M. domestica*, is closely related to *hobo* and based on structural criteria appeared functional (Warren *et al.,* 1994). *Hermes* is 2749 base pairs in length, flanked by 17 bp imperfect terminal inverted repeats, and encodes a transposase protein that is 70 kDa in size (Warren *et al.,* 1994).

The *hobo* and *Hermes* transposases are 55% identical and 70% similar at the amino acid level, and their inverted terminal repeats are identical over 10 and 11 out of 12 nucleotides (Warren *et al.,* 1994). *Hermes* elements have been found in house fly populations throughout the world. To date no housefly populations devoid of *Hermes* elements have been found (Atkinson *et al.,* 2001)

Hermes has been used to generate stable transgenic lines from six insect species: *D. melanogaster* (O'Brochta *et al.,* 1996; Pinkerton *et al.,* 2000), *Ae. aegypti* (Jasinskiene *et al.,* 1998; Pinkerton *et al.,* 2000), *Ceratitis capitata* (Michel *et al.,* 2001), *Stomoxys calcitrans* (O'Brochta *et al.,* 2000), *Tribolium castaneum* (Berghammer *et al.,* 1999) and *Culex quinquefasciatus* (Allen *et al.,* 2001). *Hermes*-mediated transformation of *D. melanogaster* can be as high as 60% but is routinely around 30%-40% (O'Brochta *et al.,* 1996; Pinkerton *et al.,* 2000).

Hermes-mediated transformation of the mosquitoes *Ae. aegypti* and *Cx. quinquefasciatus* results in integration of both the *Hermes* element and flanking

and plasmid sequences (Jasinskiene *et al.*, 1998; Pinkerton *et al.,* 2000.) *Hermes* can, however, transpose in a predicted manner in mosquitoes (Sarkar *et al.,* 1997; Zhao and Eggleston, 1998). The sequences transposed were delimited by the terminal nucleotides of the transposable element, and an 8-bp duplication was created at the target site.

Hermit (*L. cuprina*) is a 2716 bp transposable element with 15bp terminal inverted repeats. Southern blot analysis of genomic DNA indicated that *Hermit* was present once in the genome of the strain of *L. cuprina* from which it was originally isolated and in 10 other strains from Australia, New Zealand and South Africa (Coates *et al.,* 1996).

Hector from the Australian bush fly, *M. vetustissima,* is highly similar to *hobo* and *Hermes*. *Hector* is a single copy sequence in the *M. vetustissima* strain from which it was isolated (Warren *et al.,* 1995).

3.) The *mariner* element family

mariner elements are wide-spread in insects (Robertson 1993; Robertson *et al.,* 1995) including *D. simulans*, *D. sechellia, D. teissieri, D. yakuba* and *Zaprionus tuberculatus* (Capy *et al.,* 1991; Maruyama and Hartl, 1991b). However most of the elements appear to be non-functional. This made it extremely difficult to isolate the few forms of the element that may encode a functional transposase (Atkinson *et al.,* 2001). To date only two *mariner* elements have been isolated or reconstructed from insects-*Mosl* and *Himar*.

Mosl from *D. mauritiana*, is the only naturally occuring *mariner* element that has been isolated from insects. *Mosl* is 1286 bp in length with a single long ORF. The element has 28 bp inverted terminal repeats containing four mismatches and creates a 2 bp TA repeat at its insertion site (Jacobson and Hartl, 1985; Haymer and Marsh, 1986).

Mos1 has been used for transformation of *D. melanogaster* (Lidholm *et al.*, 1993) and *Ae. aegypti* (Coates *et al.,* 1997, 1998). Interplasmid transposition assays demonstrated that *Mos1* could accurately transpose in at least three species of non-drosophilid insects (Coates *et al.,* 2000): *Ae. aegypti, Lucilia cuprina*, and *Bactrocera tryoni. Mos1* has also been used for transformation of other eukaryotes including Leishmania, zebrafish, and chickens (Fadool *et al.,* 1998; Gueiros-Filho and Beverley, 1997; Sherman *et al.,* 1998).

In *D.melanogaster* transformants, in transpositions arising from interplasmid transposition assays performed in *B. tryoni, L. cuprina, and Ae. aegypti,* and in transpositions performed *in vitro*, the *Mos1* sequences that are integrated are delimited by the terminal nucleotides of the *Mos1* element (Lidholm *et al.,* 1993; Coates *et al.,* 1997, 1998; Tosi and Beverley, 2000). In *Ae. aegypti* transgenics, most (three of four) of the transformed lines contain *Mos1* elements that have been integrated in the same manner, however, one of the lines contains *Mos1* element together with flanking plasmid DNA sequences (Coates *et al.,* 1998). When *Mos1* transposase protein was used instead of helper plasmid, only these types of integration events were recovered (Coates *et al.,* 2000).

Himar is a reconstructed element based on the sequence of various copies of *mariner* elements isolated from the horn fly, *Haematobia irritans* (Lampe *et al.,* 1996). *Himar* is mobile in *D. melanogaster* but is unsuccessful as a gene vector in this species (Lampe *et al.,* 1996)

Himar1 has been genetically modified both to improve our understanding of the molecular basis of its movement and also to isolate hyperactive forms of this element (Lampe *et al.,* 1999). Two *Himar1* mutants displayed increased levels of transposition in *E. coli*. Neither of these, however, showed an increase in transpositional activity in *Drosophila*. Nevertheless this type of strategy will no doubt lead to new forms of *Himar1*, and some of these forms will most likely also have hypermobility properties in insects.

As for other members of the *mariner/Tc1* superfamily of elements, *Mos1* and *Himar1* are inserted only at TA dinucleotide sequences where they create 2-bp target site duplications. Studies performed *in vitro* for both elements have revealed that this insertional specificity is dependent on the presence of magnesium and is reduced when manganese is substituted for magnesium (Lampe *et al.,* 1998; Tosi and Beverley, 2000). The physical properties of both transposases are similar, and both displayed increased rates of transposition with increasing transposase concentration (Lampe *et al.,* 1998; Tosi and Beverley, 2000).

4.) The *Tcl* element family

The *Tcl* element is a short inverted repeat-type element originally identified and isolated from the nematode *Caenorhabditis elegans* (Emmons *et al.,* 1983). Related elements are found in a number of other organisms including insects. Four *Tcl*-like elements have been isolated from *Drosophila* species; *HB* and *Baril* from *D. melanogaster* (Caizzi *et al.,* 1993), *Uhu* from *D. heteroneura* (Brezinsky *et al.,* 1990) and *Minos* from *D. hydei* (Franz and Savakis, 1991).

Minos, is currently of the most interest of this family. It was originally identified as a dispersed repetitive sequence within the transcribed spacer of a rRNA gene of *D. hydei* (Franz and Savakis, 1991). *Minos* is 1775 bp in length, has inverted terminal repeats of 255 bp and two long non-overlapping ORFs separated by a 60-bp intron. The two open reading frames are part of the same transcription unit and result in a spliced product (Franz and Savakis, 1991). *Minos* inserts at TA residues and creates 2-bp target site duplications (Franz and Savakis, 1991). *Minos* is inserted at multiple locations within the genome of *D. hydei* and different strains of this species display insertion site polymorphisms (Franz *et al.,* 1994).

Minos has been shown to be capable of transposition in several different *An. gambiae* cell lines as well as in developing *Anopheles stephensi* embryos (Catteruccia *et al.,* 2000a). Successful transformation of *An. stephensi*, using a *Minos* element containing the *EGFP* genetic marker, has been reported

(Catteruccia *et al.,* 2000b). Two types of integration events were observed for *Minos* when it was transfected into *anopheline* cell lines (Catteruccia *et al.,* 2000a). One type of integration event involved the *Minos* element and flanking sequences. The second type of integration event occurred through the cut-and paste transposition of the *Minos* element into the *anopheline* genome and created TA target site duplications associated with this type of transposition. The reason for this difference in integration mode is unknown, although Catteruccia *et al.* (2000a) speculated that the cut-and-paste mode of transposition was perhaps more likely to occur with increasing transposase concentration.

Tcl is highly mobile in *C. elegans*, while *Minos* appears to be the only known active *Tcl*-like element from insects (Franz *et al.,* 1994). *Minos* is also mobile in non-host species of insects such as *D. melanogaster* and resulted in germ-line transformants at a frequency of approximately 2% (Loukeris *et al.,* 1995a). Integrated *Minos* elements were delimited by the inverted terminal repeats and created TA dinucleotide duplications at the integration site. *Minos* was also used as a germline transformation vector in the Medfly, *C. capitata*, which was the first successful transformation of non-*drosophilid* insects (Loukeris *et al.,* 1995b). A *Minos* vector was constructed by replacing the transposase transcription unit with the cDNA *C. capitata white*$^{+}$ gene controlled by the *hsp70* promoter of *D. melanogaster*. The *Minos* vector and transposase helper plasmids were co-injected into preblastoderm Medfly embryos homozygous for a recessive mutation in the *white* locus. The transformation rate was 1.3%.

5.) The TTAA-specific transposable element family

TTAA-specific transposable elements are a diverse group of short inverted repeat-type elements that were identified as insertion sequences in baculoviruses that infect lepidoptera cell cultures. These sequences were subsequently found within the genomes of the lepidoptera from which the baculoviruses were isolated (Beames and Summers, 1990; Fraser *et al.,* 1985). These elements share two features. First, the 5' end of their terminal inverted repeats end in two or three C residues. Second, these elements are always found

inserted in a TTAA target site. Beyond these similarities the elements have little in common. Their terminal inverted repeats are 13-15 nucleotides and their overall length can vary from 2.5 kb (*piggyBac* or IFP2) (Fraser *et al.,* 1983; Cary *et al.,* 1989) to 780 bp (*tagalong* of TFP2) (Fraser *et al.,* 1983).

Of the TTAA-specific transposable element family, *piggyBac* has been developed into an efficient gene vector in insects. The*piggyBac* element, isolated originally as an insertion sequence in the *Galleria mellonella NPV* (*GmMNPV*) following passage in the *Trichoplusia ni*-derived cell line TN-368. piggyBac is 2.5 kb in length and possesses 13-bp inverted terminal repeats (Fraser *et al.,* 1995). It contains a 2.1 kb long open reading frame that encodes a transposase with little or no structural similarity to other eukaryotic transposases. The *piggyBac* element inserts at TTAA sequences in the genome and, upon insertion, generates a duplication of this sequence (Fraser *et al.,* 1995). *piggyBac* also excises precisely from the donor site (Elick *et al.,* 1996).

piggyBac has been used for germline transformation in a range of insect species, including *D. melanogaster* (Handler and Harrell, 1999), *C. capitata* (Handler *et al*., 1998), *Anastrepha suspensa* (Handler, 2001), *M. domestica* (Hediger *et al.,* 2001), *T. castaneum* (Berghammer *et al.,* 1999), *B. mori* (Toshiki *et al.,* 2000), *P. gossypiella* (Peloquin *et al.,* 2000), and *L. cuprina* (Heindrich *et al.,* 2002). In all cases, integration has been by transpositional recombination of the *piggyBac* element. Transformation frequencies generally vary from 1-10%, although an extremely high value of 60% was observed for *T. castaneum* transformation (Berghammer *et al.,* 1999).

1.4.3 The Promoters Commonly Used in Transformation Systems

1.4.3.1 *hsp70* gene promoter

In *Drosophila*, the *hsp70* gene is the most highly conserved of seven heat shock protein-coding genes (Ashburner and Bonner, 1979). It has been shown that expression of cloned copies of the *hsp70* gene introduced into the genome of

mouse tissue culture cells can be controlled by heat shock, indicating a considerable conservation of regulatory signals (Corces *et al.,* 1981). The *hsp70* genes of *D. melanogaster* appear to be activated exclusively in response to heat shock. The expression of the *hsp70* gene is normally at a very low level and increases by more than 2 orders of magnitude during heat shock (Atkinson *et al.,* 1985). Maximum synthesis of *hsp70* occurs at 37°C (Lindquist, 1980). Induction involves the binding of a heat shock factor (HSF) to several sites located upstream from –50 with respect to the transcription start point. The heat shock elements (HSEs) within the region from -90 to -50 are sufficient to confer heat shock induction (Xiao *et al.,* 1988). Analysis of the structure of the *hsp70* promoter reveals that TFIID, RNA polymerase II, and another sequence-specific factor called the GAGA factor associate with the promoter prior to heat shock induction (Lis *et al.,* 1993; Weber *et al.,* 1997). The *hsp70* promoter of *D. melanogaster* has been an important paradigm for investigating the mechanism of transcriptional regulation (Hoch *et al.,* 1990; Chi *et al.,* 1995). However, the *hsp70* promoter appears to have low activity in *L. cuprina* embryos (Atkinson and O'Brochta, 1992).

1.4.3.2 *hsp83* gene promoter

The *hsp83* gene (or *hsp82* in some species) is one of the major heat shock genes of *D. melanogaster.* It is a single-copy gene in the *D. melanogaster* genome and encodes the largest major heat-shock polypeptide (Holmgren *et al.,* 1979). The *hsp83* gene is highly conserved with homologues in *E. coli*, yeast, *Drosophila* and humans (Farrelly *et al.,* 1984; Bardwell and Craig, 1987; Rebbe *et al.,* 1987). Studies of the *hsp83* genes from four *Drosophila* species revealed conserved DNA sequences that are postulated to be involved in the heat shock response (Blackman and Meselson, 1986). The *hsp83* gene regulatory region contains heat-responsive elements, with three overlapping copies of the heat shock consensus sequences (-88 to -49, CnnGAAnn TTCnnG) (Pelham *et al.,* 1982; Pelham, 1985) being required for full levels of heat shock-inducible expression of the *hsp83* gene (Xiao *et al.,* 1989). In contrast to the *hsp70*, the *hsp83* gene in *D. melanogaster* is expressed at high levels at all stages during normal

development of the animal (Mason *et al.,* 1984) and increases only several fold in response to heat shock (Xiao *et al.,* 1989). The maximum synthesis of *hsp83* occurs at 33°C (Lindquist, 1980). Coates *et al.* (1996) reported that *hsp83* from *D. pseudoobscura* elicits a high level of expression of the *cat* (Chloramphenicol acetyltransferase) gene in *L. cuprina* embryos. Since the *hsp70* promoter has low activity in this species, this result suggests that the *hsp83* promoter may be utilised as an exogenous promoter in other non-*drosophilid* insects in which poor expression levels are obtained from constructs containing the *hsp70* promoter.

1.4.3.3 *α1-tubulin* promoter

α1t gene encodes *α1–tubulin* protein, a subunit of microtubules, which are necessary for cell division, cell motility, intracellular transport, and maintenance of cell structure. The *α1t* gene is one of the four *α-tubulin* genes of *D. melanogaster* and is the only one that appears to be expressed at high levels in all tissues and stages of development (Kalfayan and Wensink., 1982; Natzle *et al.,* 1984; Matthews *et al.,* 1989;).

Three regions within -157 to +696 of the transcription start site are sufficient to direct the *α1t* core promoter to begin transcribing at the stage of cellular blastoderm formation and to continue thereafter at high levels in all tissues and developmental stages. The first two regions (TE1, 29bp and TE2, 68bp) are located immediately upstream (-157 to - 61) of the core promoter (-35 to -26). They are independent regulatory elements that function in all tissues and prevent chromosomal position effects. The third region is located a short distance downstream from the core promoter within the first intron. These three elements protect the *α1t* promoter from distal enhancers or silencers act in all tissues (O'Donnell *et al.,* 1994). The *α1t* promoter provides a means for ubiquitous expression of gene products independent of the site of chromosome insertion.

1.4.3.4 *armadillo* promoter

armadillo , a segment polarity gene, produces very abundant transcripts, which are found at all stages but accumulate to the highest levels during early to mid embryogenesis and early pupal development (Riggleman *et al.,* 1989). *arm* transcripts are uniformly distributed and expressed in a wide range of cell types (Riggleman *et al.,* 1989). *arm* is known to have a broad developmental requirement, and loss of *arm* function appears to affect many cell types at all stages (Gergen and Wieschaus, 1986). The *arm* promoter has been used as a constitutive promoter to drive β–galactosidase expression in all cell types in *Drosophila* (Vincent *et al.,* 1994)

1.4.4 Marker Genes for Identification of Insects

Early attempts at transformation of insects often used drug resistance gene as a marker, but the selection conditions are empirical and the false positive rate is high (Ashburner, 1995). Insecticide resistance (for example, to dieldrin or parathion) (Benedict *et al.,* 1994) genes may be better, but, best of all would be a mutant in the target insect that has a phenotype which can be complemented by a transformed gene.

1.4.4.1 The *white* genes

The white gene of *D. melanogaster* is widely used as a marker gene to identify transformants in this species. Similarly, the Medfly *white* gene functions as a transformation marker in transgenic *Drosophila* (Zwiebel *et al.,* 1995) and Medfly, *Ceratitis capitata* (Loukeris *et al.,* 1995b). Further, white-eyed mutants of *L. cuprina* exist and the *white* gene of this species has been isolated (Elizor, 1990)

1.4.4.2 *GFP* marker gene

The green fluorescent protein (GFP), from jellyfish *Aequorea victoria* (Chalfie, *et al.,* 1994), is monomeric, soluble, extremely stable and brightly fluorescent. These properties derive from its tightly packed three-dimensional structure that rigidly holds a chromophore within the core of an eleven-stranded β–barrel (Tsien, 1998). With the ability to clone and express GFP in a diverse range of cells and

organisms including bacteria, yeast, plants and higher animals, GFP has become a versatile fluorescent marker for monitoring physiological processes, visualising protein localisation and detecting the expression of transferred genes (Gerdes and Kaether, 1996). The wild-type GFP consists of 238 amino acids (Yang *et al.,* 1996). This protein fluoresces without specific cofactors when illuminated at the correct wavelengths, and can be detected in live animals provided they are reasonably transparent (Chalfie *et al.,* 1994; Wang and Hazerigg, 1994). Further, the fluorescence can be quantified using fluorimeters or imaging devices.

Since there is no enzymatic amplification step, GFP is less sensitive as a reporter gene than the bacterial β-galactosidase gene that has been widely used. However, new generations of mutant GFPs that are brighter have compensated for this shortcoming. Heim *et al.* (1995) developed a mutant GFP (S65T) that improved fluoresence intensity 4- to 6-fold upon excitation. By a molecular evolution method using a DNA shuffling technique, Crameri *et al.* (1996) obtained a mutant with a whole cell fluorescence signal that was 45-fold greater than standard.

GFP or its enhanced derivative (EGFP) has been used as a genetic marker in organisms as diverse as *D. melanogaster* (Plautz *et al.,* 1996; Handler and Harrell, 1999), *Ae. Aegypti* (Pinkerton *et al.,* 2000), pink bollworm (Lepidoptera: Gelechiidae) (Peloquin *et al.,* 2000), silk worm (Tamura *et al.,* 2000), *Caenorhabditis elegans* (Chalfie *et al.,* 1994), zebrafish (Amsterdam *et al.,* 1995) and mammals (Ikawa *et al.,* 1999).

Horn (2000) reported that an artificial promoter containing three binding sites for Pax-6 homodimers in front of a TATA box (3xP3) was hyperactive, regionally restricted and universal. This promoter can drive expression of an enhanced GFP variant (EGFP) in the eye of *D. melanogaster* and in the flour beetle *Tribolium castaneum* (Tsien, 1998). The evolutionary conservation and the 'master regulatory' function of Pax-6 in the eye development of insects and vertebrates means that the *3xP3* promoter should be active in any photoreceptor cell. The

small size of the marker gene (1.3 kb) allows for small transposon constructs, resulting in high transformation rates. Horn *et al*. (2000) constructed three vectors based on the *Hermes*, *piggyBac* and *mariner* transposons, each carrying the *3xP3-EGFP* marker. Together with helper plasmids to provide the respective transposase, these vectors were microinjected into *Drosophila* eggs of a strain mutant for the white gene. They obtained transgenic lines displaying strong fluorescence with transformation efficiencies of 4% for *mariner*, 50% for *Hermes* and 35% for *piggyBac.*

1.4.4.3 *cinnabar* marker gene

The *cinnabar* eye colour gene is one of the four genes that are absolutely required for xanthommatin synthesis (reviewed by Lizen, 1974). Xanthommatin is the main ommochrome screening pigment found in dipteran eyes which is biosynthetically derived from tryptophan via a series of oxidation reactions that involve N-formylkynurenine, kynurenine, and 3-hydroxykynurenine as intermediates (reviewed by Linzen, 1974; Summers *et al.,* 1982). The *cinnabar* (*cn*) gene encodes the third enzyme of the pathway, kynurenine 3-monooxygenase (Sullivan *et al.,* 1973; Warren *et al.,* 1996).

The *cinnabar* (*cn*) gene was successfully used as a marker gene in *D. melanogaster* a white-eyed strain has been isolated that lacks kynurenine 3-monooxygenase activity. It has also been used successfully as a marker gene in the strain of *Ae. aegypti* (Jasinskiene *et al.,* 1998; Coates *et al.,* 1998).

1.5 INDUCIBLE GENE EXPRESSION SYSTEMS

1.5.1 Overview

An inducible gene expression system involves the ability to adjust levels of gene expression by varying levels of inducer, thereby permitting correlation of phenotype with the level of expression (Jaenisch, 1988).

Several inducible systems for regulating target gene expression have been reported in the past. The earliest systems involved the use of heat shock (Bienz *et al.,* 1986), isoprotyl β-D-thiogalactoside (IPTG) (Baim *et al.,* 1991), and heavy metals (Mayo *et al.,* 1982) as inducing agents. Because of their lack of specificity (heat shock induces many other endogenous genes) and toxicity (associated with IPTG and heavy metals), several research groups reported the successful use of drug- or ligand-inducible systems *in vitro* and to some extent *in vivo.* Such inducing agents or drugs include FK1012 or rapamycin (Spencer *et. al*, 1993) mifepristone (RU 486) (Wang *et al.,* 1994, 1997), tetracycline (Gossen *et al.,* 1995, Bello *et al.,* 1998), and ecdysone (No *et al.,* 1996). A common approach with these various drug-inducible systems involves the use of a chimeric transcriptional activator that reversibly binds a target gene construct in response to the administered drug or ligand.

1.5.2 Tetracycline-Regulated Inducible Gene Expression Systems

1.5.2. 1 Mechanism of tetracycline action on bacteria

Tetracycline (tet) is assumed to enter the bacterial cell by diffusion across the cytoplasmic membrane in the neutral form. After tetracycline has diffused through the cytoplasmic membrane into bacteria of different species it chelates a divalent cation, preferentially Mg^{2+}. The complex $[MgTc]^{+}$ acts by binding to the small (30S) prokaryotic ribosomal subunit, thereby inhibiting protein biosynthesis and stalling the growth of the invaded cell (Wissmann and Hillen, 1989).

1.5.2.2 The resistance mechanisms of bacteria against the tetracycline-type antibiotics

The presently known resistance mechanisms against the tetracycline-type antibiotics involve protons that either protect the ribosome from attack by $[MgTc)^{+}$ (Burdett, 1991) or export tetracycline out of the bacteria cell (Mendez, *et al.*, 1980; Schnappinger and Hillen, 1996).

The export mechanism is most abundant among gram-negative bacteria. It relies on the transport protein TetA, which is embedded in the cytoplasmic membrane and acts as an antiporter by coupling the export of $[MgTc]^+$ out of the resistant bacterial cell with the uptake of H^+ (Eckert and Beck, 1989; Mager and Saier, 1993). The expression of TetA is tightly regulated by the tetracycline repressor (tetR). TetA would be disadvantageous for the cell in the absence of $[MgTc]^+$, since it interferes with the maintenance of the eletrostatic potential across the cell membrane (Ecker and Beck, 1989).

1.5.2.3 Induction of the tetracycline repressor (*TetR*) by [MgTc] $^+$

The tetracycline repressor occurs as the homodimer $(TetR)_2$ in which two identical helix-turn-helix (HTH) motifs bind in the absence of [MgTc] $^+$ to two adjacent major grooves of DNA. $(TetR)_2$ has only a low affinity to nonspecific DNA sequences but binds tightly to two specific DNA operator sequences, *tetO1* and *tetO2*. Binding of $[MgTc]^+$ to $(TetR)_2$ induces a conformational change that weakens its affinity to *tetO* in two steps, depending on conditions: $10^2 \pm 10^3$ fold for the first and 10^4 - 10^7 fold for the second bound $[MgTc]^+$. Thus the overall affinity of (TetR)2 to *tetO* is reduced by 6-10 orders of magnitude (Leder *et al.,* 1995), so that expression of the genes *tetR* and *tetA* can now proceed. The antiporter protein TetA is inserted into the cytolplasmic membrane and exports [MgTc] $^+$, thereby preventing attack of [MgTc] $^+$ at the ribosomal 30S subunit, which has 1000-fold lower affinity to [MgTc] $^+$ than $(TetR)_2$.

1.5.2.4 Tetracycline-regulated inducible gene expression systems

The inhibition of $(TetR)_2$ binding to the tetO DNA [MgTc] $^+$ has been utilized by molecular biologists as an easily controllable switch for the regulation of gene expression in transgenic organisms (Gatz *et al.*, 1991; Wirtz and Clatyton, 1995; Freundlieb *et al.*, 1997).

The first tetracycline-regulated gene expression system developed by Gossen and Bujard (1992), for use in mammalian cells, provides a genetic tool for controlled gene expression in eukaryotic cells. This system consists of constitutive expression of the tetracycline transactivator protein (tTA) and the tetracycline inducible promoter pTet.

tTA is a fusion protein (Gossen and Bujard, 1992) composed of a 207 amino acid sequence of tetracycline tetR and the transcriptional activation domain of the VP16 protein of herpes simplex virus. pTet contains the human cytomegalovirus (CMV) immediate early (IE) minimal promoter/enhancer preceded by the *tetO*. The gene to be regulated by transactivator tTA is placed under the control of pTet. In the absence of tetracycline, the tetracycline repressor portion of tTA mediates high affinity, specific binding to *tetO*. In the presence of tetracycline, however, a conformational change in tetR prevents tTA from binding to *tetO* (Heinrichs *et al.,* 1994). In the initial study, performed in HeLa cells stably expressing tTA, expression of luciferase was very low in the presence of ng/ml quantities of tetracycline, and removal of tetracycline resulted in as much as a 100,000-fold increase in luciferase levels (Gossen and Bujard, 1992).

This basic system has been used extensively in tissue culture for the expression of a variety of different genes (Fruh *et al.,* 1994, 1995). This system has also been used to identify the targets of a viral transcriptional transactivator, to examine the phenotype of cells over expressing a tyrosine kinase that regulates *c-src*, and to determine the consequences of disregulated expression of various cell cycle regulators both in HeLa cells and rat fibroblasts (Mendez *et al.*, 1980; Schnappinger and Hillen, 1996). This system has also been used to produce transgenic mice reversibly expressing luciferase or β–galactosidase in a variety of fetal and adult tissues (Furth *et al.,* 1994).

In an attempt to activate higher levels of gene expression than those obtained with the basic system, and to prevent possible toxic effects of constitutive *tTA* expression, Shockett *et al*. (1995) placed *tTA* under the control of *tetP*, resulting in

the autoactivation of tTA in the absence of tetracycline. This autoregulatory system appeared to have two important advantages when compared with a system constitutively expressing tTA: it yields substantially higher levels of target gene expression, and the frequency of inducible clones obtained was higher. Transgenic mice produced by coinjection of the autoregulatory *tTA* and a *tetP*-driven luciferase transgene expressed luciferase inducibly in a variety of tissues with highest levels in thymus and lung (Shockett *et al.,* 1995).

Gossen *et al.* (1995) developed a modified system by generating a mutant tetR: VP16 fusion protein (rtTA) which has the reverse property of only binding to the *tetO* and activating transcription in the presence of the tetracycline derivatives, doxycycline or hydrotetracycline. Using the CMV IE promoter to drive stable expression of rtTA in HeLa cells, luciferase activity could be induced by 3 orders of magnitude in 20 hrs after the addition of the tetracycline derivatives. This system now has many applications in various fields of medical research (Bieschke *et al.,* 1998; Xiao *et al.,* 2000).

Although the systems described above have been used successfully in many cell lines, cautions have been raised regarding the general efficacy of the systems in all cell or tissue types (How *et al.,* 1995). Because of the heterogeneity in gene expression that has been observed in some cases, it is generally agreed that success in any given cell or tissue milieu might require alternative minimal promoters and careful choice of constitutive or tissue-specific promoters for transactivator expression (Gossen and Bujar, 1995; Hennighausen *et al.,* 1995).

A study of transgenic tobacco plants by Weinmann *et al.* (1994) demonstrated the feasibility of placing the tTA and reporter genes in opposing orientations on a single vector. Streamlined single vector expression systems for mammalian cells have also been developed and provide advantages for certain applications. Baron *et al.* (1995) constructed a series of plasmids that contain two minimal promoters in opposite orientations on either side of the heptamerized *tetO*

allowing the tetracycline-regulated expression of two genes in stroichiometric amounts from a single vector.

1.5.2.5 The inducible gene expression systems in *D. melanogaster*

In *Drosophila*, two major systems have been designed to achieve the conditional expression of gene constructs integrated into the genome. One is that the target gene is placed under the control of a heat shock (*hsp*) protein gene promoter. The heat inducible promoter of the *hsp70* gene is commonly used for this purpose (Schneuwly *et al.,* 1987). A high level of expression can be obtained following a heat shock to the individuals at heat shock temperatures. Because the induction of the transgene can be precisely controlled, this offers a unique opportunity to determine at which stage the gene is required to achieve its function. This approach however has limitations. First the heat shock stress required for induction causes the activity of the genes expressed at normal temperatures to be severely repressed (Gibson and Gehring, 1988). Thus, it may be difficult to distinguish between the primary and secondary effects of over expression. Second, since heat shock causes the ectopic expression in all cells, various indirect effects, including lethality, may mask the result of the ectopic expression in the desired cell types.

Another approach to achieve the conditional expression of a gene is to engineer a gene construct inducible by a single transcription factor whose activity can be controlled *in vivo* such as tTA.

Bello *et al.* (1998) generated transgenic lines carrying the tetracycline-controlled transactivator gene controlled by the *hsp70* promoter and the *E. coli lacZ* gene fused to a tTA-responsive promoter. These authors found that expression of β--galactosidase can be efficiently inhibited in larvae with tetracycline provided in the food, and that simple removal of the larvae from tetracycline exposure results in the induction of the enzyme in a time- and concentration-dependent manner.

Bello *et al.* also made transgenic lines carrying the *antennapedia* (*ANTP*) gene controlled by the tTA responsive promoter. Treatment with tetracycline was used to prevent the lethality associated with the ectopic expression of ANTP in embryos and subsequently, to control the timing of the homeoprotein ANTP specifically in the antennal imaginal disc. Their results show that the expression of a gene placed under the control of a tetracycline-responsive promoter can be tightly controlled in insects, both spatially by the regulatory sequences driving the expression of tTA and temporally by tetracycline.

Bieschke *et al.* (1998) developed a doxycycline (dox)-induced transgene expression system ("tetracycline-on" system) in *Drosophila melanogaster*. Using the *actin5C* promoter to drive the expression of *rtTA* in transgenic flies. For the reporter gene, they made three constructs each contained the *E. coli* β-*galactosidase* (*β-gal*) gene driven by *tetOx7-adh* core promoter and *tetOx7-hsp 70* core promoter respectively. Feeding of the flies containing the *actin5C-rtTA* transgene and any one of the three reporter constructs with dox caused up to 100-fold induction of *β–gal*.

Heinrich and Scott (2000) developed a tetracycline induced female-specific lethal system in *Drosophila melanogaster*. In this system the regulator gene tTA was placed under the control of the fat body and the female-specific transcription enhancer from the *yolk protein 1* (*yp1*) gene. The targeting gene was the proapoptotic gene *hid* driven by 7 copies of *tetO* linked to a minimal promoter. Female flies died in the absence of tetracycline added to the culture medium.

1.6 RESEARCH AIMS

The main objective of this study was to develop a female-killing system for Australian sheep blowfly, *L. cuprina* based on the *D. melanogaster msl2* gene which will enhance the ability to eradicate economic pests by SIT technology.

The specific aims of this study were:

1. Develop new *piggyBac* and *Minos* transformation systems and use *Drosophila melanogaster* as a model system to evaluate their effectiveness at mediating both inter-plasmid transposition and germ-line transformation.

2. To use the *piggyBac* and /or *Minos* helper plasmids to develop a method for germline transformation of *L. cuprina*.

3. To develop and evaluate a controllable female-killing genetic system *in D. melanogaster* based on the regulation of *msl2* gene expression by tTA.

CHAPTER 2

MATERIALS AND METHODS

2.1 PLASMIDS AND BACTERIAL STRAINS

The plasmids and bacterial strains used in this study are described in Table 2.1.

2.2 BACTERIAL MEDIA

2.2.1 Luria Broth (LB)

LB 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.

2.2.2 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, $MgSO_4$.

2.2.3 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.

2.2.4 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 0.72 M K_2HPO_4.

2.2.5 Brain Heart Infusion (OXOID)

Brain Heart Infusion (BHI) (g/L): 37 g was added to 1 litre of distilled water which contained: Calf brain infusion solids, 12.5; Beef heart infusion solids, 5.0;

Table 2.1 Plasmid or bacterial strain

Plasmid or Bacterial Strain	Relevant Characteristic	Source
Plasmids		
pBluescript IIKS-	2.96 kb phagemid derived from pUC19. Contains *lacZ* gene interrupted by multiple cloning sites flanked by T3 and T7 promoters.	Stratagen
pCaSpeR-arm-βgal	11 3.75kb, pCaSpeR (pUC, mini-white, *P*-element 5' and 3' ends) containing 1 .7 kb *Eco*R I/*Kpn* I armadillo promoter and 4.4kb *lacZ* with SV40 3' UTR.	J.P Vincent
pCaSpeR4	7.9 kb, pCaSpeR with additional *Xho* I (2), *Stu* I, *Hpa* I (3), *Sal* I, *Spe* I, Sfi I, *Not* I, *Sst*I I, *Kpn* I sites, with *Sst*I and *Eco*R I sites removed.	V. Pirrotta

pTURBO(pUCsπΔ23)	7.3 kb, pUC118 containing 3.6 kb *P*-element coding sequence, *hsp70* promoter and *ry* 3´ flanking region	Rio and Rubin, (1985)
pHSShsILMi20	5 kb, *Minos* gene controlled by *hsp70* promoter.	B. Savakis
pMiwNotI	8kb,pTZ18R based vector, carrying *hsp70* promoter / *C.cw/hsp70 polyAsignal.*	B.Savakis
p3E1.2	5.962 kb, pUC18 containing an active *piggyBac* element with a *Hpa* I unique site.	P. Shirk and M. Fraser
pBAC(C.cw+)	9.6 kb, a *Not* I site was introduced into the unique *Hpa*I site of p3E1.2, and a *Not* I fragment containing the *D. melanogaster hsp70* promoter and C.c*w+* gene was inserted. The piggyBacORF has been disrupted, so won't transpose unless povided with this transposase.	A. Handler
pBspIIKS(+)-2NotI	3 kb, pBluescriptIIks(+) with another *Not* I site added between *Kpn* I/*Xho* I	J. Heinrich (our lab)

pW.T. ΔPr.	9 kb, *P* element based vector containing *w* / multi cloning sites/ *tTA*.	A. Bello, W. Gehring
pBac(C.cw+)-αl-tub	10.4 kb, pBac(C.c .w+) added *α1-tub* promoter.	J. Heinrich (our lab)
pGEM-T-α1-tub	3.9 kb, pGEM-T containing *α1-tub* promoter.	J. Heinrich (our lab)
pBsptIIKS(+)-hsp83	3.8 kb, pbluescriptIIks (+) containing *hsp83* promoter between *Eco*R V and *Bam*H1 sites.	J.Heinrich (our lab)
pXL1	5.3 kb, pBulescriptIIKS- contains *msl-2* between *Eco*R I and *Bam*H I. *Sma*I site is destroyed.	This study
pXL4	10.8 kb, pCaSpeR-arm-βgal cantains 2.3 kb *msl-2* between *Eco*R I and *Xba* I sites and SV40 polyA signal.	This study
pXL9	11.4 kb, pCaSpeR4 added 3.55kb *hs70 promoter /msl2 / SV40* poly A signal gene cassette in *Not* I site.	This study

pXL12	7.5 kb, pBluescriptKS(+)-2NotI added *D. melanogaster* 4.5 kb *mini-white* gene in *Hin*d III site.	This study
pXL13	10.05 kb, pUCBac(C.cw+) replaced *C. cw* with *D. melanogaster mini-white* between *Not* I sites.	This study
pXL14	4.55 kb, pBluescriptIIKS(-)added 1.58 kb *piggyBac* tail *Eco*RV at site.	This study
pXL15	5.03 kb, pX14 added 0.483kb piggyBac head in *Eco*R V site.	This study
pXL16	5.5 kb, pXL15 added *hsp70* promoter between *Not* I/*Sma* I sites.	This study
pXL17	6.0 kb, pXL15 added *hsp83* promoter between *Not* I/*Sma* I.	This study
pXL18	5.8 kb, pXL15 added *α1- tub* promoter between *Not* I/*Sma* I.	This study
pXL19	10.8 kb, pWT.ΔPr. added 1.8 kb *arm* promoter between *Eco*R I/*Kpn* I sites.	This study
pXL20	9.8 kb, pWT.ΔPr. added 0.8 kb *α1-tub* promoter between	This study

	*Eco*R I / *Kpn* I sites.	
pXL23	3.76 kb, pBluescriptIIKS(-) added 0.8kb α*1-tub* promoter between *Not* I/*Sma* I to get an extra *Eco*R I site.	This study
Bacterial Strain		
Esherischia coli DH5α	-F80dlacZDM15D(*lac*ZYA- *arg*F) U169*deo*R *rec*A1*hsd*R17(ρ_K^-, m_K^+) *pho*A *sup*E44λ^- *thi*-1 *gyr* *r*A96 *rel*A1	Hanahan (1983)
Max efficiency DH5α^{TM} competent cells	ϕ80d*lac*ZMΔ15 Δ (*lac*ZYA-*arg*F) U169 *deo*R *rec*A1*hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44λ^- *thi*-1*gyr* A96 *rel*A1	Life Technologies
EectroMax^{TM} DH10B^{TM} Cells	F-*mcr*AΔ (*mrr-hsd*RMS-*mcr*BC) ϕ80*dlac* ZMΔ15 Δl*ac*X74 *deo*R *rec*A1 *end*A1 *ara*D139Δ(*ara, leu*) 7697 *gal gal*Kλ^- *rpsL nup*G	Life Technologies

Proteose peptone, 10.0; Glucose, 2.0; Sodium chloride, 5.0; Di-sodium phosphate, 2.5. pH 7.4 ± 0.2. For BHI agar 25 g/L of agar (Davis) was added to BHI medium before autoclaving.

2.2.6. Antibiotics and Media Additives

Ampicillin, A 50 mg/mL stock solution of ampicillin was made and added to LB, LB agar (section 2.2.1) and BHI agar (2.2.5) to a final concentration of 50 μg/mL

Kanamycin, A 10 mg/mL stock solution of kanamycin was made and added to LB, LB agar (section 2.2.1) and BHI agar (section 2.2.5) to a final concentration of 30 μg/mL.

Chloramphenicol, A 10 mg/mL stock solution was made up and added to LB, LB agar (section 2.2.1) and BHI agar (section 2.2.5) to a final concentration of 20 μg/mL.

Tetracycline, A 12 mg/mL stock solution of tetracycline dissolved in 70% ethanol and added to LB, LB agar (section 2.2.1) and BHI agar (section 2.2.5) to a final concentration of 12 μg/mL.

X-Gal (5-Bromo-4-Chloro-3-Indyol-β-D-Galactosidase), A 25 mg/mL stock stock solution of X-gal dissolved in N, N dimethyl formamide and added to LB, LB agar (section 2.2.1) and BHI agar (section 2.2.5) to a final concentration of 25 μg/mL

IPTG (Isopropylthio-β-D-galactosidase), A 25 mg/mL stock solution of IPTG was made up and added to LB agar (section 2.2.1) and BHI agar (section 2.2.5) to a final concentration of 25 μg/mL.

2.3 MAINTENANCE OF BACTERIAL CULTURES

For long term storage, 3 mL of LB (section 2.2.1) 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 2.2.1) plate containing the appropriate antibioic (section 2.2.6) and incubated overnight at 37°C or 30°C. If necessary the plate was stored for up to two weeks at 4°C.

2.4 BUFFERS AND SOLUTIONS

All solutions were made with Milli-Q water, autoclaved if necessary and stored at room temperature unless otherwise stated.

All solutions were as described in Ausubel *et al.* (1999)

2.5 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 (pH8.0) was added. This was mixed and the phases allowed to be separated at 15-30°C. After phase separation the upper aqueous phase was removed and an equal volume of 0.1 M Tris-HCl (pH8.0) was added. The solution was repeated until the aqueous phase had a pH of 8.0. The phenol was over laid

with an equal volume of 0.1 M Tris-HCl (pH 8.0) and stored at 4°C in a foil covered bottle.

2.6 PURIFICATION OF DNA

2.6.1 Purification of DNA after PCR

Following amplification by Polymerase Chain Reaction (section 2.17), DNA was purified using the Qiagen QIAquick PCR Purification Kit according to the manufacturer´s instructions.

2.6.2 Phenol/Chloroform Extraction of DNA

The DNA solution was made up to 200 µL with TE (10 mM Tris pH8 1mM EDTA) and 20 µl 3 M NaAc (Sodium acetate pH 5.2) was added. To this solution equal volumes of Tris equilibrated phenol (section 2.5) 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 2.6.3).

2.6.3 Ethanol Precipitation of DNA

Three 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 (10 mM Tris pH 8 1mM EDTA) to the desired concentration.

2.7 DETERMINATION OF DNA CONCENTRATION

2.7.1 Determination by Comparison to a DNA Ladder

The λ *Hin*dIII/*Sst*I ladder (bacterophage lamdha DNA which had been digested with the restriction endonucleases *Hin*dIII and *Sst*I) 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 (Roche) was used for comparison.

2.7.2 Spectrophotometric Determination of DNA Concentration

The DNA of unknown concentration was diluted in TE 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.

2.7.3 Fluorometric Determination of DNA Concentration

The Hoefer Scientific TKO 100 Fluorometer was used to determine unknown concentrations of DNA according to the manufacturer's instruction.

2.8 AGAROSE GEL ELECTROPHORESIS

2.8.1 Standard Agarose Gel Electrophoresis

DNA was size fractionated by electrophoresis on agarose gels in 1x TBE (89 mM Tris-HCl, 89 mM Boric acid and 2.5 mM Na_2EDTA) buffer. The concentration of agarose ranged from 0.8 % for separation 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 (Ficoll 400 20% (w/v), EDTA (pH 8.0) 0.1 M and bromophenol blue 0.24% (w/v)) were loaded into wells in the set gel alongside 10 μl of *Hin*dIII/*Sst*I digest lambda DNA or 4 μl of DNA molecular

weight marker XIII (Roche). 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 distained in water. The gel was visualised under short wave UV light and photographed using the gel documentation system (Alpha Innotech).

2.8.2 DNA Extraction from Seaplaque Agarose

Following an appropriate restriction enzyme digest (section 2.9.3), DNA was size fractioned on a 0.8 -1 % TAE (40 mM Tris acetate, 1.4% (v/v) glacial acetic acid and 1 mM EDTA, pH 8.5) seaplaque agarose gel. The gel was stained in 2 μg/mL ethidium bromide and the required fragment was cut out with a scalpel under UV light. The DNA was purified from the agarose using Qiagen QIAquick Gel Extraction Kit or the GibcoBRL Concert Gel Extraction System according to the manufacture´s protocols.

2.9 RESTRICTION DIGESTION OF DNA

2.9.1 Restriction Enzymes

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

2.9.2 Analytical Restriction Enzyme Digestion

Restriction enzyme digestions for plasmid analysis after transformation were carried out in buffer supplied by the manufacturer. Three units of the appropriate restriction enzyme(s), constituting less than 1/10 of the final volume, was used per μg DNA. When required BSA was added to a final concentration of 0.1 mg/mL. RNase (100μg/mL) was added to final concentration of 0.2 μg/mL. Digestions were incubated at 37°C (unless other wise specified ie. *Sma* I at 25°C and *Sau*

3AI at 65°C) 4-16 hours. After digestion 1/10 volume of 10x gel loading dye was added, and DNA size frationated by agarose gel electrophoresis (section 2.8.1).

2.9.3 Preparative Restriction Digestion

For digestion of large amounts of DNA, a digest was taken after 2-3 hours incubation and checked by agrose gel electrophoresis to determine whether complete digestion has occurred. If digestion was not complete more enzyme was added and the DNA digested overnight. If digestion was complete, the enzyme was heat inactivated, if possible, at 65°C for 20 minutes or by phenol/chloroform extraction (section 2.6.2) and ethanol precipitation (section 2.6.3).

2.10 SOUTHERN BLOTTING TRANSFERRED

Following agarose gel electrophoresis, DNA was to a nylon membrane (Hybond, Amersham) according to standard procedures. The Whatman 3MM filter paper wick method was performed instead of the sponge method. One minor modification to the standard protocol was that after disassembly of the transfer pyramid, the membrane was sealed in 3MM filter paper baked at 80°C for two hours, then wrapped in gladwrap and UV crosslinked by placing on a transilluminator for 45 seconds. For Southern hybridisation analysis, the DNA was digested with *Bgl* II and *Not* I, size separated by agarose gel electrophoresis and transferred to a nylon membrane. The membrane was hybridised with a [^{32}P]-labelled probe using standard procedures (Ausubel *et al.,* 2000). The probe was a mixture of two DNA fragments that were isolated from pB[PUbnlsEGFP], a 4.3 kb *Bgl* II-*Cla* I fragment and 0.9 kb *Kpn* I fragment.

2.10.1 Preparation of Labelled Probe

Preparation of labelled probe was performed by using DNA Labelling Beads (-dCTP) (Ready-ToGo™) according to the manufacturer´s instructions.

Radiolabelled DNA was separated from unincorported [α-^{32}P]dCTP by using a ProbeQuant™ G-50 microcolumn (Amersham) according to manufacturer's instructions.

2.10.2 Hybridisation and Post Hybridisation Washes

The membrane was pre-wet in 5x SSC, then prehybridzed for 3 hrs in hybridization buffer (15 mL) with constant agitation, at 68°C. The labelled DNA probe (50 µL) was denatured by incubation at 100°C for 5 min, then added to the hybridization buffer, and incubated overnight with gentle agitation at 68°C. After the hybridization, the membrane was washed by incubating twice, 5 minutes each, in 2x SSC, 0.1% (w/v) SDS for 15 minutes, and finally 0.1x SSC, 0.1% (w/v) SDS for 2x 10 minutes at 68°C. The membrane was then briefly blotted dry, wrapped in SaranWrap and exposed to X-ray film.

2.11 SYNTHETIC OLIGONUCLEOTIDES

Synthetic oligonucleotides used in this study are described in Table 2.2.

2.12 RECOMBINATION DNA MANIPULATIONS

All recombinant DNA manipulations were carried out using standard procedures (Ausubel *et al.,* 2000) unless otherwise specified.

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

After restriction enzyme digestion (section 2.9.3) 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 2.6.2) was performed and the DNA ethanol precipitated (section 2.6.3). The DNA was then resuspended at 50 μg /mL in 1x *Eco*Pol buffer (New England Biolabs) before Klenow and dNTPs were added.

2.12.2 Removal of 5' Phosphate Groups from DNA with Calf Intestinal Phosphatase (CIP)

Removal of 5' phosphate groups prior to ligation prevents vector self-ligation. 1 U/pmol DNA ends of Calf Intestinal Alkaline Phosphatase (CIP) 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 New England Biolabs´ restriction enzyme buffers.

2.12.3 Annealing Oligonucleotides

Oligonucleotides were resuspended to 100 μM in sterile TE. 25 ng of the two oligonucleotides (section 2.12) was combined with 15 uL 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.

2.13 DNA LIGATION

Ligation of DNA fragments was carried out in 1x ligase buffer, with 1 μL of T4 DNA ligase (New England Biolabs), in a final volume of 10-25 μL. The ligation mixture contained 200 ng 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 to prevent self-ligation. Ligations were incubated overnight at 18°C.

Table 2.2 Synthetic oligonucleotides

Synthetic Oligonucleotides	
Sequencing Primers	
MinIver/primer	5'-TGT TGT TGT TGG AAA TAG AGC-3'
tub1	5'-GTTGAATTCGAGTCCCAACTAGTCCTGC-3'
tub2	5'-TCAATACGGTGAGTACTTTAAA-3'
tub3	5'-TCACCGTATTGAGTTTTTATTGG-3'
tub4	5'-ATTCACGCTGTGGATGAGGAG-3'
Mipromo	5'-AATCCCTAATCAATACTCTG-3'
JFO1	5'-CCT CGA TAT ACA GAC CGA TAA AAC ACA-3'
JF03	5'-GAG CAA TAT TTC AAG AAT GCA TGC GTC-3'
MF01	5'-GTC CAC GAG GCG TAG CCG AG-3'
MF04	5-'GTC AGT CAG AAA CAA CTT TGG CAC-3'
tetR/Rev	5'-AGC CCT CTA GCC CTT CGC TGT CCA-3'
α–tubEnd	5'-CGA TTT TCT TGG GGT GTG TAA CTA-3'
Oligonucleotide Linkers	
EcoNco-Top	5'-AAT TCA GAT CTA AAA CAA C-3'
EcoNco-Bot	5'-CAT GGT TGT TTT AGA TCT G-3'
NotNco-Top	5'-GTA CCG CGG CCG CCA TGG C-3'
NotNco-Bot	5'-TCG AGC CAT GGC GGC CGC G-3'

2.14 TRANSFORMATION OF PLASMID DNA

Esherischia coli DH5α cells were transformed with plasmid DNA (section 2.1) by the method of Hanahan (1983). DH5α cells were streaked out for single colonies from a glycerol stock onto a SOB agar (section 2.2.2) 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 2.2.2) by vortexing. A 1 litre flask containing 30 mL of SOB medium was inoculated with the cells 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 appropriate density was 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 Sorbal 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. 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 (1 M dithiothreital, 90% (v/v) dimethyl sulphoxide and 10 mM potasium) 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 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 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 2.2.3) 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 2.2.1) plus ampicillin (section 2.2.6) 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 2.1) from a freshly streaked SOB agar plate (section 2.2.2) 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 3000 g, resuspended in 28 mL of Tfb1 (100 mM $RbCl_2$; 50 mM $MnCl_2$; 30 mM KAc; 10 mM $CaCl_2$ and 15% glycerol; pH 5.8) 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 (10 mM $RbCl_2$; 80 mM $CaCl_2$; 0.2% MOPS and 15% glycerol; pH 7.0). The resuspension was divided into 200 µL aliquots and either used directly or flash frozen in liquid nitrogen, 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 2.2.3) and incubated at 37°C for 1 hour. The samples were spun for 30 seconds at 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 2.2.1) plus ampicillin (section 2.2.6.1) plates and incubated overnight at 37°C.

2.15 DNA SEQUENCING

Automated sequencing was employed for analysing DNA sequences and was provided by Massey University DNA Analysis Service. Either synthetic or standard primers were used for automated sequencing. 200 ng/µL of double stranded sample DNA and 0.8 pmol/µL of primers were supplied for sequencing.

Sequencing reactions were analysed on a ABI Prism 377 DNA sequencing machine.

2.16 β-GALACTOSIDASE ASSAYS

β-galactosidase assays were based on the method of Simon and Lis (1987). Equal number male and female flies were transferred to weighed microcentrifuge tubes and weighed. Then transferred the flies to a 1 mL homogeniser (kontes) and ground in 500 μL of assay buffer (50 mM KH_2PO_4 and 50 mM K_2HPO_4. 1 mM $MgCl_2$ was added after autoclaving). The homogenate was transferred back to the microcentrifuge tubes and additional 500 μL 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). 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. β-galactosidase activity was measured as Δabsorbance/min/μg flyweight.

2.16.1 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. 15 μL of each fly homogenate was assayed in

triplicate. The average absorbance was calculated and the µg protein/15µL determined using the standard curve.

2.17 INVERSE PCR AND CYCLE SEQUENCING OF *D.MELANOGASTER* DNA

Inverse PCR was performed essentially according to the method of J. Rehm (http://www.fruitfly.org/methods/) using the strategy of Elick *et al.* (1996) for *piggyBac* integrations.

To isolate fly DNA, anesthetized flies (30 for *D. melanogaster* or 1 for blow fly) were collected in an eppendorf tube and frozen at -80°C. Flies were homogenised in 200 µL Buffer A with disposable tissue grinder (Kontes). After several passes an additional 200 µL Buffer A (100 mM Tris-HCl, pH 7.5; 100mM EDTA; 100 mM NaCl; 0.5% SDS) was added and homogenisation continued until only cuticle remained. Samples were incubated at 65°C for 30 minutes. Then 800 µL of LiCl/Kac (Mix 1 part 5 M KAc stock: 2.5 parts 6 M LiCl stock) solution was added and incubated on ice for at least 10 minutes, then centrifuged for 15 minutes at room temperature at 13,000g. 1 mL of the supernatant was transferred into a new tube, avoiding floating debris (if debris transfers, respin.). 600 µL isopropanol was added, mixed, spun 15 minutes at room temperature at 13,000g. The pellet was washed with 70% ethanol, dried then resuspended in 150 µL TE. The DNA was stored at -20°C.

Drosophila genomic DNA was digested with either *Taq*I or *Sau* 3A, self-ligated at low DNA concentration to favour monomeric circle formation then used as a template for PCR. The primers used to amplify the left arm (*Sau 3A* digest) were *JF01* and *MF04* (Table 2.2) and for the right arm (*Taq* I digest) were *MF01* and *MS03* (Table 2.2). Reactions were heated to 94°C for 5 min then cycled 35 times (30s at 94°C; 30s at 47°C and 2 min at 68°C) in a Perkin-Elmer 9600 thermocycler.

2.18 HISTOCHEMICAL STAINING FOR β-GALACTOSIDASE ACTIVITY IN *DROSOPHILA* TISSUE

Wandering third instar larvae were dissected in insect Ringer´s medium. The Ringer medium was removed by using a piece of 3 MM paper as wick and the tissues then incubated for 15-20 minutes in fixative solution. The fixative solution was then removed and rinsed twice with PBS (10 x PBS contained 1.4 M NaCl; 27 mM KCl; 101 mM Na_2HPO_4 and 18 mM NaH_2PO_4, pH7.3). Tissues were transferred to an eppendorf tube containing 150 µl X-gal solution and incubated overnight at 30°C. Tissues were then transferred to a glass slide and mounted in 90% glycerol in PBS. The staining tissues were checked under Olympus BX50 microscope and Photographs of blue staining tissues were taken with a digital camera.

2.19 ANTIBODY STAINING OF POLYTENE CHROMOSOME SPREADS

Antibody staining of polytene chromosome spreads used the procedure essentially as described by Lyman *et al.* (1997). Goat anti-MSL3 was used at a dilution of 1:50 and secondary incubation was with fluorescein isothiocyanate-conjugated anti-goat (Sigma Chemical Co.). DNA was counterstained with 4´,6-diamidino-2-phenyl-indole (DAPI)

2.20 INTERPLASMID TRANSPOSITION ASSAYS IN *D. MELANOGASTER*

Interplasmid transposition assays were performed essentially as described by Sarkar *et al.* (1997). Manually dechorionated *y w* embryos were injected with a mixture of helper (100µg/mL), pB[KOa] donor (Thibaut *et al.,* 1999) (400µg/mL) and pBC/SacRB target plasmids (Klinakis *et al.,* 2000) (800 µg/mL) in injection

buffer. For the RNA helper experiments, the RNA was at a final concentration of 100 μg/mL. DNA was isolated from injected embryos based on a modification of the Hirt method (Hirt, 1967). 20 to 30 embryos were homogenized in 40-60 μL of Hirt lysis buffer (0.6% SDS, 10mM EDTA pH8.0.), then incubated at room temperature for 10 min for complete lysis. 1/4th volume 5 M NaCl was added, mixed then stored at 4°C overnight. Samples were spun at 13,000g for 10 min. The supernatant was then transferred to a clean tube. Phenol and then chloroform extractions were performed and the DNA precipitated by adding 0.1 volume 3 M NaAc and 3 volume ethanol then precipitate with 3 M Na Acetate and ethanol. DNA was re-suspended in 1 μL water per 5 embryos. Aerosol barrier tips were used throughout the procedure to minimise contamination. The extracted plasmid DNA used to transform ElectroMAX DH10B cell (Life Technologies). Approximately 25 embryo equivalents of DNA was used per transformation. A 100 μL aliquot of the transformation was plated onto Brain Heart Infusion (BHI) (Oxoid) (37 g/L)/ agar medium supplemented with chloramphenicol (20 μg/mL) or kanamycin (30 μg/mL) to estimate the number of transformants containing target plasmids or donor plasmids respectively. The remainder of the transformation was plated onto triple selection BHI/agar medium (5% sucrose, 20 μg/mL chloramphenicol, 30 μg/mL kanamycin) to estimate the number of transpositions. Plasmid DNA was isolated for rapid analysis using the CloneChecker system (Life Technologies), according to the manufacturer´s instructions.

2.21 RNA SYNTHESIS

RNA synthesis was performed using the description of SP6 Cap-Scribe Kit SP6 (Roche), according to the manufacturer´s instructions.

2.22 FLY STOCKS AND TRANSGENIC FLY STOCKS

Fly stocks and transgenic fly stocks used in this study are described in Table 2.3.

2.23 MAINTENANCE OF FLY STOCKS

All stocks were kept in 20 mL cornmeal agar vials (section 2.23) 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.

2.23.1 Fly Media

2.23.1.1 Cornmeal agar

Cornmeal agar contained (g/L): agar (Davis), 10.7; cornmeal, 107.1; yeast, 32. These were combined with 1 L of water 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).

2.23.1.2 Formula 4-24 (Instant fly food)

7 mL of water was added to 1.5 g of Formula 4-24 (Cardina Biologicals) in a vial and mixed.

2.23.2 Collection of Virgin Females

Virgins were collected from bottles in which flies were beginning to eclose. 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.

2.23.3 Setting Fly Crosses

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

2.24 MICROINJECTION OF *D. MELANOGASTER* EMBRYOS

2.24.1 Co-Precipitation of Plasmid DNA

Target plasmid DNA and "helper" (transposase) DNA was mixed and the volume was made up to 100 - 200 µL with TE. The DNA was then ethanol precipitated (section 2.6.3) and resuspended in injection buffer (0.1 mM NaH_2PO_4; 0.1 mM Na_2HPO_4 and 5 mM KCl) to the appropriate concentration. Before use, the DNA solution was centrifuged at 13 000 rpm for 20 minutes to remove any particles that may block the microinjection needle. 3µL of DNA in injection buffer was loaded into a Femtotip using a microloader (Eppendorf).

2.24.2 Collection of Embryos

Approximately 200 flies were placed in a plastic tripour beaker and inverted onto a 60 x 15 mm plate of cornmeal agar (section 2.23.1.1), 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.

Table 2.3 *Drosophila melanogaster* fly stocks and transgenic fly stocks

Fly strain	Use/Chromosomal Linkage	Source
***D.melanogaster* Fly Stocks**		
yw	Microinjection recipient strain.	J. Lucches
W;In(3LR)TM3, Sb	Determination of chromosomal linkage.	J. Lucches
yw; L^2/CyO, Cy pr cn^2 y^+	Determination of chromosomal linkage.	J. Lucches
Transgenic Fly Stocks		
ptTA-α-tub #1; *yw*; (*w^+*)	X	This study
ptTA-α-tub #2; *yw*; (*w^+*)	2nd	This study
ptTA-α-tub #3; *yw*; (*w^+*)	3rd	This study
ptTA-arm #1; *yw*; (*w^+*)	3rd	This study
ptTA-83 #1; *yw*; (*w^+*)		This study
ptTA-83 #2; *yw*; (*w^+*)		This study

ptTA-83 #3; *yw*; (w^+)		This study
pMiCcw+Hsp70 #1; *yw*; (w^+ /w^+)		This study
pMiCcw+Hsp70 #2; *yw*; (w^+ /w^+)		This study
pWHT-arm #1; *yw*; (w^+ /w^+)		This study
pWHT-arm #2; *yw*; (w^+ /w^+)		This study
pWHT-83 #1; *yw*; (w^+ /w^+)		This study
pWHT-83 #2; *yw*(w^+ /w^+)		This study
ptetO-msl-2 #1; *yw*; ((w^+ /w^+)	2nd	This study
ptetO-msl-2 #2; *yw*; (w^+ /w^+)	2nd	This study
ptetO-msl-2 #3; *yw*; (w^+ /w^+)	X	This study
ptetO-msl-2 #4; *yw*; (w^+ /w^+)	2nd	This study

pBac D.mw #1; *yw*; (w^+)	This study
pBac D.mw #1; *yw*; (w^+)	This study
pBac D.mw #2; *yw*; (w^+)	This study
pBac D.mw #3; *yw*; (w^+)	This study
pBac D.mw #4; *yw*; (w^+)	This study
pBac D.mw #5; *yw*; (w^+)	This study
pBac D.mw #6; *yw*; (w^+)	This study
pBac D.mw #7; *yw*; (w^+)	This study
pBac D.mw #8; *yw*; ((w^+)	This study
pBac D.mw #9; *yw*; ((w^+)	This study
pBac D.mw #10; *yw*; (w^+)	This study
pBac D.mw #11; *yw*; (w^+)	This study
pBac D.mw #12; *yw*; (w^+)	This study
pBac D.mw #13; *yw*; (w^+)	This study
pBac D.mw #14; *yw*; (w^+)	This study
prtTA; *yw*; (w^+)	Mitzi. I. Kuroda lab

2.24.3 Dechorionation of Embryos

Using fine forceps under a dissection 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.

2.24.4 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 embyos were covered with halocarbon oil (Series 700, Halocarbon Products Corporation) or paraffin oil (Witerex 334, Pauling Industries Ltd) to stop further dehydration.

2.24.5 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 2-24 food (section 2.23.1.2), approximately 40 per vial and incubated at 25°C until the flies emerge. These first flies are termed the G_0 generation for future crosses.

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

2.25 IDENTIFICATION AND CHROMOSOME LINKAGE OF TRANSGENIC *DROSOPHILA* LINES

2.25.1 Crossing Adult Survivors

After hatching the G_0 flies were mated to the *yw* (table 2.3) recipient stock individually. Each G_0 female was mated with two *yw* males and each G_0 male was mated with five *yw* virgin females and incubated at 25°C. The adults were removed after one week and vials incubated at 25°C until the G_1 progeny hatched.

2.25.2 Identification of Transformants and Establishment of a Transformant Stock

G_1 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 *yw* virgin females. If no transformant males were found a transformant female was mated with two *yw* males. Heterozygous G_2 males and virgin *yw* females (or heterozygous F_2 virgin females and *yw* males) were crossed to produce heterozygous G_3 progeny for use in β-galactosidase and total protein assays. Heterozygous G_2 males and G_2 virgin females were also crossed, then a

single homozygous male (identificated by a dark eye colour) and virgin female were crossed to establish a homozygous line.

2.25.3 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*+ male from each transformant line were mated separately with either five w; *In (3LR)TM3,Sb ry*^{rk}*e/In(3LR)TM6, Tb e* or five *y w; L*^{2}*/CyO, Cy pr cn*^{2} *y*+ virgin females. *Sb w*+ and *Cy w*+ male progeny from these crosses were mated with *yw* virgin females. If the insert was on the 2nd chromosome, all the non-*Cy* progeny would be *w*+. Alternatively if 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 *yw* virgin females. If the insertion was on the X chromosome, only female progeny would *w*+, due to males receiving their X chromosome from the female parent.

2.26 *L. CUPRINA* MAINTENANCE

Lucilia stocks and transgenic blowfly stocks used in this study are listed in Table 2.4.

2.26.1 *Lucilia* Stocks

Lucilia cuprina adults were kept in mesh cages and fed sugar-water diet supplemented with either a liver feed and/or a protein rich biscuit. Embryos were collected from liver and tranferred to commercial jelly meat pet food. The stocks used were *ru w* which has rusty body colour and white eyes (Weller and Foster, 1993), y which has yellow eyes and "Munro" a wild-type strain of New Zealand origin.

2.26.2 *L. curprina* Egg Collection

About a week after the flies have emerged, flies were fed liver to allow the fertilised eggs to complete development. A second liver feed was given about one week later for egg collection.

To collect eggs for injections, a piece of liver, which is placed on wet paper towels in a small petri dish, is put in the cage in the morning of the injections. Usually the flies will lay fresh eggs after an hour or so. These eggs are collected off the liver and washed with cold water in a plastic container with a mesh lid.

The washed eggs which have an intact chorion were lined up on piece of double-stick tape on a microscope slide. The posterior end of embryos was hanging over the edge of the tape for injection.

2.27 *L. CUPRINA* GERM-LINE TRANSFORMATION

ru w or Munro *Lucilia* embryos were injected through the chorion with a mixture of plasmid DNA carrying the marker gene (either pMihsCcw (Loukeris *et al.,* 1995), pMi[PUbIsEGFP], pB[Ccw] (Handler *et al.,* 1998), pB[a1-tubCcw] or pB[PUbnIsEGFP] (Handler and Harrel, 1999)) at 400 ug/mL and either helper plasmid DNA (either pHSS6hsILMi20 (Klinakis *et al.,* 2000) phsp70pBac, phsp83pBac or pα1-tubpBac (Li *et al.,* 2001) (100 μg/mL) or RNA (50 μg/mL) using procedures similar as for Drosophila embryos (section 2.24).

The main difference was that since the *L. cuprina* embryos were not dechorionated the needles used for injection had to be both very sharp and strong, so they did not bend easily. The needles used were either Femtotips II (Eppendorf), or obtained from Mr.Rod Bycroft (North Shore Fertility). The latter were made from thick walled glass capillaries [Clark electromedical instruments, 1.0 mm OD x 0.58 mm ID, standard wall without filament] using a horizontal Sutter P87 Puller. The programme parameters were heat 620, pull 250, velocity 200,

time 100 and pressure 400. The hole opening was less than 10 μm. Single G_0 *ru w* adults were mated with *ru w* and offspring of these crosses were examined for non-white eye colour. Single G_0 Munro adults were mated with Munro and offspring (late embryos, early larvae) examined for EGFP expression using a Leica MZFLIII fluorescence stereomicroscope. Single G_1 transformants were backcrossed with Munro strain. Images were captured using a Leica digital camera.

Table 2.4 Blowfly (*Lucilia cuprina*) stocks and transgenic fly stocks

Fly strain	characteristic	Source
Blow Fly Stocks		
ru w	rusty body, white eyes	Weller & Foster,1993
y	yellow eyes	Weller & Foster,1993
Munro	wild type	NZ origin
Transgenic Fly Stocks		
A18 Bac *EGFP*	3xpBac *EGFP* insertions	This study
A54 Bac *EGFP*	1xpBac *EGFP* insertions	This study

CHAPTER 3

EVALUATION OF *piggyBac*-MEDIATED TRANSPOSITION IN *D. MELANOGASTER*

3.1 INTRODUCTION

Transgenic insects are made following the *P*-element paradigm (Figure 3.1) where pre-cellular embryos are injected with a mixture of two plasmids, one that encodes a transposase (so-called helper) and the other with the ends of the transposon bracketing the gene(s) that is to be integrated into a chromosome. Since the *P*-element is only active in Drosophilids (O'Brochta and Handler, 1988), transgenic insects have been made using other transposons that have a broader host range, such as *Minos* (Loukeris *et al.*, 1995b), *Hermes* (Jasinskiene *et al.*, 1998), *mariner* (Coates *et al.*, 1998) and *piggyBac* (Fraser *et al.*, 1995) (section 1.4.2.3). All four broader host-range transposons have been used for germ-line transformation of *Drosophila melanogaster* (Loukeris *et al.*, 1995a; O'Brochta *et al.*, 1996; Lidholm *et al.*, 1993; Handler and Harrell, 1999).

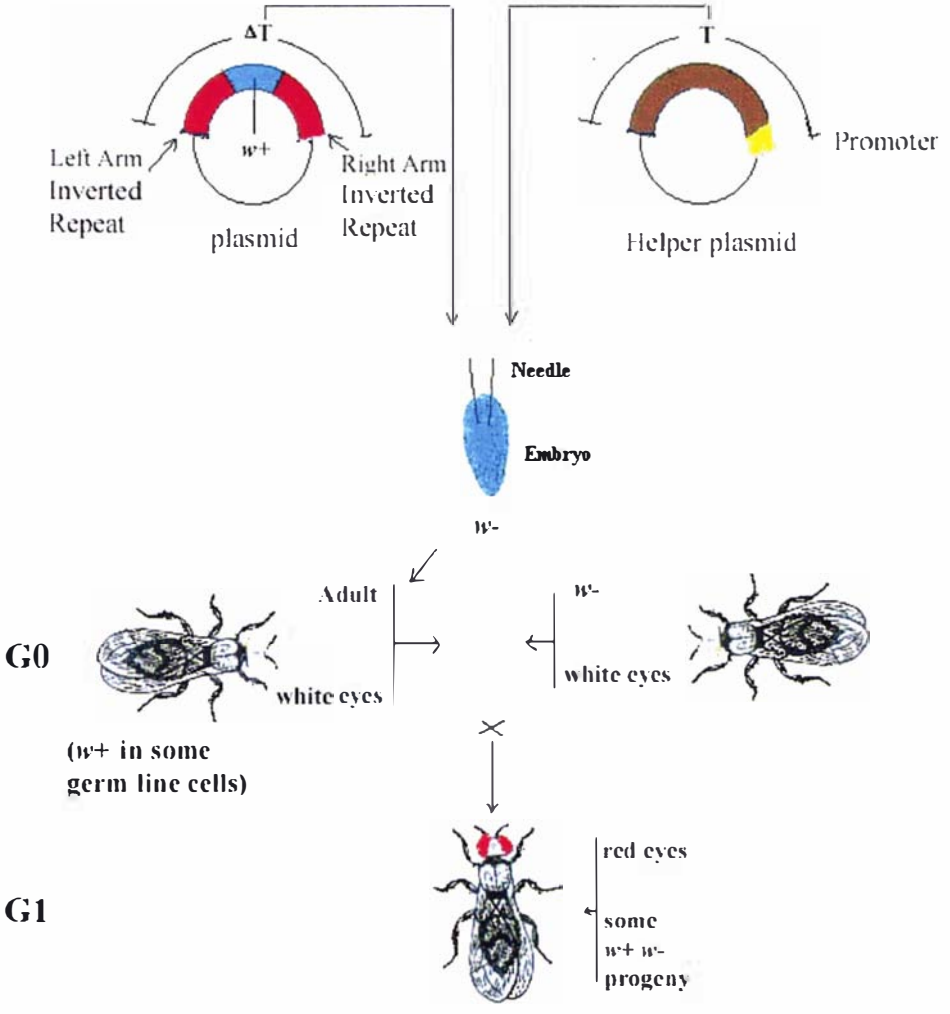
Although tremendous progress has been made in germ-line transformation of non-Drosophilids, the frequency of adults that develop from injected embryos which produce transgenic offspring is often very low (Peloquin *et al.*, 2000; Catteruccia *et al.*, 2000; O'Brochta *et al.*, 2000). This problem is compounded by the difficulty in obtaining sufficient numbers of fertile adults following microinjection of DNA for some insect species.

The low frequency of transformation could be due to several factors such as low marker gene expression due to negative chromosomal effects (Handler and Harrell, 1999) and host-specific factors that affect the rate of transposition (Sarkar *et al.*, 1997). The level of expression of the transposase may also affect the rate of transposition. For example, overproduction of the *mariner* transposase lowers the rate of excision of a target element suggesting a decrease in the level of active transposase (Lohe and Hartl, 1996). The level of production of transposase depends upon the activity of the promoter used to drive expression of the gene and on the stability of the mRNA and protein. In previous studies either the *piggyBac* or the heat-inducible *hsp70* promoter

Figure 3.1 Microinjecting procedure for making transgenic insects

Pre-cellular embryos were injected with a mixture of two plasmids. One, called the "helper", encodes a transposase and the other with the ends of the transposon bracketing the target gene(s) (w^+). During development of an injected embryo, a transposon may jump and insert into the genomic DNA of a germline cell. This transposition is detected by crossing the adult survivors (G_0) with a white eye strain (w^-) and determining if any offspring (G_1) have coloured eyes due to integration of the w^+ gene.

Microinjection Method For Making Transgenic Insects



from *D. melanogaster* was used to control expression of the *piggyBac* transposase gene (Handler and Harrell, 1999).

One aim of this study was to determine the effectiveness of helper plasmids that used constitutive promoters to control expression of the *piggyBac* transposase gene. Transposition in *D. melanogaster* embryos was measured by using both interplasmid transposition assays and by selecting for germ-line transformants.

3.2 RESULTS

3.2.1 Development of *piggyBac* Helpers and Transposon

3.2.1.1 Construction of pXL15 plasmid carrying *piggyBac* open reading frame (ORF)

To construct pXL15 plasmid, a 1583 bp *Eco*R V – *Ase* I fragment from p3E1.2 (Appendix II) which contains most of the transposase ORF and polyadenylation signal was filled-in with T4 DNA polymerase (BioLabs), and then was inserted into the *Eco*R V site of pBluescript II KS (-) (Stratagene) (Appendix II). The resulting plasmid was digested with *Eco* RV and ligated with a 484 bp *Eco* R V fragment from p3E1.2 that contains the remainder of the ORF and 22 bp upstream of the translation initiation codon. The outline of the construction procedures is shown in Figure 3.2. The plasmid with correct orientation of the *Sma* I - *Eco*R V fragment, called pXL15, was identified by *Xba* I/*Hin*d III and *Xba* I/*Bgl* II restriction endonuclease mapping (Figure 3.3C) and confirmed by DNA sequencing (Figure 3.4) from a T7 primer.

3.2.1.2 Construction of pXL16 containing *piggyBac* ORF driven by *hsp70* promoter

To make the *piggyBac* helper with the *hsp70* promoter, a *Not* I – *Cla* I (filled-in with T4 DNA polymerase) fragment from pHSS6hsILMi20 which contains the *hsp70* promoter (Klinakis *et al.*, 2000) was ligated with pXL15 that had been digested with *Not* I and *Sma* I. The outline of the construction procedures is shown in figure 3.5.

3.2.1.3 Construction of pXL17 containing *piggyBac* ORF driven by *hsp83* promoter

To make the *hsp83* helper, a 900 bp fragment containing the promoter was excised from the plasmid of pGEM-hsp83 (a gift from Dr. Heinrich) by digestion

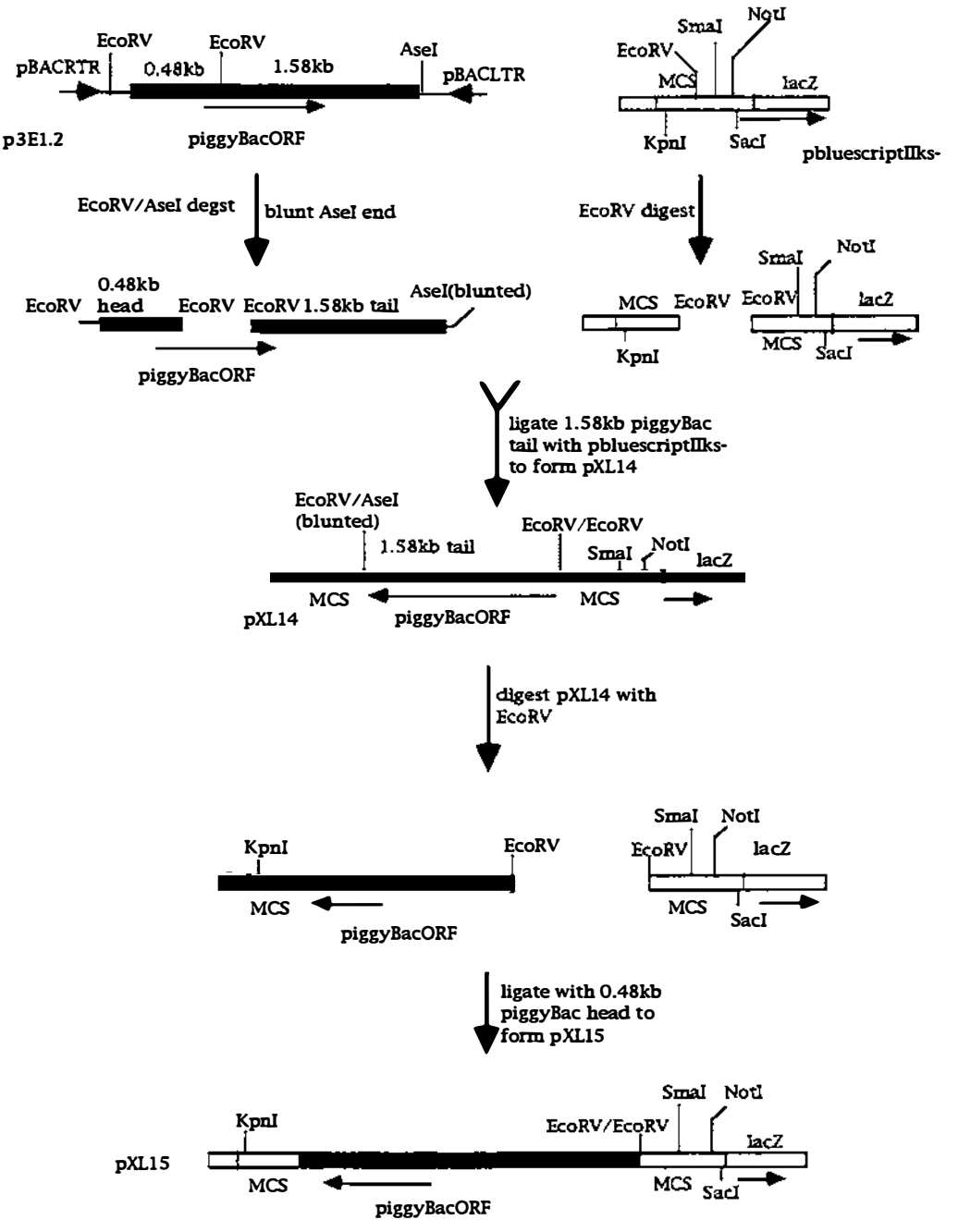


Figure 3.2 Insertion of *piggyBac* gene into pBluescript II KS-

1. p3E1.2 was digested with *Eco*R V/*Ase* I and *piggyBac* 1.58 kb tail and 0.48 kb head fragments were isolated. 2. Bluescript II KS- was digested with *Eco*R V and ligated with *piggyBac* 1.58 kb tail to form pXL14. 3. pXL14 was digested with *Eco*R V and ligated with 0.48 kb head to form pXL15.

Figure 3.3 Identification of pXL15 and pB[Dmm-w] by restriction endonuclease mapping on 1% agarose gel.

M: λ *Hin*d III/*Sac* II ladder

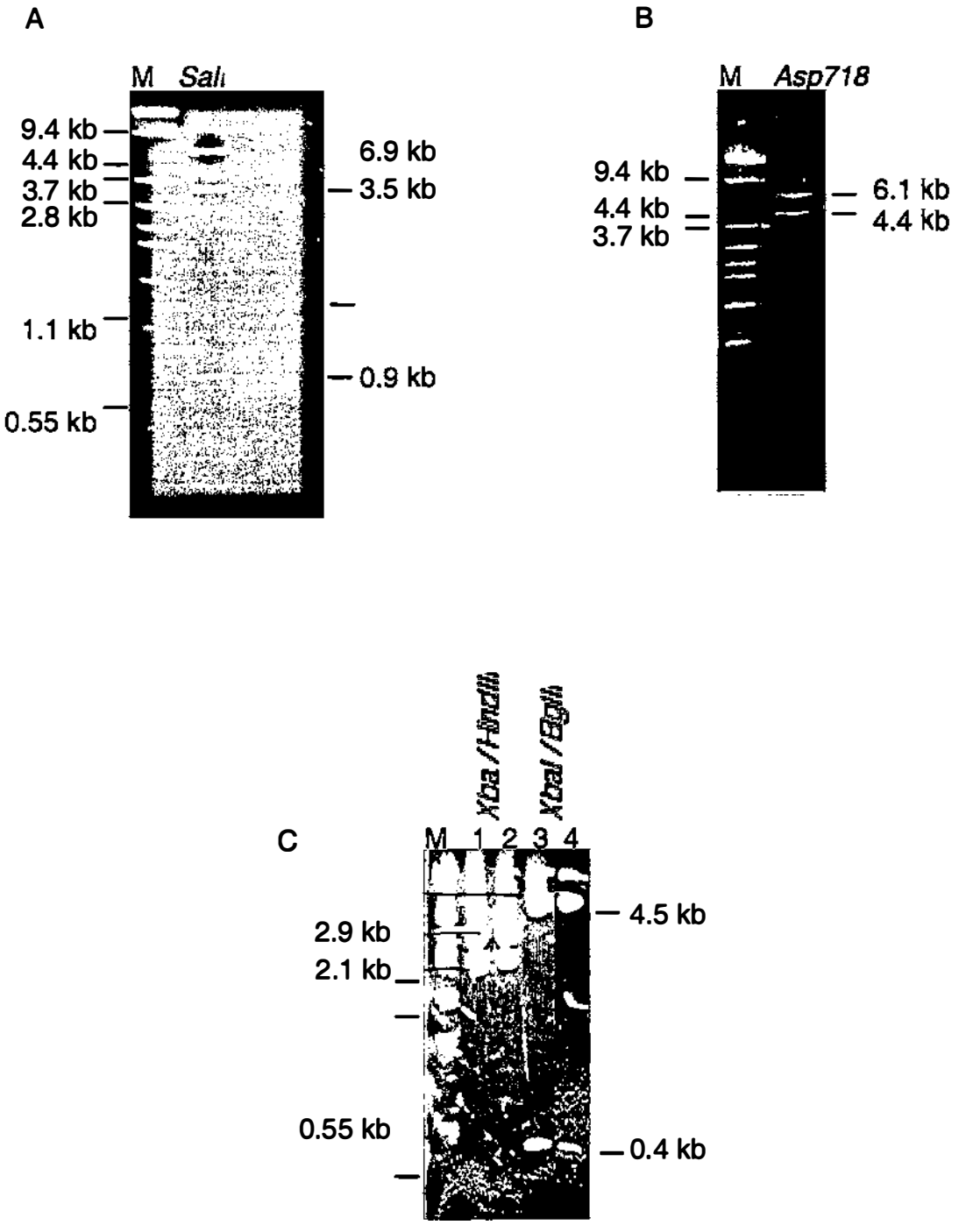
A. *Sal* I digestion of pB[Dmm-w] plasmid DNA to form 6.9 kb, 3.5 kb and 0.9 kb fragments. This was to confirm the correct size of the plasmid.

B. *Asp* 718 digestion of pB[Dmm-w] plasmid to form 6.1 kb and 4.4 kb fragment. This was to confirm the correct orientation of insertion of the *Drosophila white* gene.

C. *Xba* I/*Hin*d III and *Xba* I/ *Bgl* II digestion of pXL15 respectively.

Lane 1, 2: *Xba* I/*Hin*d III digestion of pXL15 to form 2.1 kb and 2.9 kb fragments. This was to confirm the mono copy insertion of 0.48 kb *piggyBac* head into pXL14.

Lane 3, 4: *Xba* I/*Bgl* II digestion of pXL15 to form 4.5 kb and 0.4 kb fragments. This was to confirm the correction orientation of the 0.48 kb *piggyBac* fragment insertion into pXL14.


A
M SalI
9.4 kb
4.4 kb
3.7 kb
2.8 kb
1.1 kb
0.55 kb
6.9 kb
3.5 kb
0.9 kb
B
M Asp718
9.4 kb
4.4 kb
3.7 kb
6.1 kb
4.4 kb
C
XbaI / HindIII
XbaI / BglII
M 1 2 3 4
2.9 kb
2.1 kb
0.55 kb
4.5 kb
0.4 kb

*Eco*R I *Eco*R V

gcccgctctagaactagtgcgatccccgggctgcaGGAATTCGGTATCgtgactaatatataata

TRL start

a aATGggtagttctttagacgatgagcatatcctctctgggggtctgcaaagcgatgacnagcttgntggt

gaggattctgacagtgaaatatcagatcacgtaagtgaagatgacgtncagagcgatacagaagaagcgttt

atagatgaggtacatgaagtgcanccaacgtcaagcggtagtgaaatattagacnaacaaaatgttattgaa

caaccaggttcttcattggcttctaacagaatcttgaccttgccacagaggactattagaggtaagaataaacat

tgttggtcaacttcaaagtccacgaggcgtagccgagtctctgcactgaacattgtcagatctcaaagaggtcc

gacgcgtatgtgccgcaatatatatgacccacttttatgcttcaaactatttttttactgatgagataatttcggaaatt

gtaaaatggacaaatgctgagatatcattgaaacgtcgggaatctatgacaggtgctcatttcgtgacacgaat

gaagatgaaatctatgctttctttggtattctggtaatgacagcagtgagaaaagattaccacatgtccacagat

gacctctttgatcgatctttgcaatggtgtcgtctctgnatgagccntgatcgtttggatttttgatc

Figure 3.4. Partial sequence of pXL15 confirming orientation of 0.48 kb *Eco*R V fragment.

The sequence showed that the junction point of the *Eco*R V site between the 0.48 kb head of *piggyBac* and the *Eco*R V of pBluescript II KS(-) was exactly 22 bp upstream of the translation(TRL) start site of *piggyBac*.

with *Not* I and *Hin*c II and ligated with pXL15 that had been digested with *Not* I and *Sma* I (Figure 3.5).

3.2.1.4 Construction of pXL18 containing *piggyBac* ORF driven by the *α1-tubulin* promoter

To make the helper with the *α1-tubulin* promoter, a *Not* I and *Sac* II (made blunt ended using T4 DNA polymerase) fragment from pGEM-α1T (a gift from Dr. Heinrich) was ligated with pXL15 that had been digested with *Not* I and *Sma* I (Figure 3.5).

3.2.1.5 Construction of *piggyBac* transposon of pB[Dmm-w] carrying *mini-white* gene

To make a *piggyBac* vector carrying the *Drosophila melanogaster* mini-*white* gene, pB[Dmm-w], a 4.5 kb *Hin*d III fragment containing the *mini-white* gene was isolated from pCaSpeR4 (Thummel and Pirrotta, 1991) (Appendix II) and inserted into the *Hin*d III site of pBluescript2Not I (a gift from Dr. Heinrich which was a modified version of pBluescript II KS (-) that has two *Not* I sites). The *mini-white* gene was then excised from the resulting plasmid by *Not* I digestion and ligated with *Not* I digested pB[Ccw] (Handler *et al.*, 1998) to form the vector pB[Dmm-w]. This essentially replaces the *C. capitata white* gene in pB[Ccw] with the Drosophila *mini-white* gene (Figure 3.6).

3.2.2 Construction of pSP64PBORF and Synthesis of *piggyBac* RNA

To make the plasmid template for *piggyBac* RNA synthesis, pSP64PBORF, the *piggyBac* ORF was excised from pXL15 by digestion with *Hin*d III and *Eco*R I, the ends were filled-in with T4 DNA polymerase and then the fragment was inserted into the *Hin*c II site of pSP64 Poly(A) (Promega) (Appendix II) which has a poly A sequence (A_{30}) immediately downstream of the multiple cloning site.

Capped *piggyBac* RNA was synthesed as described in methods (section 2.21).

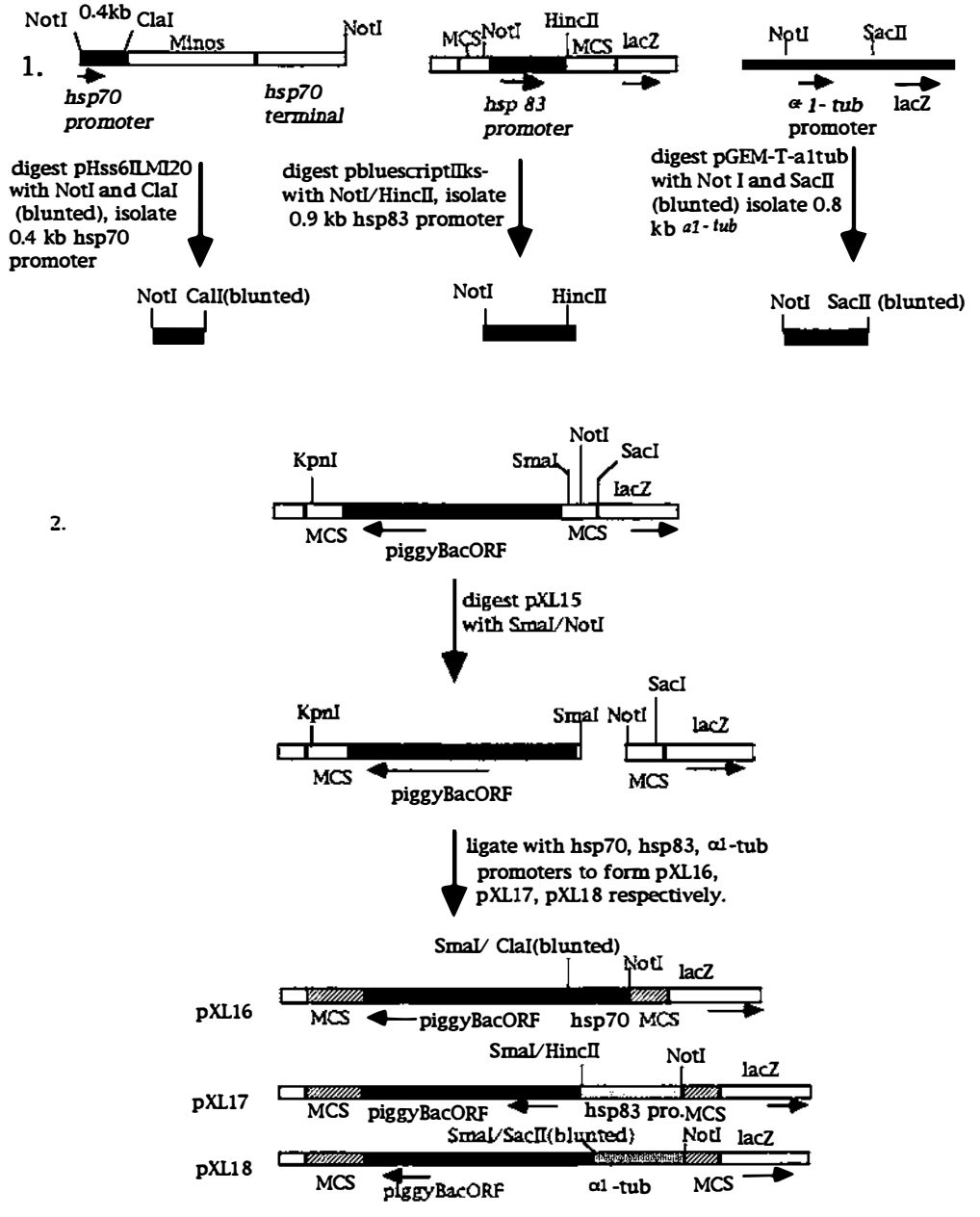


Figure 3.5 Insertion of *hsp70*, *hsp83* and *α1-tub* promoters into pXL15 respectively.

1. Digested pHSS6hsILMi20 with *Not* I/ *Cla* I, blunted *Cla* I end and then isolated 0.4 kb *hsp70* promoter; digested pBluescipt II KS-*hsp83* with *Not* I/*Hinc*II and isolated 0.9 kb *hsp83* promoter; digested pGEM-T-α1tub with *Not* I / *Sac* II and blunt *Sac* II then isolated 0.8 kb *α1-tub* promoter. **2.** Digested pXL15 with *Not* I/*Sma* I and ligated with *hsp70*, *hsp83*, *α1-tub* promoters respectively to form pXL16, pXL17, pXL18 plamids.

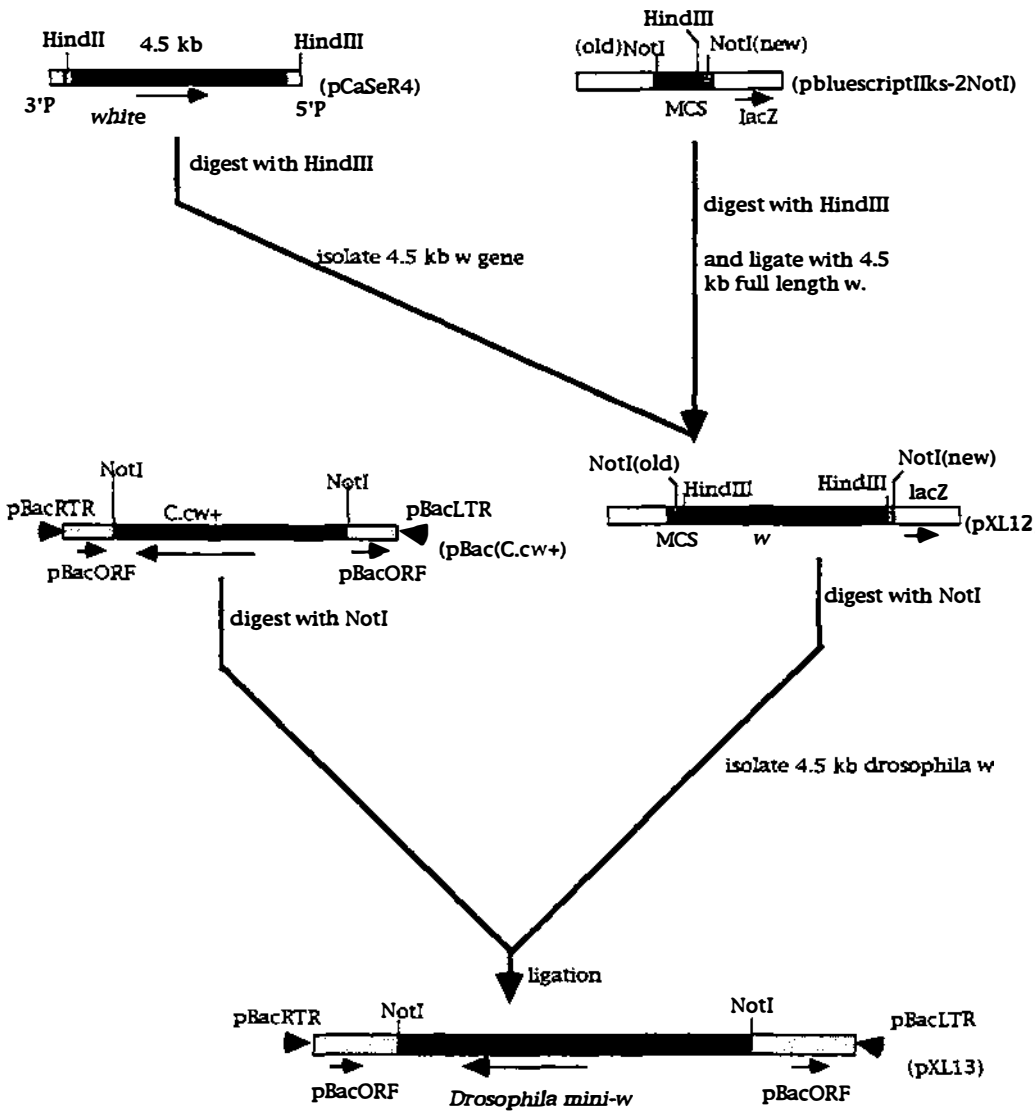


Figure 3.6 Procedures of construction of pB (D.mm-w) transposon.

1. Digested pCaSpeR4 with *Hind* III and isolated 4.5 kb *mini-white* gene. **2.** Digested pBuescript-2Not I with *Hind* III and ligated with 4.5 kb *mini-white* gene to form pXL12. **3.** Digested pXL12 with *Not* I and isolated 4.5 kb Drosophila *mini-white* gene. **4.** Digested pBac (C.cw+) with *Not* I and ligated with 4.5 kb Dosophila *mini-white* gene instead of *C.cw+* to form pB[D.mmw] (pXL13).

The size and concentration of the RNA was estimated by formadehyde agarose electrophoresis with known RNA standards (Figure 3.7).

3.2.3 Interplasmid Transposition

Interplasmid transposition assays followed the scheme outlined in Figure 3.8. A mixture of three plasmids, "helper", "donor" and "target" were injected into pre-blastoderm Drosophila embryos. Helper plasmids contained the *piggyBac* open reading frame (ORF) and polyadenylation signal and either the Drosophila *hsp70*, *hsp83* or *α1–tubulin* promoters directly upstream of the ORF.

Both the *hsp70* and *hsp83* promoters are heat inducible but the *hsp83* promoter has a significantly higher basal level of activity and is particularly active in adult ovaries (Xiao and Lis, 1989). The *α1–tubulin* promoter is a strong constitutive promoter that has high activity at all stages in all living tissues (O'Donnell *et al.*, 1994). The promoter is active throughout embryogenesis beginning at the cellular blastoderm stage. Full activity of the promoter requires DNA sequences in the first intron in addition to the sequences immediately upstream of the transcription start site (O'Donnell *et al.*, 1994). Since a promoter that had maximal activity was required a fragment of the *α1-tubulin* gene that included upstream sequences, first exon, first intron and part of the second exon was used. The *α1-tubulin* translation start codon, which is present in the first exon, was changed to ACG so that translation would begin at the *piggyBac* start codon.

In addition to the helper plasmids, an RNA helper that encodes the *piggyBac* transposase was also tested. An RNA helper could be advantageous in insect species where the Drosophila promoters may be poorly recognised by the transcription apparatus and consequently have low activity.

In the donor plasmid, p[KOα] (Appendix II) the *piggyBac* transposon carries

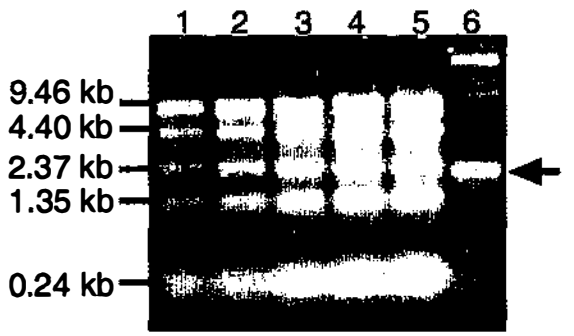


Figure 3.7. Estimation of the concentration of the RNA by 1% formaldehyde-agarose denaturing gel electrophoresis with RNA standards to check the size.

Lanes 1-5 were all loaded with standard RNA. The amount of RNA of per band for each lane was: 1 = 52 ng/band, 2 = 86 ng/band, 3 = 168 ng/band, 4 = 251 ng/band and 5 = 333 ng/band.

Lane 6 was loaded with 1 μL injection solution contained *piggyBac* RNA. The sample was estimated to contain approximately 200 ng/μL of RNA which was of the correct size (arrow).

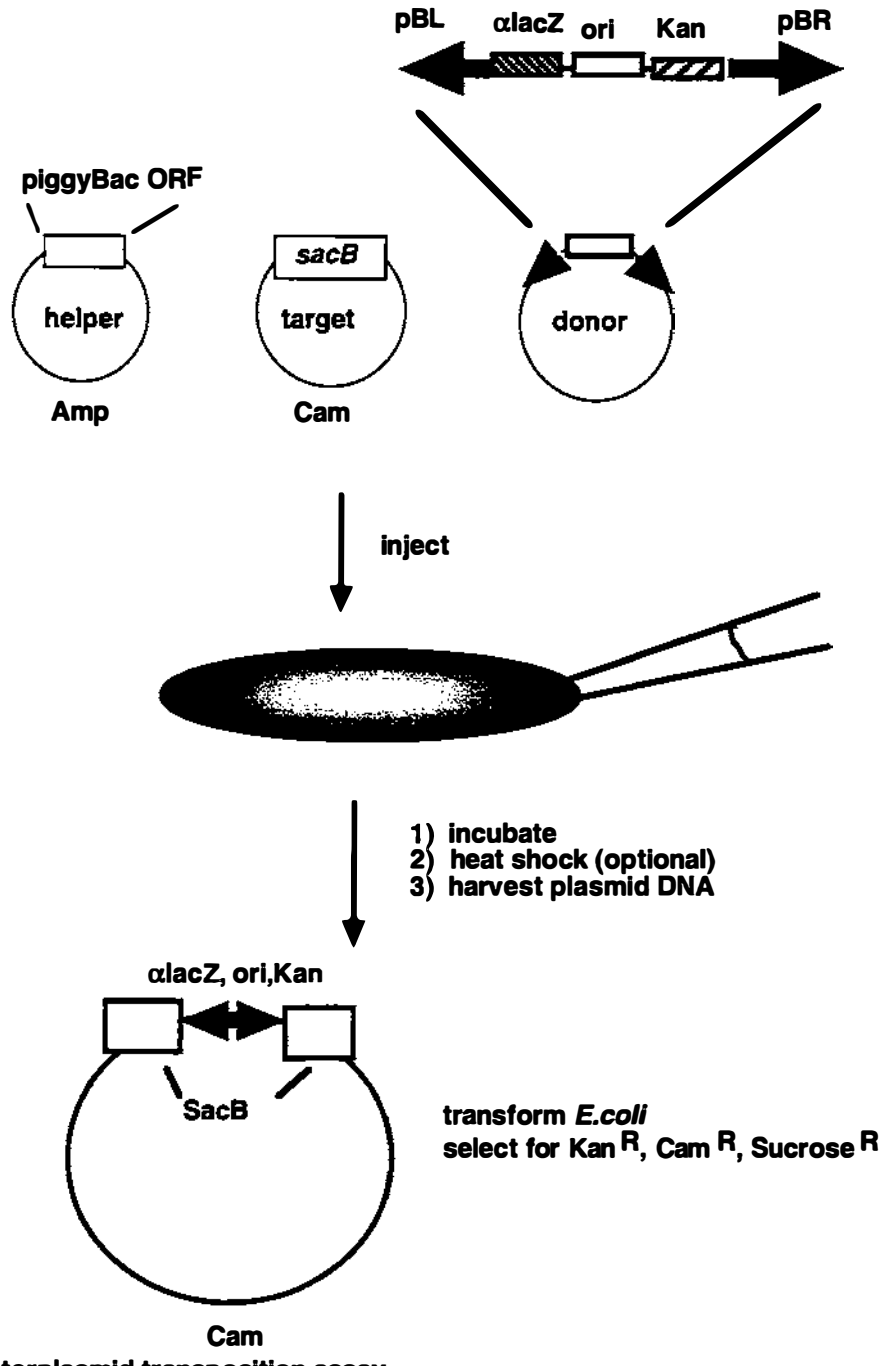


Figure 3.8 Interplasmid transposition assay

Drosophila embryos were injected with a mixture of 3 plasmid DNAs, helper, donor and target. After incubation and an optional heat shock treatment, low molecular weight DNA was isolated from the embryos and used to transform *E. coli*. Transposition in the embryo of the *piggyBac* transposon carrying the kanamycin resistance marker to the target plasmid, disrupting the *sucrase* (*sacB*) gene, results in a plasmid that can be identified by triple selection (kanamycin, chloramphenicol and sucrose) of transformed *E. coli*.

kanamycin resistance and *αlacZ* genes for selection (Thibault *et al.*, 1999).

The target plasmid pBC/SacRB (Appendix II) contains the *sacB* gene from *B. subtilis* and a chloramphenicol resistance gene. *E. coli* carrying the target plasmid die in the presence of 5% sucrose due to the induction of levansucrase (*sacB* gene product) synthesis (Gay *et al.*, 1985).

A triple selection of chloramphenicol, kanamycin and sucrose resistance was used to identify plasmids that result from *piggyBac*-mediated transposition of the *αlacZ-ori-kan*^{R} gene cassette from donor to target disrupting the *sucrase* gene. In initial experiments few colonies were obtained due to transposition and the colonies grew very slowly. The yield of transpositions detected was improved significantly by using commercial high efficiency electrocompetent cells and a rich plating medium BHI agar (section 2.2.5).

The results of an experiment using the above conditions are shown in Table 3.1. With the exception of the *hsp83* helper, all helpers produced at least 40 colonies on triple selection plates. Plasmid DNA was isolated from either all of the colonies (*hsp83* and RNA helper) or from 50 colonies (*hsp70* and *α1-tub*) and analysed by rapid restriction endonuclease digestion followed by agarose gel electrophoresis. Some of the results are shown in Figure 3.9. The results showed that 85-100% of the colonies obtained by triple selection were due to a canonical cut and paste transposition of *piggyBac* from donor to target. The number of transposition colonies shown in Table 3.1 is either the exact number shown to be due to disruption of *sacB* (e.g. *hsp83* and RNA helpers) or inferred from analysis of 50 colonies (e.g. *hsp70* helper with heat shock, 300 colonies were obtained on triple selection plates but only 45 of 50 colonies were shown to be due to disruption of *sacB*, so the corrected value is 270 colonies).

The results show that, with the exception of the plasmid with the *hsp83*

Table 3.1. Interplasmid transposition assay in *D. melanogaster*.

Helper	Heat shock	*Targets	@Donors	Transpositions#	Freq per 10^4 (T^a)	Freq per 10^4 (D^b)
hsp70	No	38,000	51,000	93	25	18.2
hsp70	Yes	80,000	106,000	270	34	25.5
hsp83	No	59,000	70,000	1	0.17	0.14
α1-tub	No	139,000	164,000	113	8.1	6.7
RNA	No	47,000	42,000	42	8.9	7.9

*Colonies obtained on chloramphenicol plates (Cam^R)..

@Colonies obtained on kanamycin plates (Kan^R).

#Colonies obtained on triple selection plates (chloramphenicol, kanamycin and sucrose) and confirmed to be due to disruption of *sacB* by restriction endonuclease analysis of plasmid DNA isolated either from all colonies (*hsp83*, RNA) or a sample of 50 colonies.

[a] Targets; [b] Donor.

Figure 3.9 Rapid restriction endonuclease digestion of plasmid DNA by using CloneChecker kit (Life Technology) identify "transposition" plasmids

Single colonies were picked from triple selection plates and dispersed in lysis buffer (Life Technology). Plasmid DNA was isolated and digested by *Not* I enzyme which cuts at a unique restriction site in the transposition plasmid to form a 11.1 kb DNA fragment. In contrast *Not* I digestion of donor or target plasmid would yield fragments of 8.9 kb and 6.1 kb respectively.

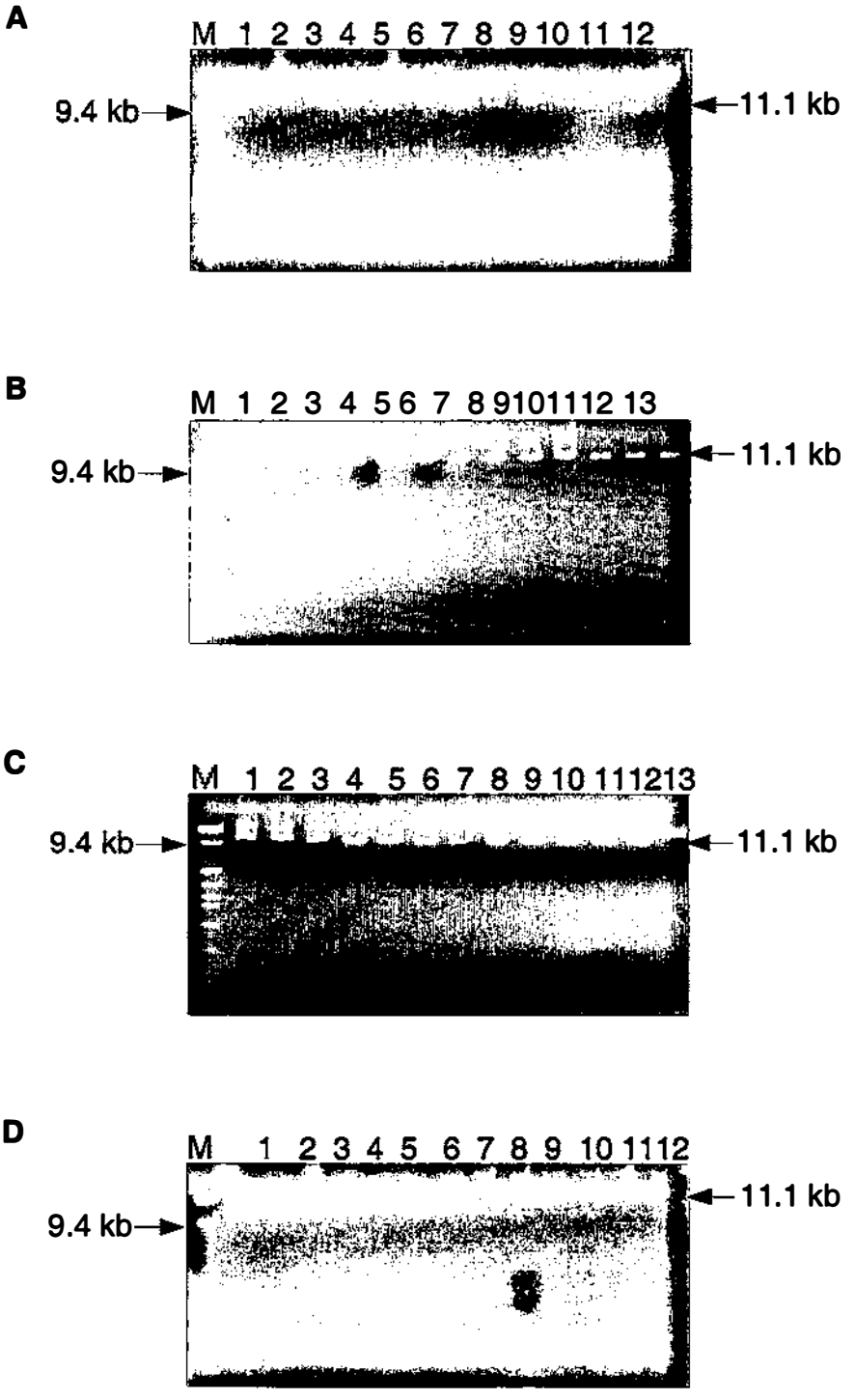
M. λ *Hin*d III/*Sac* II ladder

A. Plasmids obtained from non-heat shocked embryos injected with the hsp70-piggyBac helper. Sample 1-10 and 12 were identified as likely transposition plasmids.

B. Plasmids obtained from heat shocked embryos injected with the hsp70-piggyBac helper. Sample 1-13 were identified as likely transposition plasmids.

C. Plasmids obtained from embryos injected with the *α-tub1*-piggyBac helper. Sample 1-12 were identified as likely transposition plasmids.

D. Plasmids obtained from embryos injected with the *piggyBac* RNA helper. Sample 2, 3, 5, 6 and 9-12 were identified as likely transposition plasmids.


A
M 1 2 3 4 5 6 7 8 9 10 11 12
9.4 kb
11.1 kb
B
M 1 2 3 4 5 6 7 8 9 10 11 12 13
9.4 kb
11.1 kb
C
M 1 2 3 4 5 6 7 8 9 10 11 12 13
9.4 kb
11.1 kb
D
M 1 2 3 4 5 6 7 8 9 10 11 12
9.4 kb
11.1 kb

promoter, all of the helpers were effective at promoting transposition from donor to target in Drosophila embryos. It's unclear why so few colonies were obtained with the *hsp83* helper, as this is an effective helper for germ-line transformation (see below). The *hsp70* helper, with or without heat shock, was 3 to 4 times more efficient than either the *α1-tubulin* or RNA helpers, suggesting that this was the best helper in this assay. However, although the difference was statistically significant (chi-square test, $p<0.001$) this result would need to be confirmed by further experiments. The frequency of transposition obtained with the *hsp70* helper is comparable to that obtained by using a double promoter (*hsp70* and *piggyBac*) *piggyBac* helper and the pGDV1 target plasmid (Lobo *et al.*, 1999).

In Drosophila embryos, *piggyBac* preferentially integrates into only two of 21 potential TTAA integration sites in the pGDV1 target plasmid (Lobo *et al.*, 1999). To determine if *piggyBac* has preferred sites of integration in the *sucrase* gene, the insertion sites for 15 "transposition" plasmid DNAs were determined by direct sequencing with primers specific for the left and/or right ends of *piggyBac* . We found *piggyBac* integrated into 5 of 10 potential TTAA insertion sites within the *sucrase* open reading frame (Table 3.2). There was a pronounced preference for three sites in particular. The sequences surrounding the TTAA sites were aligned (12 nucleotides on either side) to determine if there was a consensus sequence for the preferred sites of integration. The preferred integration sites showed a bias for A or T at positions –3, -1, +1 and +3 relative to the TTAA site. That is the consensus for preferred sites was A/TNA/T**TTAA**A/TNA/T. While the consensus is based on only three preferred integration sites, a similar sequence bias was found for genomic integrations (see below).

Table 3.2 Sites of *piggyBac* insertion in the *sucrase* target gene.

TTAA site*	Sequence	Number of insertions (for/ rev)+
500	CAGTA**TTAA**CCTTT	0
681	CACAA**TTAA**AAATA	0
891	CGTCT**TTAA**AGACA	4 (4/0)
1053	ACAAG**TTAA**CGTAT	1 (1/0)
1296	TTTAT**TTAA**CAAAG	4 (3/1)
1503	CGTCT**TTAA**AATGA	5 (3/2)
1613	ATTCT**TTAA**CTGGC	0
1655	TTGTG**TTAA**AAATG	1 (1/0)
1868	GACAA**TTAA**CAGTT	0
1875	AACAG**TTAA**CAAAT	0

* Nucleotide number according to Genbank submission for *sacB* gene (X02730). The *sacB* open reading frame is from 464 to 1885

+Orientation of *piggyBac* relative to *sacB* where in the forward orientation piggyBac and sacB are in the same 5' to 3' direction.

3.2.4 Germ-line Transformation

3.2.4.1 Germ-line transformation rates

Transgenic Drosophila were obtained with all three plasmid helpers (Table 3.3). The frequency of fertile G_0s that produced transgenic offspring with the *α1-tubulin* helper was about 2.5 times higher than with the *hsp83* helper and 7 times higher than with the *hsp70* (no heat shock) helper. Although the difference between the helpers is statistically significant (chi square test, p=0.015 *α1-tub* relative to *hsp83* and p=0.0008 for *hsp70*) (the results were obtained from only two or three independent injection experiments and would need to be confirmed with further experimentation. Surprisingly no transgenics were obtained with the RNA helper although this helper was active in interplasmid transposition assays. However, since only 30 fertile G_0 were obtained with the higher concentration of RNA, it is possible that this helper could catalyse germ-line transformation but at a low rate (<3%).

3.2.4.2 Analysis of transformants

3.2.4.2.1 Analysis of transformants by Southern DNA hybridisation

DNA was prepared from 20 lines and analysed by Southern DNA hybridisation (Fig. 3.10). The genomic DNA was digested with either *Sac* I (Fig. 3.10B) or *Hin*d III (Fig. 3.10C) and hybridised with the probes indicated (Fig. 3.10A). If a transformant line had a single integration, two bands would be detected with *Sac* I digested DNA, one of 4.1 kb and the other at least 3 kb, and a single band at least 1.4 kb in size would be detected with *Hin*d III digested DNA. The results show that all but four lines have a single *piggyBac* insertion, which is consistent with a previous report that Drosophila *piggyBac* transformants generally carry a single copy of the transposon (Handler and Harrell, 1999). The exceptions are lines *hsp70*(15), *hsp70*(19), *hsp83*(25) and *α1-tub*(41). Lines *hsp70*(15) and *hsp70*(19) clearly show 3 bands of hybridisation with *Sac* I digested DNA and two bands with *Hin*d III digested DNA indicating these lines carry two insertions. Line *hsp83* (25) shows two clear bands of hybridisation with *Hin*d III digested DNA but only two strong bands of hybridisation with *Sac* I

Table 3.3. Germ-line transformation of Drosophila

Helper	Expt.	Heat Shock	No. G_0	Fertile G_0	No. of Transformants (Frequency)
hsp70	1	No	58	47	0
	2	No	93	86	3
Total			151	133	3 (2.3%)
hsp70	1	Yes	80	63	4 (6.3%)
hsp83	1	No	35	30	0
	2	No	25	19	1
	3	No	85	69	6
Total			145	118	7 (5.9%)
α1-tub	1	No	64	60	10
	2	No	55	43	7
Total			119	107	17 (15.9%)
RNA (20ng/μl)	1	No	11	10	0
RNA (200ng/μl)	1	No	35	30	0

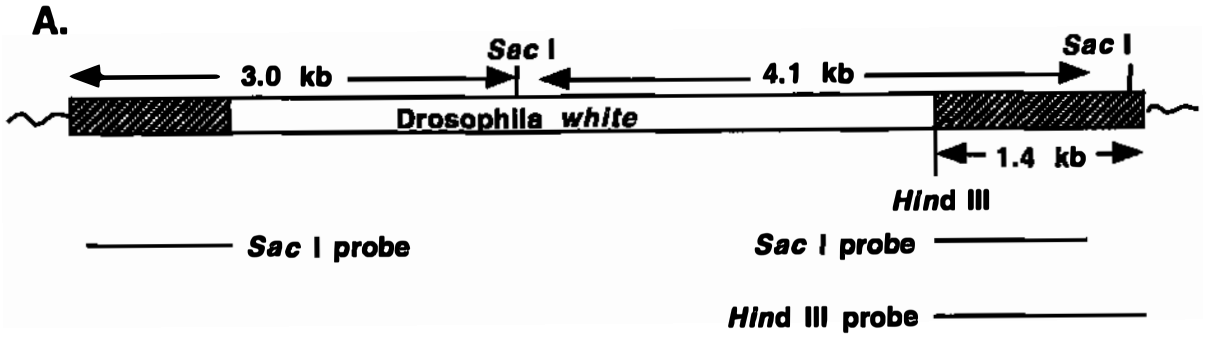
Figure 3.10 Southern hybridisation analysis of Drosophila transgenic lines

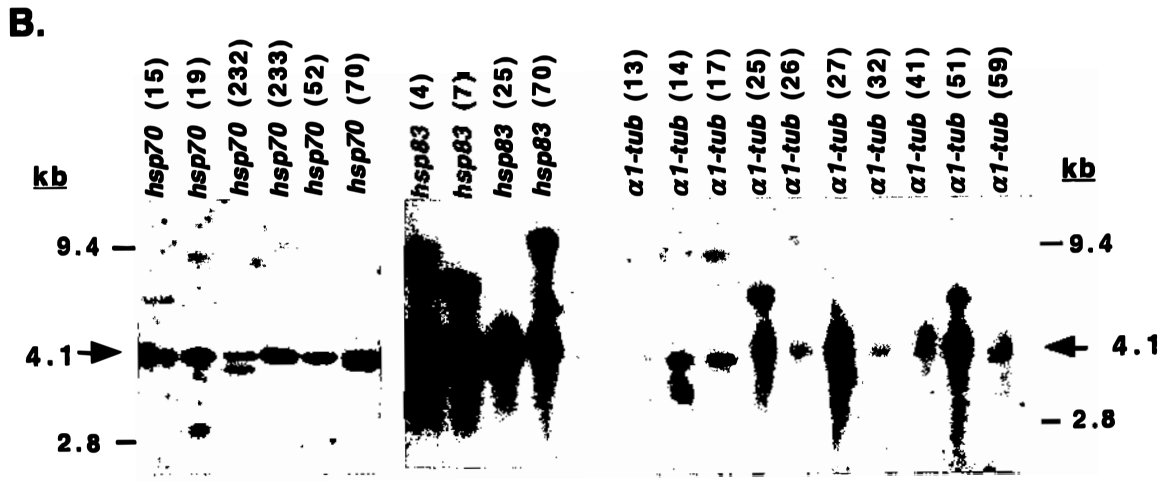
A. Schematic illustration of an integrated transgene.

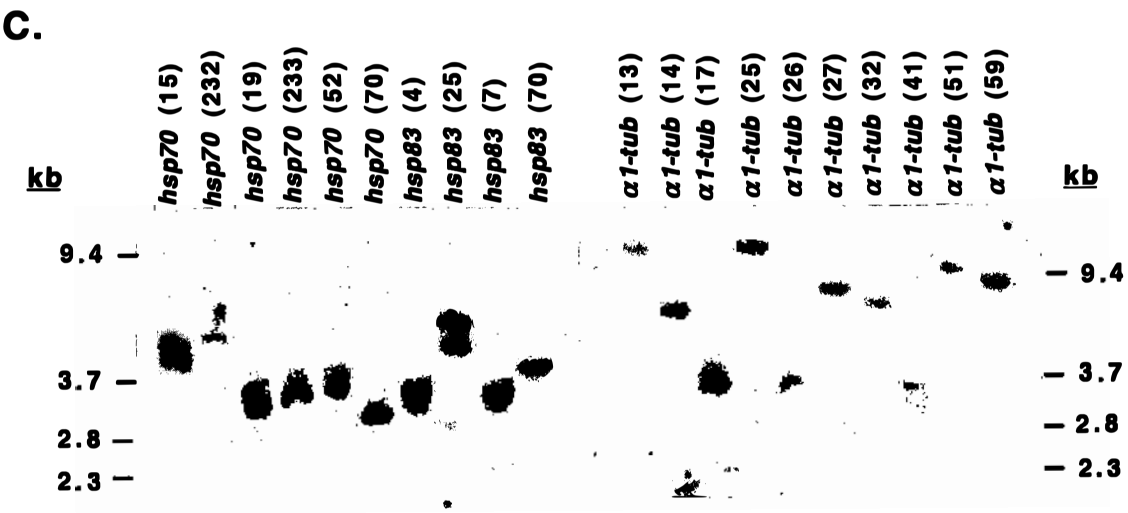
Drosophila genomic sequences are wavy lines, *piggyBac* sequences are cross-hatched and the Drosophila *mini-white* gene with adjacent 5' P is an open box. The fragment used as a probe against *Sac* I digested genomic DNA contains the entire *piggyBac* transposase ORF and detects both left and right *piggyBac* arms. When the genomic DNA is digested with *Sac* I, the probe will detect a constant 4.1 kb band for all transformant lines and a second variably-sized band that is at least 3.0 kb in size. The probe used with *Hin*d III digested genomic DNA detects a variably sized band of at least 1.4 kb in size.

B and C. Autoradiograms showing hybridisation analysis for genomic DNA from 20 lines.

In panel B, the DNA was digested with *Sac* I and in panel C the DNA was digested with *Hin*d III. DNA size markers are shown to the sides of the autoradiograms. Note that the loading order of samples *hsp70*(19) and *hsp70*(232) and samples *hsp83*(7) and *hsp83*(25) are reversed in panel C compared to B.


A.
Sac I
Sac I
3.0 kb
4.1 kb
Drosophila white
1.4 kb
Hind III
Sac I probe
Sac I probe
Hind III probe


B.
kb
hsp70 (15)
hsp70 (19)
hsp70 (232)
hsp70 (233)
hsp70 (52)
hsp70 (70)
hsp83 (4)
hsp83 (7)
hsp83 (25)
hsp83 (70)
α1-tub (13)
α1-tub (14)
α1-tub (17)
α1-tub (25)
α1-tub (26)
α1-tub (27)
α1-tub (32)
α1-tub (41)
α1-tub (51)
α1-tub (59)
kb
9.4
4.1
2.8
9.4
4.1
2.8


C.
kb
hsp70 (15)
hsp70 (232)
hsp70 (19)
hsp70 (233)
hsp70 (52)
hsp70 (70)
hsp83 (4)
hsp83 (25)
hsp83 (7)
hsp83 (70)
α1-tub (13)
α1-tub (14)
α1-tub (17)
α1-tub (25)
α1-tub (26)
α1-tub (27)
α1-tub (32)
α1-tub (41)
α1-tub (51)
α1-tub (59)
kb
9.4
3.7
2.8
2.3
9.4
3.7
2.8
2.3

digested DNA. However, on very short exposures the 4.1kb band with *Sac* I digested DNA is resolved into two bands of 4.1 and 4.2 kb. Thus this line also carries two copies of *piggyBac*. Line *α1-tub* (41) shows three bands of hybridisation with *Sac* I digested DNA but also 3 bands with *Hin*d III digested DNA. The former indicates two insertions the latter three. Thus from this analysis, we cannot determine the number of copies of *piggyBac* that are present in this line.

3.2.4.2.2 Analysis of transformants by inverse PCR

The sites of insertion for 10 lines were determined by inverse PCR, using primer combinations that would amplify DNA flanking either left or right sides of the transposon (Table 4). If sequence could only be obtained for one side of the insertion (lines *hsp70* (15), *hsp70* (23), *hsp83* (4), *α1–tub* (14), *α1–tub* (27)), the sequence from the other side was inferred from the genome sequence. With one exception, the flanking sequences were identical to the published genome sequence. The exception was line *α1–tub* (59) where there are two nucleotide differences for both left and right flanking sequences and the insertion site TTAA is TTTA in the published genome sequence. This probably reflects single nucleotide polymorphisms between the strain sequenced by Celera and the strain used to make transformants in this study. In the interplasmid transposition assays it was noted that the preferred integration sites showed a bias for A or T at positions –3, -1, +1 and +3 relative to the TTAA site. Inspection of the sequences flanking the integration sites in the transformant lines show a similar bias except that there is no preference for A or T at the +3 position.

The results are consistent with *piggyBac* transposition occurring via a canonical cut and paste mechanism. In none of the lines analysed has *piggyBac* disrupted a gene. Further, all of the *hsp83 and α1-tub* lines (the *hsp70* lines were not evaluated) were homozygous viable, although in four of these lines, *piggyBac* had inserted within 800 bp upstream of the 5' end of a gene. Thus, either the insertion has not sufficiently disrupted transcription to cause a mutant

Table 3.4 *piggyBac* insertion sites in the transgenic Drosophila lines

Line	Insertion site sequence	Nearest Gene	Relationship of site to gene
hsp70 (15)	CCATATTTTTTATTA**TTAA**AGTCATACTTAACCA	CG17520	3,534 bp downstream of 3'end
hsp70 (23)	GCTAATCAAACGTGC**TTAA**AGCGAATTATTCTTA	CG9175	2,518 bp upstream of 5' end
hsp70 (52)	TACCAAACCAAGTTA**TTAA**ATCAAAAACTAGCTT	CG8122	57 bp upstream of 5'end
hsp83 (4)	CTTTTTGA**TTAA**GCTCTT**TTAA**GCGTGCATTGTTGTG	U23485	170 bp upstream of 5' end
hsp83 (7)	GTTACTGAGC**TTAA**TCTT**TTAA**TGCCGCTAAAATAAT	CG10564	431 bp upstream of 5' end
hsp83 (70)	CGCGTTGTTGTGCA**TTAA**TTCTTCATTGTGGTC	Cyp6v1	306 bp upstream of 5' end
α1-tub (14)	TA**TTAA**TGTAGTTA**TTAA**TGCGTGTTATTTTTT	CG15167	1,510 bp downstream of 3' end
α1-tub (25)	CACACTCGGTTACC**TTAA**ACACAACCATTGAGA	CG2191	156 bp downstream of 3' end
"	"	CG2187	721 bp upstream of 5' end
α1-tub (27)	CACGGACAAGTTTTA**TTAA**CTCTATGAAATTTCA	CG12443	4,551 bp downstream of 3' end
α1-tub (59)	AGCTGGTACTTGATA**TTAA**TATCCGACAATAACG	CG7548	1,488 bp downstream of 3' end

phenotype or the gene is not essential for viability. None of the genes are known hotspots for *P* insertion (Spradling *et al.*, 1999). The closest hotspot in any of the lines is the *mam* gene, but this is over 12 kb from the site of insertion in line *hsp70* (52). Thus, from this limited analysis, *piggyBac* does not appear to have the same gene preference as *P*. This result would need to be confirmed with a much larger sample size.

3.3 DISCUSSION

3.3.1 Use of Constitutive Promoters for Driving *piggyBac* Transposase Expression

In this study we have shown that helper plasmids that used constitutive promoters to drive expression of the *piggyBac* transposase gene were effective at mediating transposition in Drosophila. Thus, for *piggyBac*-mediated transformation of non-Drosophilids, it may be advantageous to use either the *α1-tubulin* or *hsp83* helpers, particularly in those species such as the Australian sheep blowfly *Lucilia cuprina* where the *D. melanogaster hsp70* promoter has low activity (Atkinson and O'Brochta, 1992). It may also be worthwhile to isolate a gene with a constitutive promoter from the species of interest. In this regard it's noteworthy that germ-line transformation of the silk moth *Bombyx mori* was accomplished using a *piggyBac* helper with a *B. mori* actin promoter to control transposase expression (Tamura *et al.*, 2000).

3.3.2 The Preference of *piggyBac* Insertion Sites

As expected, *piggyBac* inserted into TTAA target sites in both interplasmid transposition assays and germ-line transformation. Integrations into the *sacB* target gene and into genomic DNA showed a preference for AT-rich DNA where the 8 bp consensus sequence was A/TNA/T**TTAA**A/T (target site in bold). This may in part explain the integration site bias as some of the less-preferred TTAA sites do not match the consensus. For example, the TTAA at position 1053 in *sacB* is preceded by a G and followed by a C. Inspection of the sequences surrounding the TTAA sites in the pGDV1 plasmid shows that the highly preferred sites at position 363 (AGT**TTAA**A) and 491 (TGG**TTAA**A) match the consensus except that the latter site has a G at the –1 position. Other sites in pGDV1 such as 945 (TGATTAAA) are a better match to the consensus than the site at position 491, yet these target sites are not used in Drosophila. This suggests that factors other than just the primary sequence must determine

integration site bias. As suggested by Lobo *et al* (Lobo *et al.*, 1999) one such factor could be a host-specific accessory protein(s) that is required for *piggyBac* transposition. It has also been suggested that at preferred integration sites, flanking sequences adopt a conformation that causes a high degree of DNA bending at or near the target site (Saville *et al.*, 1999; Lampe *et al.*, 1998).

3.3.3 *piggyBac* - Mediated Germline Transformation

The frequency of germ-line transformation was higher using the helper with the strong *α1-tubulin* promoter than with the helper that had the *hsp70* promoter (no heat shock) which has a low basal activity. These results suggest that for germ-line transformation it may be an advantage to use a helper that expresses high levels of the transposase. However, although the difference in germ-line transformation frequencies was statistically significant these results would need to be confirmed by additional experiments as each helper was only tested twice.

Further, it would be necessary to measure the level of transposase RNA produced from each helper at different stages of early Drosophila development. In the interplasmid transposition assays, the frequency of transposition due to basal activity of the *hsp70* helper (embryos not heat shocked) was higher than that obtained with the *α1-tubulin* helper. While the difference was statistically significant it would need to be confirmed with further experimentation. However, this does highlight that there is a difference between interplasmid transposition assays which primarily measure activity in somatic cells and transformation which reflects transposon activity in the germ-line. A promoter that is active in somatic cells may not necessarily be active in germ cells. Similarly a transposon that is active in somatic cells may not necessarily be active in germ cells if transposition requires a host-specific factor that is limiting in germ cells. In this regard it is worth noting that *Hermes* shows high activity in medfly embryos as measured by interplasmid transposition assays (Sarkar *et al.*, 1997) yet mediates germ-line transformation of medfly at a relatively low frequency (Michel *et al.*, 2001).

3.3.4 *piggyBac* Insertion Site Preference in the Drosophila Genome

In none of the ten transgenic Drosophila lines where the site of insertion was determined had the transgene disrupted a gene. This is not surprising as the insertions were not selected on the basis of mutant phenotype. Further, since exons account for only 24.1 Mb of the 120 Mb of euchromatic sequence in the Drosophila genome (Adams *et al.*, 2000) and have a lower AT content than intergenic regions (and so fewer TTAA target sites), *piggyBac* is less likely to insert into an exon than other regions. However, in 4 lines (hsp70(52), hsp83(4), hsp83(7) and hsp83(70)) *piggyBac* had inserted in the promoter regions of genes, within 500 bp of the 5' end. All of the *hsp83* lines (the *hsp70* lines were not evaluated) were homozygous viable. This may indicate that *piggyBac* has a preference for insertion into gene promoter regions but this would need to be confirmed by analysis of a much larger number of transformant lines. Mutation-causing *P*-elements also show a preference for insertion into gene promoter regions (Spradling *et al.*, 1995). However, in none of the *piggyBac* transformant lines has the transgene inserted near a known *P* "hotspot". While this is a very limited analysis it suggests that a large-scale screen for *piggyBac* disruption mutations would complement the existing *P*-element screens. A transgenic strain carrying the *piggyBac* transposase ORF controlled by either the *α1-tubulin* or *hsp83* promoters such as we have developed, would be ideal for this purpose.

CHAPTER 4

GERM-LINE TRANSFORMATION OF THE AUSTRALIAN SHEEP BLOWFLY *LUCILIA CUPRINA*

4.1 INTRODUCTION

The Australian sheep blowfly, *L. cuprina* is the most economically important pest species involved in flystrike of sheep in Australia (Watts *et al.*, 1979) and New Zealand (Heath and Bishop, 1995). (section 1.2). In New Zealand, *L. cuprina* is primarily controlled through the application of insecticides. However, increasing resistance to some insecticides coupled with a demand for wool with low insecticide residue has resulted in an increased interest in biological methods of control.

One biological method that has been proven to be effective in the field is the sterile insect technique (SIT) (Krafsur, 1998) (section 1.1). SIT has been shown to be most effective in medfly, *Ceratitis capitata* when only sterile males are released in the field (McInnis *et al.*, 1994). This is accomplished by using so-called "genetic sexing strains". The development of male-only strains represents a potentially important step toward improving SIT. Genetic sexing strains have been normally made by classical genetic methods (Franz *et al.*, 1994) (section 1.1.3).

An alternative method of making a genetic sexing strain is to use genetic engineering (O'Brochta and Atkinson, 1998) (1.1.3.2). The aim of this study is to use a genetic engineering method to make a genetic sexing strain of *L. cuprina* that would be suitable for a SIT program. However, in order to introduce a female-specific lethal genetic system into *Lucilia,* such as a tetracycline repressible female-specific lethal genetic system (section 1.5.2.1), a method for making germ-line transformants needed to be developed.

Several transposable elements have become available over the past few years. These transposable elements have been shown to be active in different insect hosts (section 1.4.2). In this study the focus was on using the *piggyBac* and *Minos* transposable elements for making transgenic *L. cuprina.* This is because *piggyBac* and *Minos* have a broad host range (see section 1.4.2.3).

Interplasmid transposition assays have been performed to determine a potential high efficiency transposase for germ-line transformation of *L. cuprina*. Several genes have been tested as potential markers (section 1.4.4) for identifying transgenic *L. cuprina*. Finally, the isolation and characterisation of two transgenic *L. cuprina* lines is described.

4.2 RESULTS

4.2.1 Synthesis of *piggyBac* and *Minos* RNAs

In order to use a transposase RNA as a helper for transformation of *L. cuprina*, *Minos* and *piggyBac* RNA were synthesized using the SP6 Cap-Scribe kit (Roche). After resuspension in DEPC treated embryo injection buffer (section 2.24.1), an aliquot of the RNA was analysed by formaldehyde-agarose denaturing gel electrophoresis with RNA standards (50-330 ng per band) to check the size (Figure 3.7, 4.1) and concentration. Both RNA preparations were of the correct size and concentration suitable for microinjection.

4.2.2 Interplasmid Transposition Assays

4.2.2.1 General description of the assays

The interplasmid transposition assays were in general similar to that described by Sarkar *et al.* (1997) and more specifically the procedure for *Minos* was according to Klinakis *et al.* (2000) and for *piggyBac* as described previously in section 3.2.

In brief, *L. cuprina* embryos were injected with a mixture of 3 plasmid DNAs, "helper", "donor" and "target".

For *piggyBac* based interplasmid transposition assays, the plasmids of the "helper", "donor" and "target" were exactly the same as those described in 3.2. The *piggyBac* helper plasmids use the Drosophila *hsp70*, *hsp83* or *α1-tubulin* promoters to control expression of the transposase. These helpers have been previously shown to mediate germ-line transformation of Drosophila (section 3.2). In addition to the helper plasmids *in vitro* transcribed and *in vitro* capped RNA helper encoding *piggyBac* transposase was tested. Disruption of *sacB* due to *piggyBac*-mediated transposition in Lucilia embryos produces a "transposition" plasmid (Figure 4.2A) that is identified by plating transformed *E.*

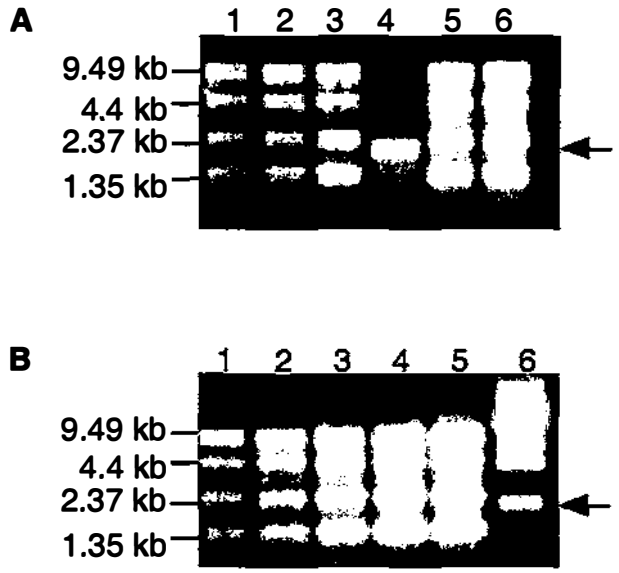


Figure 4.1 Estimation of the concentration of the *Minos* RNA for germline transformation of *L. cuprina* by formaldehyde-agarose denaturing gel electrophoresis with RNA standards to check the size.

A. Lanes 1-3 and 5-6 were loaded with standard RNA. The amount of RNA of per band for each lane was: 1 = 50 ng/band, 2 = 75 ng/band, 3 = 100 ng/band, 5 = 200 ng/band and 6 = 300 ng/band.
Line 4 was loaded with 1 μL injection solution containing *in vitro* synthesised *Minos* RNA.

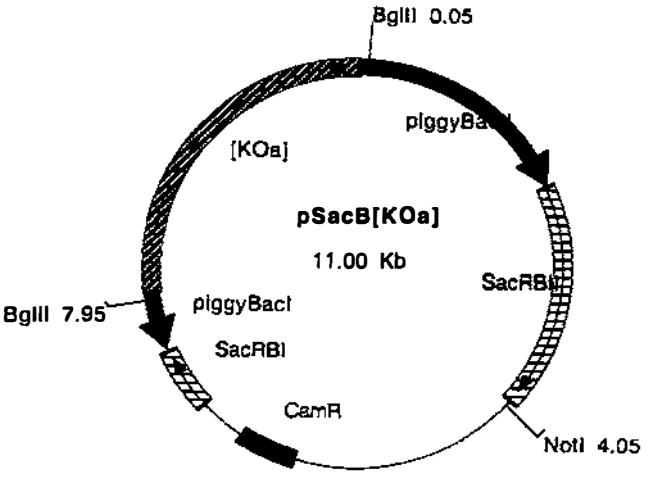
B. Lanes 1-5 were loaded with standard RNA. The amount of RNA of per band for each lane was: 1 = 52 ng/band, 2 = 86 ng/band, 3 = 168 ng/band, 4 = 251 ng/band and 5 = 333 ng/band.
Line 6 was loaded with 2 μL injection solution containing *Minos* RNA, pMiLRTet and pBC/SacRB plasmid.
The sample was estimated to contain approximately 50 ng/uL of *Minos* RNA which was of the correct size (arrow).

Figure 4.2 Maps of transposition plasmids due to *piggyBac* or *Minos* mediated transposition in Lucilia embryos.

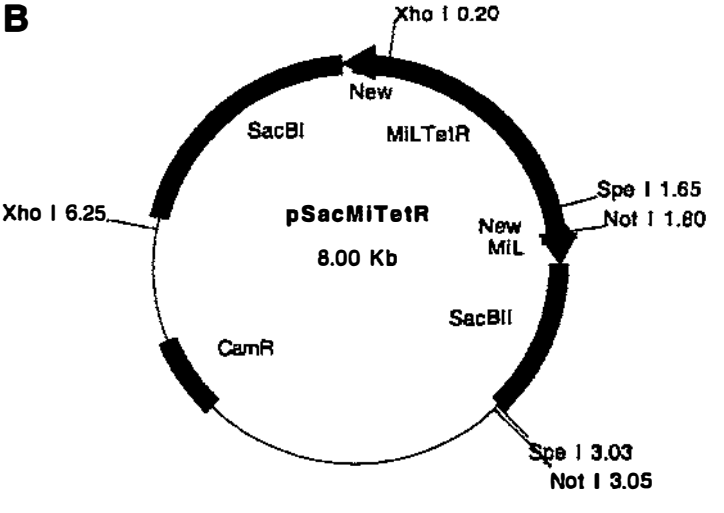
A. pSacB[kOα] plasmid was formed via disruption of *SacB* of pBC/SacRB (appendix II) with a 5.5 kb DNA fragment (*B[kOα]*) from pB[kOα]. This fragment contains *piggyBac* gene disrupted with kanamycin resistance and *αlacZ* genes (Thibault *et al.*, 1999). The total plasmid is 11.1 kb.

B. pSacMiTetR plasmid was formed via disruption of *SacB* of pBC/SacRB (II) with a 2.4 kb DNA fragment (*MiTetR*) from pMiLRTetR (appendix II). This fragment contains the ends of the *Minos* transposon bracketing the tetracycline resistance gene. The total plasmid is 8 kb.

A


BglII 0.05
piggyBac I
[KOa]
pSacB[KOa]
11.00 Kb
SacRBII
BglII 7.95
piggyBacI
SacRBI
CamR
NotI 4.05

B


Xho I 0.20
New
SacBI
MiLTetR
Spe I 1.65
Not I 1.80
Xho I 6.25
pSacMiTetR
8.00 Kb
New
MiL
SacBII
CamR
Spe I 3.03
Not I 3.05

Coli on triple media containing sucrose, chloramphenicol, kanamycin.

For *Minos* based transposition assays, *Minos* helper plasmid (pHSS6hsILMi20) (appendix II) uses the Drosophila *hsp70* promoter to drive expression of the intron-less version of the transposase gene. This helper has been used for germ-line transformation of *Anopheles stephansi* (Catterucci *et al.*, 2000). Donor plasmid, pMiLRTetR (Lorist6) (appendix II), contains the ends of the *Minos* transposon bracketing the tetracycline resistance gene that confers a selectable phenotype of tetracycline resistance in *E. coli*. The target, pBC/SacRB (appendix II) contains the *sacB* gene from *B. subtilis* and a chloramphenicol resistance gene. Disruption of *sacB* due to *Minos*-mediated transposition in Lucilia embryos produces a "transposition" plasmid (Figure 4.2B) that is identified by plating transformed *E.coli* on triple selection media sucrose/chloramphenicol/ tetracycline. In addition to the helper plasmids *in vitro* transcribed and *in vitro* capped RNA helper encoded *Minos* transposase was tested.

4.2.2.2 Determination of transposition efficiency

Colonies were obtained on triple selection medium for each of the *E. coli* transformations as shown in Table 4.1. Plasmid DNA was isolated from the colonies (section 2.20) or released from the colonies using the CloneChecker kit (Life Technologies).

Colonies obtained from *piggyBac* based transpositions were analysed by *Not* I (Figure 4.3.) and *Bgl* II (Figure 4.4) restriction endonuclease respectively. Colonies obtained from *Minos* based transpositions were analysed by *Not* I (Figure 4.5), *Xho* I and *Spe* I/*Xho* I (Figure 4.6) restriction endonuclease digestion respectively.

Table 4.1 Interplasmid Transposition Assays in *L. cuprina* embryos

Helper	Heat shock	No. Targets*	No. Donors@	No. Transpositions#	No. Transpositions confirmed	Frequency per 10^4 Targets[a]	Frequency per 10^4 Donors[b]
hsp70.Minos	No	406 000	268 000	109	104	2.6	3.9
Minos RNA	No	106 000	31 000	94	89	8.4	29
hsp70.piggyBac	No	32 000	65 000	20	12	3.8	1.8
hsp70.piggyBac	Yes	20 000	23 000	29	27	14	12
hsp83.piggyBac	No	314 000	241 000	11	1	0.03	0.04
α1-tub.piggyBac	No	55 000	38 000	8	3	0.5	0.8
piggyBac RNA	No	39 000	11 000	26	4	1.0	3.6

*Colonies obtained on chloramphenicol plates (Cam^R)..

@Colonies obtained on kanamycin plates (Kan^R).

#Colonies obtained on triple selection plates (chloramphenicol, kanamycin and sucrose for *piggyBac* and chloramphenicol, tetracycline and sucrose for *Minos*) and confirmed to be due to disruption of *sacB* by restriction endonuclease analysis of plasmid DNA isolated either from all colonies or a sample of 50 colonies (all *Minos*) .

[a] Targets; [b] Donor.

Figure 4.3 Colonies obtained from *piggyBac* based transpositions were analyzed by *Not* I restriction endonuclease digestion.

Plasmid DNA was released from the colonies using CloneChecker kit (Life Technologies) and digested by *Not* I restriction endonuclease and detected by agarose gel eletrophoresis. A unique 11.1 kb fragment was expected for each transposition plasmid.

A. Helper = piggyBac transposase was driven by *hsp70* promoter with heat shock post injection.
Lane 1: λ *Hin*d III/ *Sac* II ladder.
Lane 2-13: plasmid DNA was digested by *Not* I and each lane gave expected sized bands.

B. Helper = piggyBac transposase was driven by *hsp70* promoter without heat shock post injection.
Lane 1: λ *Hin*d III/ *Sac* II ladder.
Lane 2-13: plasmid DNA was digested by *Not* I and except lane 12 each lane gave expected sized bands.

C. Helper= piggyBac transposase was driven by *hsp83* promoter.
Lane 1: λ *Hin*d III/ *Sac* II ladder.
Lane 2-12: plasmid DNA was digested *by Not* I, lane 2 and 3 gave expected sized bands.

D. Helper = piggyBac transposase was driven by α–tubulin promoter.
Lane 1: λ *Hin*d III/ *Sac* II ladder.
Lane 2-9: plasmid DNA was digested by *Not* I, lane 5-7 and 9 gave expected sized bands.

E. Helper = piggyBac RNA.
Lane 1: λ *Hin*d III/ *Sac* II ladder.
Lane 2-13: plasmid DNA was digested by *Not* I, lane 4-6, 9-11gave expected sized bands.

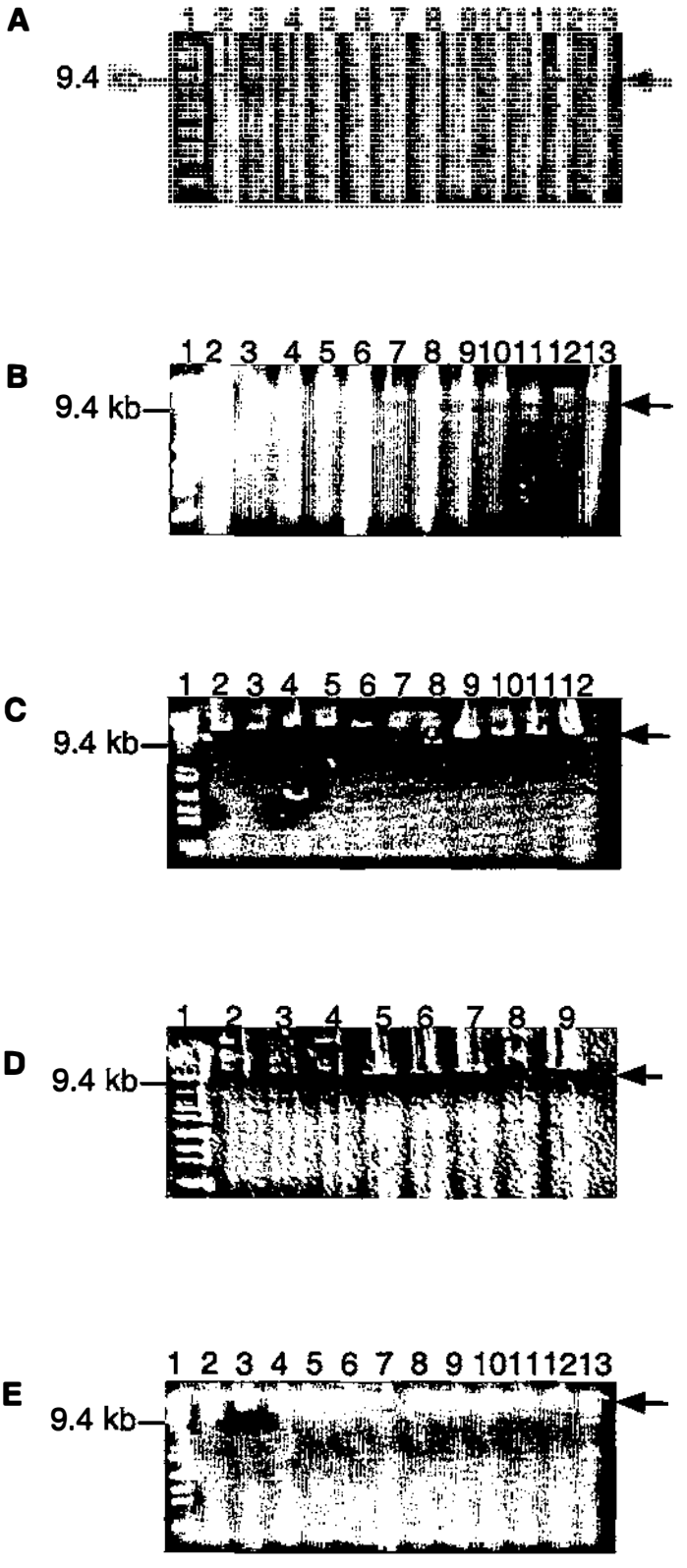

A
9.4
B
1 2 3 4 5 6 7 8 9 10 11 12 13
9.4 kb
C
1 2 3 4 5 6 7 8 9 10 11 12
9.4 kb
D
1 2 3 4 5 6 7 8 9
9.4 kb
E
1 2 3 4 5 6 7 8 9 10 11 12 13
9.4 kb

Figure 4.4 Colonies obtained from *piggyBac* based transpositions were analyzed by *Bgl* II restriction endonuclease digestion.

Plasmid DNA was released from the colonies using CloneChecker kit (Life Technologies) and digested by *Bgl* II restriction endonuclease and detected by agarose gel eletrophoresis. 3 kb and 8.1 kb fragments were expected for each transposition plasmid.

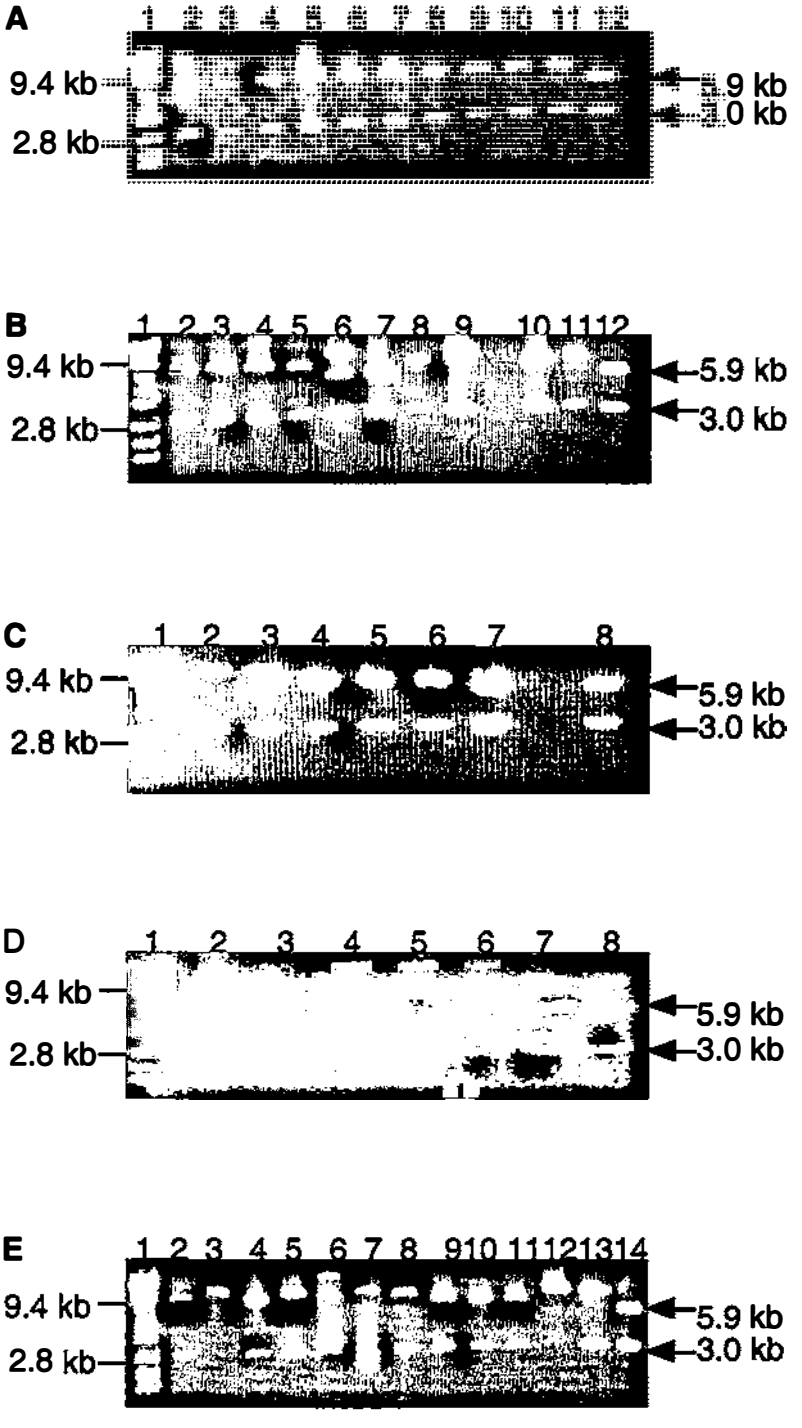
A. Helper = piggyBac transposase was driven by *hsp70* promoter with heat shock post injection.
Lane 1: λ *Hin*d III/ *Sac* II ladder.
Lane 2-11: plasmid DNA was digested by *Bgl* II and each lane gave expected sized bands.
Lane 12: control = pB[KOα] digested by *Bgl* II, gave two expected bands of 3.0 kb and 5.9 kb.

B. Helper = piggyBac transposase was driven by *hsp70* promoter without heat shock injection.
Lane 1: λ *Hin*d III/ *Sac* II ladder.
Lane 2-11: Plasmid DNA was digested by *Bgl* II. Except lane 6 and 7, each lane gave the expected sized bands.
Lane 12: control = pB[KOα] digested by *Bgl* II, gave two expected bands of 3.0 kb and 5.9 kb.

C. Helper= piggyBac transposase was driven by *hsp83* promoter.
Lane 1: λ *Hin*d III/ *Sac* II ladder.
Lane 2-7: plasmid DNA was digested by *Bgl* II. Just lane 2 gave the expected sized bands.
Lane 8: control = pB[KOα] digested by *Bgl* II, gave two expected bands of 3.0 kb and 5.9 kb.

D. Helper = piggyBac transposase was driven by *α–tubulin* promoter.
Lane 1: λ *Hin*d III/ *Sac* II ladder.
Lane 2-7: plasmid DNA was digested by *Bgl* II. Lane 4-6 gave the expected sized bands.
Lane 8: control = pB[KOα] digested by *Bgl* II, gave two expected bands of 3.0 kb and 5.9 kb.

E. Helper = *piggyBac* RNA.
Lane 1: λ *Hin*d III/ *Sac* II ladder.
Lane 2-14: plasmid DNA was digested by *Bgl* II. The bands in Lane 2, 4 and 9-11 gave the expected sized bands.
Lane 14: control = pB[KOα] digested by *Bgl* II, gave two expected bands of 3.0 kb and 5.9 kb.


A
1 2 3 4 5 6 7 8 9 10 11 12
9.4 kb
2.8 kb
9 kb
0 kb
B
1 2 3 4 5 6 7 8 9 10 11 12
9.4 kb
2.8 kb
5.9 kb
3.0 kb
C
1 2 3 4 5 6 7 8
9.4 kb
2.8 kb
5.9 kb
3.0 kb
D
1 2 3 4 5 6 7 8
9.4 kb
2.8 kb
5.9 kb
3.0 kb
E
1 2 3 4 5 6 7 8 9 10 11 12 13 14
9.4 kb
2.8 kb
5.9 kb
3.0 kb

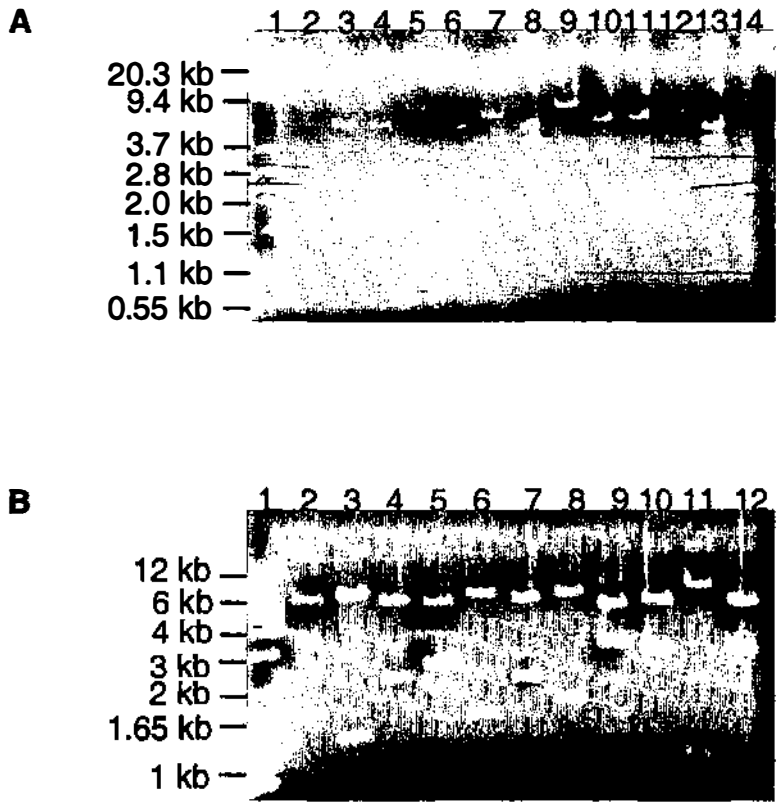


Figure 4.5 Colonies obtained from *Minos* based transpositions were analyzed by *Not* I restriction endonuclease digestion.

Plasmid DNA was released from the colonies using CloneChecker kit (Life Technologies) and digested by Not I restriction endonuclease and detected by agarose gel eletrophoresis. Two fragments were expected for each transposition plasmid in a total of 7.5 kb (6.1SacB + 1.4 MLtet).

E. Helper = Minos transposase was driven by *hsp70* promoter.
Lane 1: λ *Hind*III / *Sac* II ladder.
Lane 2-14: plasmid DNA was digested by *Not* I. Each lane gave the expected sized bands.

B. Helper = *Minos* RNA.
Lane 1: 1 kb ladder.
Lane 2-12: plasmid DNA was digested by *Not* I. Each lane gave the expected sized bands.

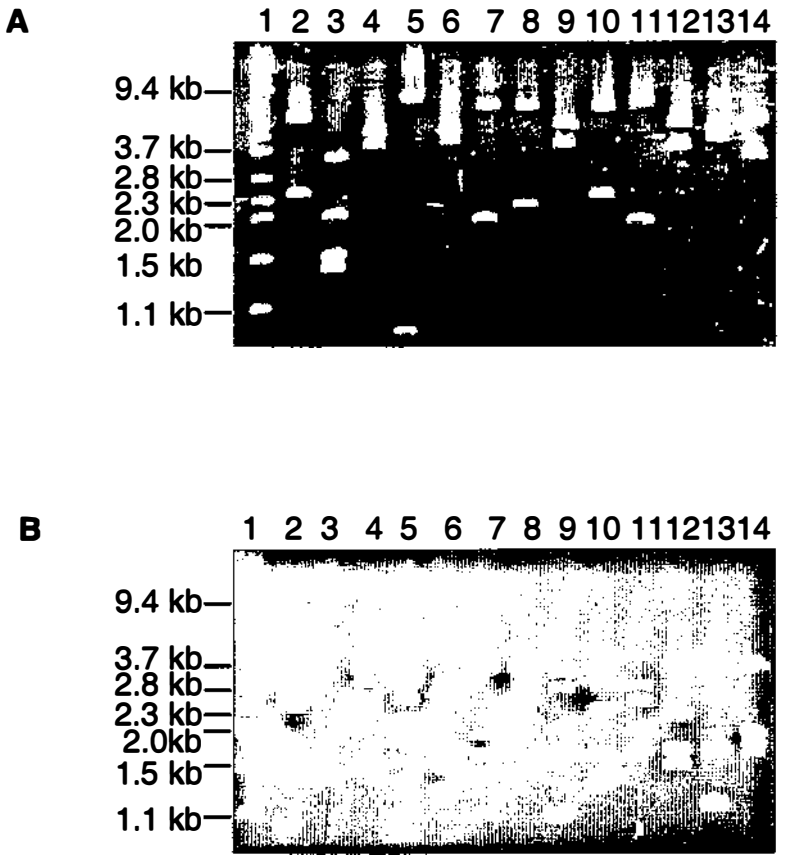


Figure 4.6 Colonies obtained from *Minos* based transpositions were analyzed by *Xho* I and *Spe*I/*Xho* I restriction endonuclease respectively.

Minos transposase was controlled by the *hsp70* promoter in transposition assay.
Plasmid DNA was isolated from the colonies and digested by *Xho* I and *Spe* I/*Xho* I restriction endonuclease respectively and detected by agarose gel eletrophoresis. For *Xho* I digestion, it was expected to give 2 bands in a total of 7.5 kb. For *Spe* I/*Xho* I digestion, it was expected to give 4 bands in a total of 7.5 kb

A. Lane 1: λ *Hin*d III/ *Sac* II ladder.
Lane 2,4-14: plasmid DNA was digested by *Xho* I.
Lane 3: plasmid DNA was digested by *Xho* I/*Spe* I.

B. Lane 1: λ *Hin*d III/ *Sac* II ladder.
Lane 2,4-14: plasmid DNA was digested by *Xho* I/*Spe* I
Lane 3: plasmid DNA was digested by *Xho* I

Nearly all of the transposition plasmids obtained using either *Minos* helper gave DNA fragments of the expected sizes for canonical "cut and paste" transposition events (confirmed transpositions in Table 4.1) (Appendix III).

Similarly most of the plasmids obtained with the *hsp70.piggyBac* helper were due to transposition into *sacB.* However, for the other *piggyBac* helpers, most of the transposition plasmids gave DNA fragments that were of an incorrect size. Several of the "false" plasmids gave digestion patterns consistent with being the donor plasmid. Further, in the experiment that used the *piggyBac* RNA helper, several of the transposition plasmids obtained were much larger than expected. The mechanism by which these plasmids arose was unable to be determined but sequence analysis suggests that they were not due to true transposition events.

The main conclusion from these assays is that both *piggyBac* and *Minos* can transpose in *Lucilia* embryos. The most active helpers are the *Minos* RNA and *piggyBac* plasmid with the *hsp70* promoter.

Of the *Minos* helpers, significantly more transpositions were obtained with the RNA than with the helper plasmid with the *hsp70* promoter ($p<0.0001$, chi square on 1 df).

Of the piggyBac helpers, significantly more transpositions were obtained with the *hsp70.piggyBac* helper than with any of the other *piggyBac* helpers (chi square, $p<0.001$). Further the number of transpositions increased significantly in embryos that were heat shocked after injection ($p= 0.0002$). However it should be noted that this is the result of a single experiment.

4.2.2.3 Determination of the insertion sites of the traspositions

The insertion sites for 17 *piggyBac* transposition plasmids (11 *hsp70*, 1 *hsp83*, 3 *α1-tub*, 2 RNA) was determined by direct sequencing (Table 4.2)

Table 4.2 Sites of *piggyBac* insertion in the *sucrase* target gene.

TTAA site*	Number of insertions (for/rev)[+]
500	2 (2/0)
681	0
891	2 (1/1)
1053	2 (0/2)
1296	6 (2/4)
1503	5 (2/3)
1613	1(1/0)
1655	0
1868	0
1875	0

*Nucleotide number according to GENBANK submission no. for the *sacB* gene (X02730). The *sacB* open reading frame is from 464 to 1885.

[+]Orientation of *piggyBac* relative to *sacB* where in the forward orientation *piggyBac* and *sacB* are in the same 5' to 3' direction.

with JF01 primer (section 2.11). As expected, in all of the plasmids the *piggyBac–αlacZ-ori-kan* gene cassette had inserted into one of the TTAA target sites in *sacB*. As previously found in Drosophila embryos (section 3.2) *piggyBac* preferentially inserted into the TTAA sites at positions 1296 and 1503.

The insertion sites for 17 *Minos* transposition plasmids was determined by directly sequencing with MiInv primer (Table 2.2). As expected all insertions occurred in a TA target site in *sacB*. With the exception of the known preferred site at 572 (Klinakis *et al.*, 2000) (2 hits), the insertion sites were different for each of the transposition plasmids. The positions were 598, 613, 732, 836, 970, 998, 1192, 1239, 1242, 1473, 1625, 1748 and 1750.

4.2.3 Germ-line Transformation

All germ-line transformation experiments were carried out in collaboration with Dr. J.C. Heinrich. Additionally Ms. R. Henry and Dr. Allen Heath carried out the fly work.

4.2.3.1 Evaluation of eye colour genes as marker

Identification of transformants requires a marker gene that encodes a product that can be readily detected and used to distinguish the transgenic flies from the much more plentiful non-transgenic siblings. One of the apparent advantages of working with *L. cuprina* is the wealth of eye colour mutant strains that have been isolated (Weller and Foster, 1993). Genes that encode proteins that are required for eye pigment synthesis such as *white*, *cinnabar* and *rosy* have been very useful for identifying transgenic Drosophila, medfly and the mosquito *Aedes aegypti* (Rubin and Spradling, 1982; Hazelrigg *et al.*, 1984; Loukeris *et al.*, 1995; Coates *et al.*, 1998). The *white* (*w*) mutant strain of *L. cuprina*, which has white eyes, is believed to be homozygous for a loss-of-function mutation in the *white* gene that is the homolog of the Drosophila and medfly *white* genes (Summers and Howells, 1980; Elizur *et al.*, 1990).

In initial attempts to make transgenic *Lucilia*, the medfly *white* cDNA driven by the Drosophila *hsp70* promoter was used as a marker gene. The medfly *white* rather than Drosophila *white* was used as the codon bias for medfly is more similar to that of *Lucilia* than *Drosophila*. Further, at the time these experiments were performed a full-length *Lucilia white* cDNA was not available. We used both *Minos* (controlled with the *hsp70* promoter) and *piggyBac* (controlled with either the Drosophila *hsp83* or *α1-tubulin* promoters) helpers as these had both been shown to be active in *Lucilia* embryos using interplasmid transposition assays. The G_0s that were obtained were mated in batches of either 5 or 10 per mating and consequently the percentage of fertile G_0s is not known. No transformants were identified from any of the G_0 matings (Table 4.3).

There are several possible explanations for the apparent failure to make transformants (see Discussion). One possibility was that the Drosophila *hsp70* promoter was not sufficiently active during eye development in *Lucilia* to produce enough *C. capitata white*$^+$ to rescue the *w* mutant. The *hsp70* promoter was replaced with the strong constitutive *α1-tubulin* promoter from Drosophila. No Lucilia transformants were obtained using this marker gene and a *piggyBac* helper (Table 4.3).

The Drosophila *cinnabar* (*cn*) gene has been used as a marker to identify transgenic *A. aegypti* (Coates *et al*., 1998). The *yellow eye* Lucilia mutant strain has been shown to lack the *cinnabar* gene product, kynurenine 3-monooxygenase (Summers and Howells, 1978). Since *cn* is non-autonomous, transient expression of microinjected DNA containing the *cn* gene is sufficient to partially rescue the eye colour of the *cn* strain of Drosophila and the *white* strain of *A. aegypti* (Warren *et al*., 1996; Cornel *et al*., 1997). We injected *Lucilia ye* embryos with plasmid DNA containing the Drosophila *cn* gene but none of the adults that developed showed any rescue of the yellow eye colour. This result indicated that the Drosophila *cn* gene may not be expressed at a high enough level to be used as a marker gene in *Lucilia*.

Table 4.3 Attempts at germ-line transformation of *Lucilia* using *C. capitata white*$^+$ as a marker gene.

Helper	Marker	Expt.	No. embryos injected	No. G0	No. G0 per mating	No. of transformants
hsp70.Minos	*hsp70.Cc w*$^+$	1	400	70	10	0
		2	400	33	10	0
		3	400	110	10	0
		4	400	65	10	0
		5	200	23	2-5	0
		6	200	20	2-5	0
Total			2000	317		0
hsp83.piggyBac	*hsp70.Cc w*$^+$	1	400	42	5	0
α1-tub.piggyBac	*hsp70.Cc w*$^+$	1	400	145	5	0
α1-tub.piggyBac	*α1-tub.Ccw*$^+$	1	400	60	5	0
		2	400	140	5	0
Total			800	200		0

4.2.3.2 Transient expression of *EGFP* gene

The gene encoding the jellyfish green fluorescent protein (GFP) or its enhanced derivative (EGFP) has been used a marker to identify a range of transgenic insects including Diptera and Lepidoptera (Handler and Harrell, 1999; Peloquin *et al.*, 2000; Tamura *et al.*, 2000; Pinkerton *et al.*, 2000). It is a sensitive marker that has the advantage that it can be used with any strain. A transient expression assay was used to determine whether an EGFP construct was active in *L. cuprina* embryos.

Embryos were microinjected with plasmid DNA containing the EGFP coding region and either a *Autographa californica* nuclear polyhedrosis virus *hr5-ie1* enhancer promoter (Huynh and Zieler, 1999) or the *D. melanogaster actin 5C* (Pinkerton *et al.*, 2000) or *polyubiquitin* (Davis *et al.*, 1995) promoters. Embryos were examined for EGFP expression using a fluorescence microscope, approximately 16–24 h post-injection. Transient expression was only observed in embryos injected with the *polyubiquitin-EGFP* construct (*PUbnlsEGFP*) and a high proportion of embryos (40%) showed EGFP expression.

4.2.3.3 Establishment of the transgenic lines of *L. cuprina* using *polyubiquitin-EGFP* as a marker

A series of transformation experiments was carried using *PUbnlsEGFP* as a marker gene and either *piggyBac* (controlled by the *Drosophila hsp70*, *hsp83* or *α1-tubulin* promoters) or *Minos* (RNA) helpers (Table 4.4). The concentration of *Minos* RNA was estimated by formaldehyde-agarose denaturing gel electrophoresis (Figure 4.7). RNA standards were used to check the size. More experiments were performed using the *hsp70.piggyBac* helper as this had the highest activity in interplasmid transposition assays. With this helper several EGFP positive embryos were identified among the G_1 offspring of two crosses (A18 and A54) where each cross had a single G_0 male. No transformants were identified with either the *hsp83.piggyBac*, *α1-tub.piggyBac* or *Minos* RNA helpers.

EGFP expression in line A18 and A54 appears to be stable. EGFP was detected in most tissues in the embryo and larvae but was generally absent in adult. The exception was the ovaries in adult female that showed strong EGFP expression (Fig 4.8).

4.2.3.4 Confirmation of the integration of pB[PUbnlsEGFP]

To confirm the integration of pB[PUbnlsEGFP] into the genome of the transgenic lines, Southern DNA hybridisation experiments were performed (Fig. 4.9). The genomic DNAs of both transformant lines A18 and A54 were isolated (section2.20) from the adults of 6-8th generations. The DNA was digested for overnight with *Not* I and *Bgl* II restriction endonucleases.

Two probes were used for the hybridisation. One was obtained by digestion of pB[PUbnlsEGFP] with *Cla* I/*Bgl* II restriction endonucleases and isolation of a 4.4 kb fragment, the other was obtained by digestion of pB[PUbnlsEGFP] with *Asp*718 restriction endonuclease and isolation of a 0.85 kb fragment. The probes contain most of the PUbnlsEGFP construct (Fig. 4.9B).

A transformant with a single insertion would give three bands, one of 3.0 kb and the other two at least 2.2 kb and 0.7 kb. Line A54 gives the number of bands expected for a single insertion of the *EGFP* construct. However, 7 strong bands and one weak band of hybridisation were seen with A18 line indicating at least 3 and possibly 4 separate insertions.

4.2.3.5 Determination of the copies of integration of pB[PUbnlsEGFP]

To determine the number of copies of integration of pB[PUbnlsEGFP], genomic DNA from both lines was digested with *Hin*d III restriction endonuclease. The membrane was hybridised with a probe which was obtained by digestion of pB[PUbnlsEGFP] with *Asp*718 restriction endonucleases and isolation of a 0.85 kb fragment.

Table 4.4 Germline transformation (TF) of *Lucilia* using *polyubiquitin.EGFP* as a marker gene.

Helper	Expt	.No. embryos injected	No. G_0	No. fertile	No. of TF (frequency)
α1-tub.piggyBac	1	400	15	9	0
	2	400	34	18	0
Total		800	49	27	0
hsp83.piggyBac	1	400	24	10	0
	2	400	27	19	0
Total		800	51	29	0
hsp70.piggyBac	1	400	6	3	0
	2	400	48	19-23	0
	3	400	12	6-9	0
	4	400	70	25-33	0
	5	400	19	9-13	0
	6	400	92	42-58	2
Total		2400	247	104-139	2 (1.4 –1.9%)
Minos RNA	1	400	85	54-72	0

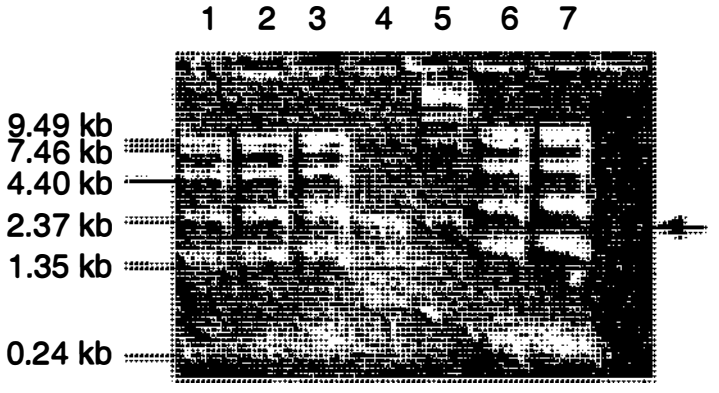


Figure 4.7 Estimation of the concentration of the *Minos* RNA which mixed with pMIEGFP plasmid for germline transformation of *L. cuprina* by formaldehyde-agarose denaturing gel electrophoresis. RNA standards were used to check the size.

Lanes 1-3 and 6-7 were all loaded with standard RNA. The amount of RNA of per band for each lane was: 1 = 50 ng/band, 2 = 75 ng/band, 3 = 100 ng/band, 6 = 150 ng/band and 7 = 250 ng/band.

Lane 4 was load with 1 μL of *in vitro* synthesised *Minos* RNA (arrow).

Lane 5 was loaded with 1 μL injection solution which contained *Minos* RNA and pMiEGFP plasmid DNA.

The sample was estimated to contain approximately 60 ng/μL of *Minos* RNA which was of the correct size (arrow).

Figure 4.8 EGFP expression in transgenic *Lucilia* lines.

A. Embryo from line A54.

B. Embryos from line A18.

C. Third instar larva from line A18.

D. Third instar larva from line A54.

E. Line A18 adult female. Note pale green abdomen that is due to EGFP expression in ovaries.

F. Immature ovaries from line A18.

G. Mature ovary from liver-fed A18 female.

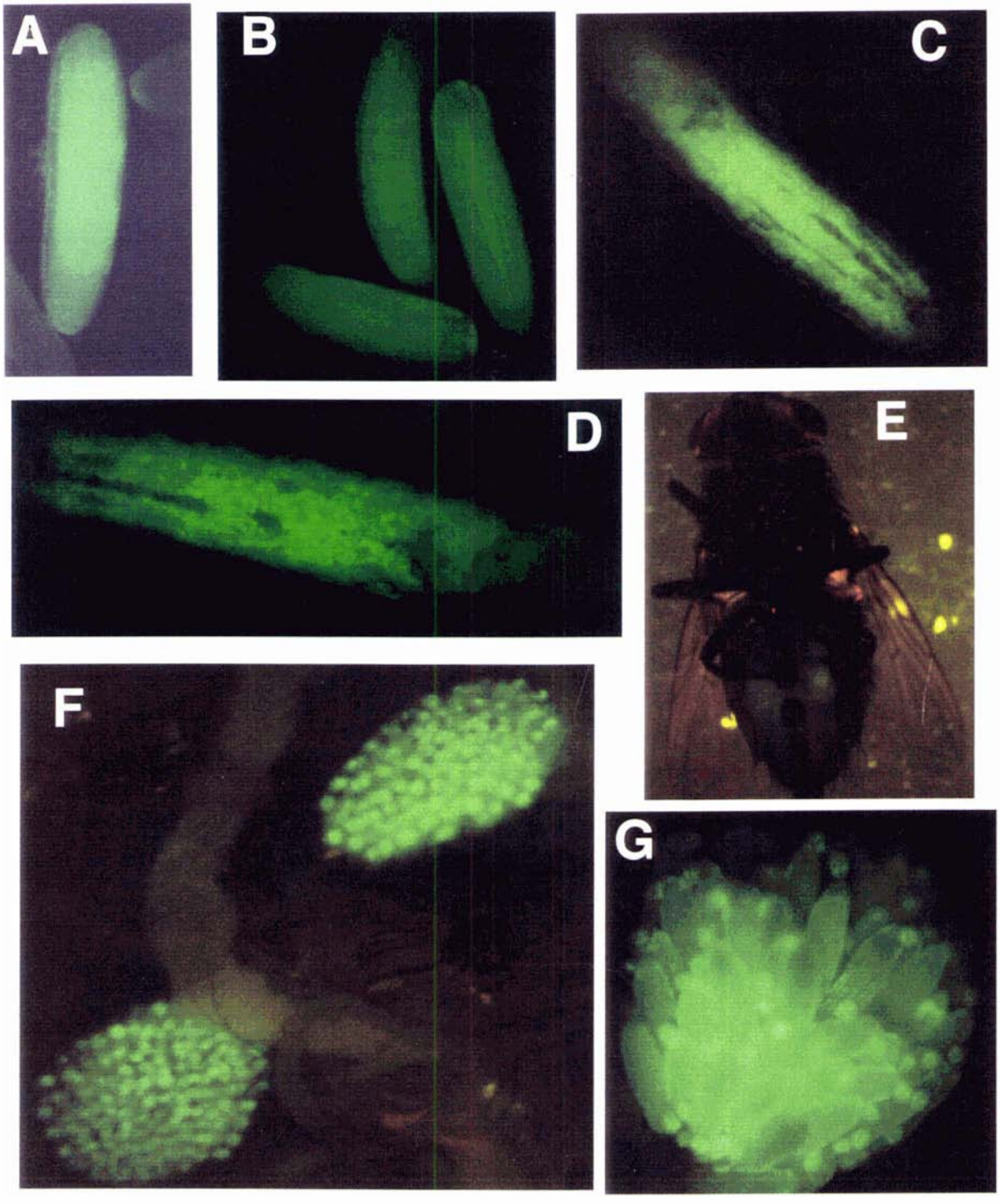
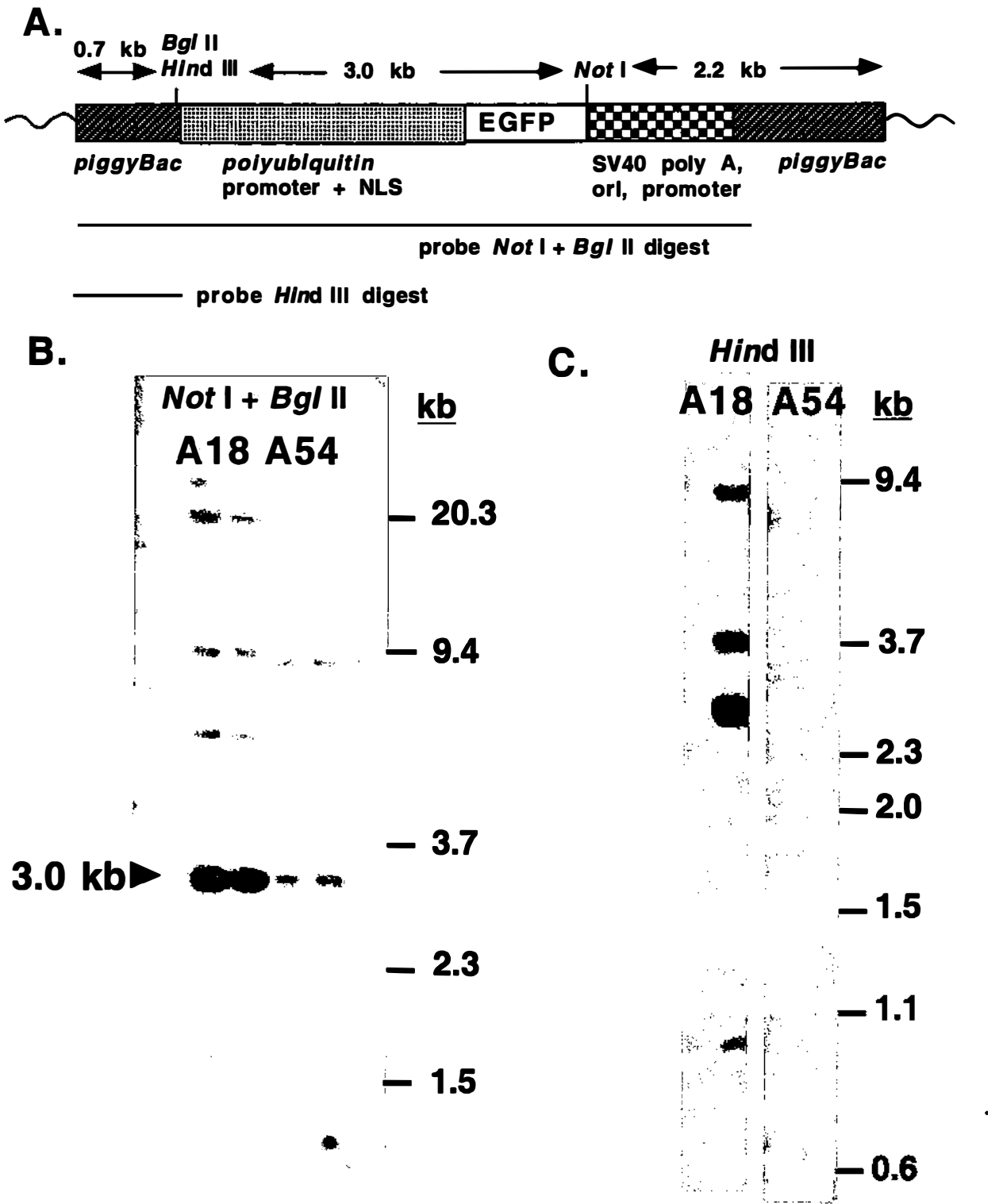

A
B
C
D
E
F
G

Figure 4.9 Southern DNA hybridisation analysis of *L. cuprina* pB[PUbnlsEGFP] transformant lines.

A. Schematic diagram of the pB[PUbnlsEGFP] vector showing the location of the *Not* I and *Bgl* II restriction sites and fragments that were used as hybridisation probe. Note that an internal fragment of 3.0 kb is generated by *Not* I and *Bgl* II digestion.

B. Genomic DNA from each of two single individuals of the A18 and A54 transformant lines was digested with *Not* I and *Bgl* II. The DNA was size separated by 1% agarose gel electrophoresis, transferred to a nylon membrane and hybridised under conditions of high stringency with [^{32}P] labelled DNA probe (A.). A line with a single insertion should give 3 bands one of which should be 3.0 kb

C. Genomic DNA from A18 and A54 lines digested with *Hin*d III was hybridised with [^{32}P] labelled DNA probe shown in A. Each insertion should give a single band of at least 0.7 kb. The data indicate that line A54 has a single insertion whereas line A18 has four separate insertions.


A.
0.7 kb
Bgl II
Hind III
3.0 kb
Not I
2.2 kb
EGFP
piggyBac
polyubiquitin
promoter + NLS
SV40 poly A,
ori, promoter
piggyBac
probe Not I + Bgl II digest
probe Hind III digest
B.
Not I + Bgl II
A18 A54
kb
20.3
9.4
3.7
2.3
1.5
3.0 kb
C.
Hind III
A18 A54
kb
9.4
3.7
2.3
2.0
1.5
1.1
0.6

This 0.85 kb probe would detect a single variably sized band larger than 0.7 kb for each insertion present in the line (Fig. 4.9C). Line A54 gave a single band confirming that this line carries a single insertion of pB[PUbnlsEGFP]. With line A18 DNA 4 bands were seen indicating that this line has 4 separate insertions of pB[PUbnlsEGFP], consistent with the previous hybridisation data.

4.2.3.6 Determination of the insertion sites of pB[PUbnlsEGFP] by inverse PCR

The sites of insertion for the two lines were determined using inverse PCR (section 2.17). Genomic DNA was digested with *Taq* I for left arm circles and *Sau* 3A for right arm circles. Sequence flanking both sides of the A54 insertion was obtained (Figure 4.10) For line A18 only 3 of 4 possible flanking sequences were obtained with both *Taq* I and *Sau* 3A-digested DNA. In all cases, as expected *piggyBac* has inserted into a TTAA target site.

In Drosophila it was noticed that there was a preference for either A or T at the –3, -1 and +1 positions relative to the TTAA site (section 3.2). The *Lucilia* insertion sites did not show the same sequence bias, with the possible exception of the –1 position where 3 of 4 sequences match the Drosophila consensus.

Using BLAST, the Genbank nucleotide database was searched for sequences similar to the sequences flanking the sites of insertion. No similarities were found with the exception of one of the left flanking sequences (*Taq* I digest) of the A18 line. This sequence showed similarity to the Lu-P1 sequence, one of two P-like sequences discovered in *Lucilia cuprina* (Perkins and Howells, 1992) (Fig. 4.11). The similarity begins 66 bp from the TTAA insertion site and extends for 169 bp to the *Taq* I restriction site. There is 75% identity between the A18 flanking sequence and Lu-P1, which is higher than the similarity between Lu-P1 and Lu-P2 (Perkins and Howells, 1992). The region of similarity

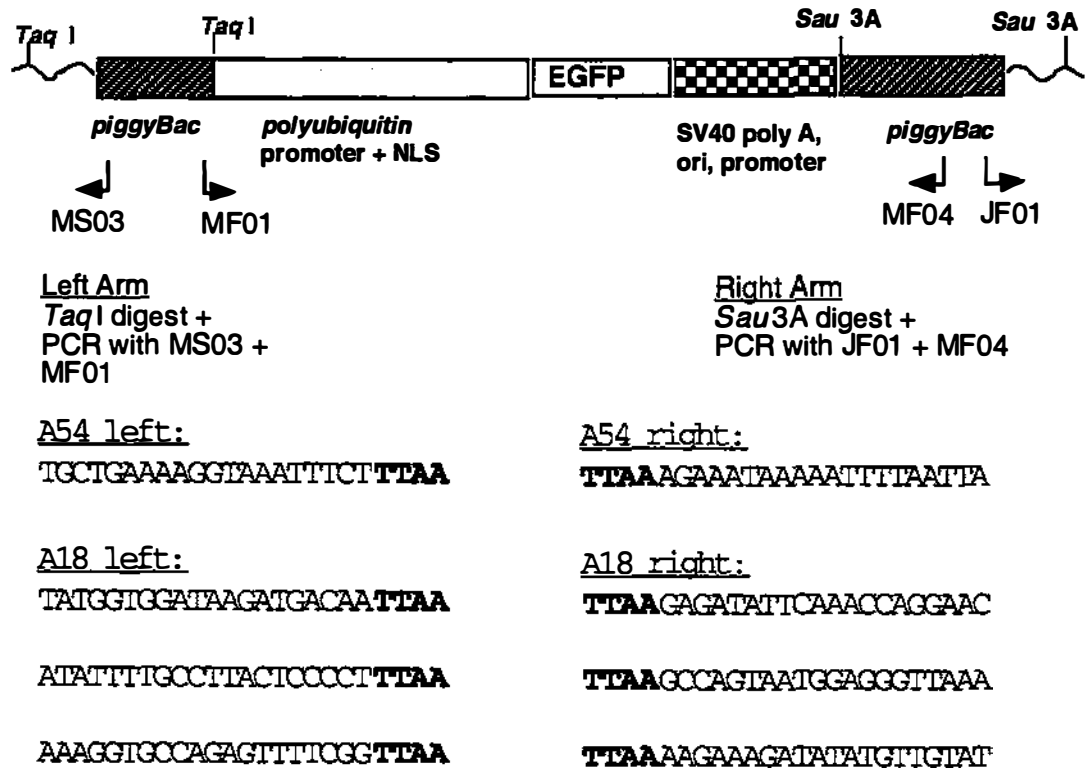


Figure 4.10 Flanking sequences of the *L. cuprina* transformant lines determined by Inverse PCR.

The inverse PCR strategy was essentially the same as that of Elick *et al.* (1997) On the top is shown a schematic diagram of the pB[PUbnlsEGFP] vector showing the location of the *Taq* I and *Sau* 3A restriction sites and PCR primers. Genomic DNA from the A18 and A54 lines was digested with *Taq* I and also with *Sau* 3A and ligated at low DNA concentration. PCR was performed with primers indicated as described in experimental procedures. The PCR products were either sequenced directly with one of the PCR primers or cloned into a plasmid vector. In all lines the vector has inserted into and duplicated a TTAA target site as expected for standard *piggyBac*-mediated transposition. Note that for the A18 line insertion sites, It is not known which left flanking sequence matches should be paired with which right sequence.

begins almost 600 bp upstream of the proposed exon 0 of Lu-P1. Good candidates for inverted repeat termini in Lu-P1 were not found previously (Perkins and Howells, 1992). The sequence similarity between Lu-P1 and A18 flanking sequence suggests that the 5' end of the Lu-P repetitive sequence is at least 600 bp upstream of the first exon.

The Southern DNA hybridisation and inverse PCR experiments were all performed by the candidate.

```
A18      70 AATATTATTTATA-TAGAATTTAAA-AGTGTTATTAGTGTTTGTAAT-TG  116
            ||||||||||  |  | ||| || |  |  | ||||||||||| || | ||
Lu-P1   253 AATATTATTG-TGCT-GAAATTCATCAC-GATATTAGTGTTTATA-TGTG  298

A18     117 AATTTTG-ACCTTTAAGTCATTTTCTGAAGGGGAGTTTGTATGATGGAT-  164
            ||||||| || || ||||||| |||||||||||| || |||||  |||
Lu-P1   299 AATTTTGGACATTCAAGTCATATTCTGAAGGGGACTTGGTATG--GGAGC  346

A18     165 AGGGTCAAATGAAGCCCGATCATTACAAAAATCGGGA-GTGTCATTTAAA  213
            |||||||||||| | |||||| |||  ||| | || | | |||||  ||
Lu-P1   347 AGGGTCAAATGAGGTCCGATCTTTATGAAATTTGGCAAG-GTCATC-AAG  394

A18     214 G-TTGTATAAAACTAAGTTT-TGTCGA.....................  238
            | |||||||||||| | ||| || |||
Lu-P1   395 GCTTGTATAAAACTTA-TTTGTGCCGA.....................  420
```

Figure 4.11 Alignment of the left flanking sequence of one of the line A18 insertion sites with Lu-P1, a *P*-like element from *Lucilia cuprina*.

The flanking sequence shows 75% identity with a 168 bp region of the Lu-P1 sequence which begins almost 600 bp upstream of the proposed exon 0 (Perkins and Howells, 1992). The similarity ends at the *Taq* I restriction site in the A18 sequence, as this was the enzyme that was used to digest the genomic DNA.

4.3 DISCUSSION

4.3.1 *piggyBac* Can Mediated Germ-line Transformation of *Lucilia cuprina*

The results of the above experiments have shown that *piggyBac* can mediate germ-line transformation of *Lucilia cuprina*, thus extending the number of insect species that can be transformed with this element.

4.3.2 Transformation Frequency of *piggyBac* Mediated Germ-line Transformation of *Lucilia cuprina*

Using the helper plasmid with the *Drosophila melanogaster hsp70* promoter controlling expression of the transposase and the *PUbnlsEGFP* marker gene two transgenic lines were obtained from 104 to 139 fertile G_0 adults giving a transformation frequency of 1.4 to 1.9%. The frequency is similar to that obtained for *piggyBac* mediated transformation of silkworm (Tamura *et al.*, 2000), medfly (Handler *et al.*, 1998) and pink bollworm (Peloquin *et al.*, 2000). Atkinson and O'Brochta (1992) showed the *hsp70* promoter appears to have low activity in *L. cuprina* embryos, so may not make much transposase. This may explain the low transformation frequency in this study. Both transformant lines were obtained from a single injection experiment where a high percentage of injected embryos developed into G_0 adults (23%). This may simply have been due to chance as almost half of the fertile adults were obtained from this experiment. Alternatively it may indicate the importance of post-injection survival in obtaining transgenic *Lucilia*. Catteruccia *et al.* (2000) noted that a high percentage adult survival was a good indicator of the likelihood of successful transformation of the mosquito *Anopheles stephensi*.

PUbnlsEGFP was used as a marker gene and either *piggyBac* (controlled by the *Drosophila hsp70*, *hsp83* or *α1-tubulin* promoters) or *Minos* (RNA) helpers to transform *L. cuprina*. No transformants were identified with either the *hsp83.piggyBac*, *α1-tub.piggyBac* or *Minos* RNA helpers (Table 4.4). However,

this does not necessarily indicate that these helpers are significantly less effective than the *hsp70.piggyBac* helper as the number of fertile G_0s obtained was much less than with the *hsp70* helper.

4.3.3 The Survival of Transformation

The reasons for the percentage adult survival being so variable is not known, although it is probable that the quality of the injection needles is an important factor. In any case, for routine transformation of *Lucilia* it would be desirable if a higher percentage of injection experiments produced transformants and/or a higher frequency of transformation was obtained from those experiments that did yield transformants.

4.3.4 Improvement of Transformation Efficiency

The frequency of transformation could potentially be improved by using a native Lucilia promoter from a constitutively expressed gene to control expression of the transposase gene. Helper plasmids with the constitutive *α1-tubulin* and *hsp83* promoters from Drosophila controlling *piggyBac* transposase expression were effective at mediating germ-line transformation in *D. melanogaster* (section 3.2).

Alternatively the frequency of transformants detected could potentially be increased by using a different marker gene. Use of a promoter from a *Lucilia* gene that is expressed in early development (so embryos can be screened) or insulator elements to block any negative chromosomal effects at the site of integration (Bell and Felsenfeld, 1999) could potentially improve marker gene expression. Further DsRed (Clontech) which emits red light upon excitation, could be a more sensitive marker than EGFP as Lucilia embryos show a low level of background yellow-green fluorescence.

4.3.5 The Copies of Integration of *PUbnlsEGFP*

Of the two transformant lines obtained, one had a single copy of the transgene and the other most likely had four copies. The latter was unexpected, since most *piggyBac*-mediated *D. melanogaster* transformant lines have a single copy of the transgene (Handler and Harrell, 1999) and medfly lines have one or two integrations (Handler *et al.*, 1998). However, it has recently been reported that *piggyBac*-mediated *Anastrepha suspensa* transformant lines have between one and four integrations of the *PUbnlsEGFP* marker gene (Handler and Harrell, 2001). Multiple integrations suggest that either there was a high level of transposition in the pole cell that took up the plasmid DNAs, possibly due to a burst of transposase synthesis, or that there were successive rounds of transposition throughout development. If the latter, then either the plasmid DNAs and/or the *piggyBac* transposase must be stable in *Lucilia*. Alternatively, if expression of the integrated marker gene was poor, for example due to position-effects, then by chance detection of a transformant line with multiple integrations would be more likely. This possibility could be tested by successive backcrossing of the line that has multiple integrations with the wild-type strain to obtain lines that carry single integrations (if on separate chromosomes). If marker gene expression is subject to strong position-effects in *Lucilia*, then some of the sub-lines would show little EGFP expression.

4.3.6 The Expression of *EFGP* in *L. cuprina*

In transformed *D. melanogaster* lines, the *PUbnlsEGFP* marker gene is expressed throughout most of development and in most cells (Handler and Harrell, 1999; Davis *et al.*, 1995). In a sus-pensa transformant lines, *PUbnlsEGFP* gene expression was detectable at all stages and in adults was most obvious in the thoracic region (Handler and Harrell, 2001). Similarly, in the *L. cuprina* transformant lines EGFP expression was detected in most cells in embryos and larvae. However, in most adult tissues EGFP expression was undetectable, with the exception of the ovaries and thoracic flight muscles.

Thoracic expression was relatively weak and was only detected in the A18 line. The lack of EGFP expression in most adult tissues could be due to position effects or one or more of the transcription factors, that recognize the *D. melanogaster polyubiquitin* promoter, may be absent in most *L. cuprina* adult cells. Additional *L. cuprina* transformant lines carrying the *PUbnlsEGFP* marker will be needed to distinguish between these possibilities.

CHAPTER 5

REGULATED EXPRESSION OF *msl2* IN *DROSOPHILA MELANOGASTER*

5.1 INTRODUCTION

The goal of this project was to develop strains suitable for male-only sterile release programs. An ideal strain for SIT would be one where female lethality could be specifically induced early in development, leaving only a population of males.

The aim of the study in this chapter was to use the fruit fly *Drosophila melanogaster* as a model insect for evaluating molecular genetic strategies for inducing female-specific lethality. *Drosophila* was used because it is easily transformed and there is a wealth of knowledge on the genetics and molecular biology of this species of insect. However, the recent development of means for transforming other insects suggest that, in the long term, these results could be used to improve SIT for controlling insect pests.

The simplest means for inducing female lethality would be to control the expression of a female-lethal gene. In *Drosophila* one such gene is *male-specific lethal-2* (*msl2*) (section1.3.2). Several gene expression controlling systems are available as reviewed in section 1.5. In this study we controlled the expression of *msl-2* via a tetracycline regulated inducible expression system.

A tetracycline regulated inducible gene expression system for *Drosophila* had been developed by Prof. W. Gehring and colleagues (Bello *et al.*, 1998). The system involved two fly strains, one expressing a chimeric tetracycline dependent transactivator (*tTA*) which was generated by fusing the tetracycline repressor (*tetR*) of *E. coli* with the strong transcriptional activation domain of the herpes simplex virus protein VP16 (Gossen and Bujard, 1992). The other strain contained a target gene (such as *lacZ*, *msl2*) placed under the control of a promoter bearing seven copies of tetracycline operator sequences (*tetO*) (Bello *et al.*, 1998). Crossing of the two strains generated a tetracycline regulated inducible gene expression system. The binding of tetR to *tetO* is inhibited by

tetracycline. Thus high levels of target gene expression occur in the absence but not presence of tetracycline (Hillen and Wissmann, 1989).

In this study several *tTA* containing constructs were developed. The *tTA* gene was controlled by different promoters (*arm*, *hsp83* and *α1-tubulin*) and carried by two different vectors. A *tetO-msl2* construct was also developed and the efficiency of expression of *msl2* induced by tTA was evaluated.

5.2 RESULTS

5.2.1 Development and Analysis of Strains Carrying *tTA* Constructs Based on Plasmid pW.TΔPr

In order to generate fly strains which constitutively express tTA, plasmids pW.T–αtub, pWT-arm and pWT-hsp83 were constructed.

The *α1-tub* (*Eco*R I/*Sac* II blunted) promoter (section 1.4.3.3) from pGEM-T–α1tub (see Table 2.1) was subcloned into pW.TΔPr plasmid (appendix II) (*Eco*R I/*Kpn* II) to form pW.T–αtub. pW.TΔPr is a *P* element based vector containing multiple sites upstream of *tTA*.

The constitutive *armadillo* (*arm Eco*R I/*Kpn* I) promoter (section 1.4.3.4) from pCaSpeR-arm–Bgal (appendix II) was subcloned into pW.TΔPr (*Eco*R I/*Kpn* I) (appendix II) to form pWT-arm.

The *hsp83* (*Not* I/*Kpn* I) promoter (section 1.4.3.2) from pBS-h83 (see Table 2.1) was subcloned into pW.TΔPr (*Not* I/*Kpn* I) (appendix II) to form pWT-h83.

D. melanogaster embryos were coinjected with pWT-αtub, pWT-arm or pWT-h83 and helper plasmid pTurBO (contains *P* transposase ORF) (see Table 2.1) to generate strains containing *tTA* driven by the *α1-tub*, *arm* and *hsp83* promoter respectively.

For the pW.T–α1tub construct, 5 lines of transformants were obtained from 37 G_1 adults with a transformation rate of 13.5%. The eye color of the transformants varied from pale yellow to wild type.

For the pW.T-arm construct, 13 lines of transformants were obtained from 80 G_1 adults with a transformation rate of 16.5%. For the pW.T-h83 construct 13

lines of transformants were obtained from 42 G_1 adults with a transformation rate of 31%. 4 lines were selected for each construct and bred to homozygosity.

To evaluate the expression of tTA, strains carrying *α1tub-tTA*, *arm-tTA* and *hsp83-tTA* genes were each crossed with a *tetO-lacZ* reporter strain (seeTable 2.1) to generate a tetracycline inducible gene expression system. The progeny from the crosses, which contained both *tTA* (driven by different promoter) and *tetO-lacZ* genes, were reared on media either without or with tetracycline (10 μg/mL). Climbing 3rd instar larvae were dissected and stained for β-galactosidase activity (section 2.18). It was expected that there would be a high level of lacZ product produced (strong blue color) in the larvae raised on normal media and there would be no (or very little) lacZ product produced (no color or weak blue) in the larvae raised on media containing tetracycline. However a weak blue color was observed in the tissues of larvae in both the presence and absence of tetracycline (data not shown). A low level of β–galactosidase activity indicated that none of the larvae were producing significant levels of tTA.

To further confirm these results, quantitative spectrophotometric assays (section 2.16) were performed. The results are shown in Tables 5.1 and 5.2. For *WT-hsp83* I found that the β–galactosidase activity was similar when tetracycline was present or absent in the culture medium. Further, the level of activity was similar to a strain that had only the *tetO-lacZ* transgene (Table 5.4). This suggested there was a lack constitutive expression of *tTA* in either the pWT-arm or pWT-hsp83 construct. However with the WT-arm lines, particularly 13 and 27 there was a small but significant increase in β–galactosidase activity in flies raised on medium that lacked tetracycline. This indicates that the WT-arm lines could be expressing a low level of tTA.

Table 5.1 β–galactosidase assays (section 2.16) of progeny of crosses between strains carrying *WT-arm* and strains carrying *tetO-lacZ* either with (+) or without (-) tetracycline (tet) in the medium.

Line no of *arm-tTA*	tet (10 μg/mL)	β–gal activity [a] Δabs/min/mg wet fly weight x10^4	n	95% [b] confidence limit
13	+	0.34	3	0.02
13	-	0.72	3	0.14
19	+	0.50	3	0.040
19	-	0.86	3	0.11
27	+	0.20	3	0.094
27	-	0.50	3	0.036

[a] β–gal activity (Δabs/min/mg wet fly weight x10^4) is the mean of β–galactosidase activity from n number of independent experiments.
[b]95% confidence limits was determined by using Microsoft Excel 98.

Table 5.2 β–galactosIdase assays (section 2.16) of progeny of crosses between strain carrying *WT-hsp83* and strain carrying *tetO-lacZ* either with (+) or without (-) tetracycline (tet) in the medium.

Line no Of *WT-hsp83*	tet (10µg/mL)	β–gal activity[a] Δabs/min/mg wet fly weight x10^4	n	95%[b] confidence limit
8	+	0.24	3	0.054
8	-	0.23	3	0.042
30	+	0.59	3	0.19
30	-	0.64	3	0.26
35	+	0.19	3	0.011
35	-	0.35	3	0.062
40	+	0.32	3	0.074
40	-	0.43	3	0.036

[a] β–gal activity (Δabs/min/mg wet fly weight x10^4) is the mean of β–galactosidase activity from n number of independent experiments.
[b]95% confidence limits was determined by using Microsoft Excel 98.

5.2.2 Development and Analysis of Strains Carrying pWHT (Appendix II) Based *tTA* Genes

Since none of the constructs based on the pWTΔPr vector appear to make little if any tTA, this suggested that there may be a fault in the vector. The vector was made by Dr. Bruno Bello but was not tested by him. Dr. Bello has thoroughly tested another vector pWHT (appendix II), which has a minimal promoter (TATA box) upstream of the *tTA* coding sequences. Dr. Jorg Heinrich had also successfully used the pWHT vector (Heinrich and Scott, 2000). Therefore new *arm-tTA* and *hsp83-tTA* constructs were prepared based on the pWHT plasmid. These constructs would thus potentially have 2 transcription start sites, one from the inserted promoter (*ie. arm* or *hsp83*) and one from the minimal promoter.

The *hsp83* (*PsPOM* I/*Not* I) promoter from pBS-hsp83 (see Table 2.1) was subcloned into pWHT (*Not* I) (appendix II) to form pWH-hsp83. The constitutive *armadillo* (*arm Eco*R I/*Kpn* I) promoter from pCaSpeR-arm-Bgal (appendix II) was subcloned into pWHT (*Eco*R I/*Kpn* I) (appendix II) to form pWH-arm. *D. melanogaster* embryos were coinjected with each of pWH-arm and pWH-h83 respectively and helper plasmid pTurBO (Table 2.1) to generate new strains containing *tTA* driven by the *arm* and *hsp83* promoter respectively. For the pWH-arm construct, 2 lines of transformants were obtained from 24 G_1 adults with a transformation rate of 8.3%. For the pWH-h83 construct, 2 lines of transformants were obtained from 22 G_1 adults with a transformation rate of 9.1%. All 4 lines were bred to homozygosity.

To confirm constitutive expression tTA, pWH-arm and pWH-h83 lines were crossed with a *tetO-lacZ* reporter strain (obtained from Dr.B. Bello see Table 2.1) to generate a tetracycline inducible gene expression system. The progeny the crosses containing both *tTA* and *tetO-lacZ* genes were reared on media without or with tetracycline (10 μg/mL). Climbing third instar larvae were collected and stained for β-galactosidase activity (section 2.18).

For crosses between *WH-arm* and *tetO-lacZ* a strong blue color was observed in most of the tissues in the larval offspring when tetracycline was absent, suggesting a high level of *lacZ* product produced. In contrast, there was no color or weak blue in the tissue of the larvae when tetracycline was present, suggesting the production of no or very little lacZ product. These results are shown in Figure 5.1.

For crosses between *WH-hsp83* and *tetO-lacZ* a blue color was observed in most of the tissues in the larvae when tetracycline was absent (Figure 5.2). However, the level of staining observed was less strong than the one in crosses between *WH-arm* and *tetO-lacZ*. There was no or weak blue color in the tissue of the larvae when tetracycline was present, suggesting the production of no or very little lacZ product.

To further confirm that the *WH-arm* and *WH-h83* strains constitutively expressed tTA, the new *tTA* lines were crossed with the *tetO-lacZ* strain and cultured in medium with or without tetracycline. Quantifiable spectrophotometric β–galactosidase assays (section 2.16) were performed using the adult progeny. The results of the assays are shown in Tables 5.3 and 5.4. The results showed that the β–galactosidase activity was significantly elevated when tetracycline was absent from the culture medium, indicating that there was constitutive tTA expression in both the *WH-arm* and *WH-h83* strains. The *WH-arm* lines appear to produce higher tTA levels than the *WH-h83* lines.

To address the induction of lethality by the *WH-arm* and *WH-h83* lines, a strain carrying *tetO-hid* (a gift from Dr. Heinrich) was crossed with these lines. Only the cross between line 19 of *WH-arm* and *tetO-hid* caused lethality (Table 5.5) indicating that there was constitutive tTA expressed in this *WH-arm* line.

Figure 5.1 Histochemical β–galactosidase assays of third instar larvae carrying both *WH-arm* and *tetO-lacZ* constructs

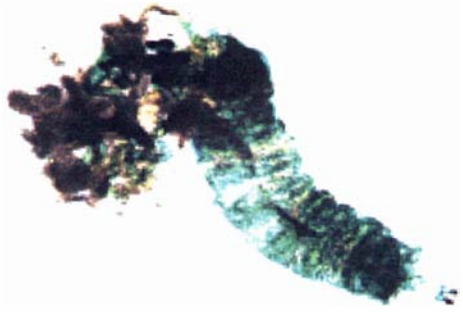
Fly strains (line 19 and 28) containing the *WH-arm* construct were crossed with a strain containing *tetO-LacZ* genes. Third instar larvae of the crosses were dissected and stained using a histochemical method for β–galactosidase activity (section 2.18).

Larvae from *arm-tTA* line 19 (A) or line 28 (C) raised on normal instant food showing β–galatosidase expression in skin and gut.

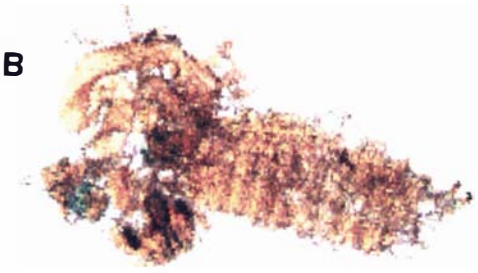
Larvae from *arm-tTA* line 19 (B) or line 28 (D) raised on instant food contained 10 μg/mL tetracycline, showing no β–galatosidase expression in any tissue except in some gut. This activity is probably due to endogenous Drosophila β–galactosidase.

E and F. β–galactosidase staining of third instar larvae carrying a *lacZ* gene driven by arm promoter. β–galactosidase is expressed constitutively in this strain.

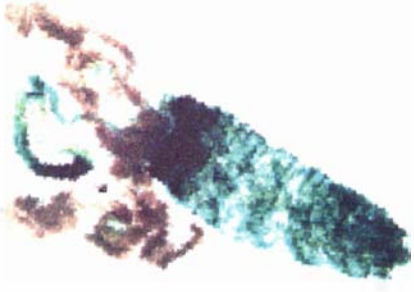
A



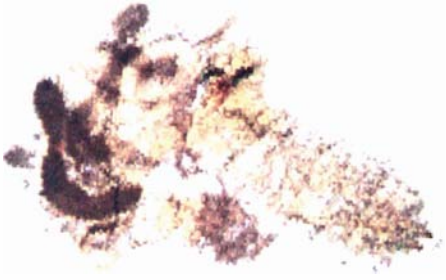
B



C



D



E

F

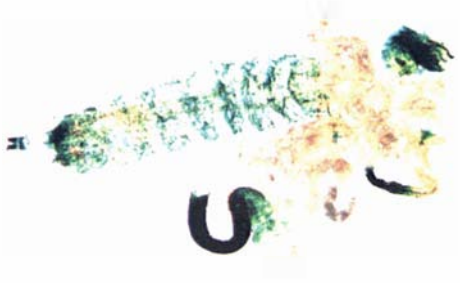
Figure 5.2 Histochemical β–galactosIdase assays of third instar larvae carrying both *WH-hsp83* and *tetO-lacZ* constructs

Fly strains (line 18 and 3) containing the *WH-hsp83* construct were crossed to a strain containing *tetO-LacZ* genes. Third instar larvae of the crosses were dissected and stained using a histochemical method for β–galactosidase activity (section 2.18).

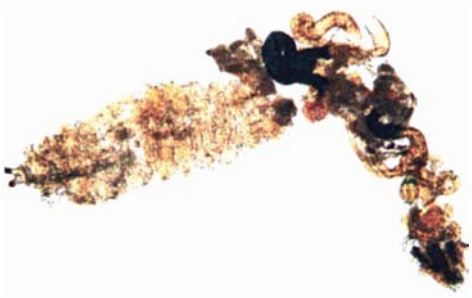
Larvae from *hsp83-tTA* line 18 (A) or line 3 (C) raised on normal instant food showing β–galatosidase expression in skin and gut.

Larvae from *hsp83-tTA* line 18(B) or line 3 (D) raised on instant food contained 10 μg/mL tetracycline, showing no β–galatosidase expressed in any tissues except in some gut. This activity is probably due to endogenous Drosophila β–galactosidase.

A



B



C

D

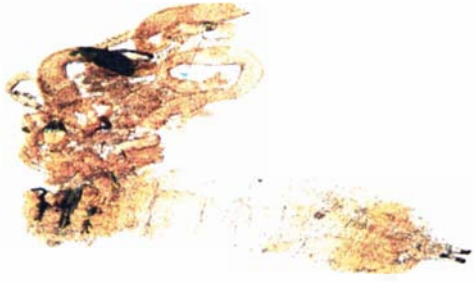


Table 5.3 β –galactosidase assays (section 2.16) of progeny of cross between strains carrying new *WH-arm* and strain carrying *tetO-lacZ* either with (+) or without (-) tetracycline (tet) in the culture medium.

Line no of *arm-tTA*	tet (10μg/mL)	β–gal activity [a] Δabs/min/mg wet fly weight x10^4	n	95% [b] confidence limit
19	+	0.54	3	0.046
19	-	2.1	3	0.27
28	+	0.30	3	0.04
28	-	3.1	3	0.39
arm-lacZ		14	3	0.43

[a] β–gal activity (Δabs/min/mg wet fly weight x10^4) is the mean of β–galactosidase activity from n number of independent experiments.
[b]95% confidence limits was determined by using Microsoft Excel 98.

Table 5.4 β–galactosidase assays (section 2.16) of progeny of crosses between strains carrying *WH-hsp83* and strain carrying *tetO-lacZ* either with (+) or without (-) tetracycline (tet) in the culture medium.

Line no of *hsp83-tTA*	tet (10μg/mL)	β–gal activity[a] Δabs/min/mg wet fly weight x10^4	n	95%[b] confidence limit
3	+	0.35	3	0.028
3	-	1.6	3	0.34
18	+	0.44	3	0.16
18	-	1.7	3	0.21
[c]*pgd-lacZ* (control)		3.5	3	0.32
tetO-LacZ (control)		0.26	3	0.02

[a] β–gal activity (Δabs/min/mg wet fly weight x10^4) is the mean of β–galactosidase activity from n number of independent experiments.
[b]95% confidence limits was determined by using Microsoft Excel 98.
[c] *pgd-lacZ* line strongly expresses β–galactosidase in several tissues (Scott and Lucchesi, 1991)

Table 5.5 Number of male and female offspring of crosses between lines *WH-arm* or *WH-hsp83* and lines carrying *tetO-hid* genes raised on instant food (section 2.23.1.2) either with (+) or without (-) tetracycline (tet) in the culture medium.

Line no.	tet (10μg/mL)	No. of male	No. of female
WH-arm			
19	+	36	33
19	-	0	0
28	+	35	37
28	-	29	35
WH-hsp83			
3	+	35	40
3	-	35	39
18	+	31	34
18	-	43	37

5.2.3 Development and Evaluation of a Tetracycline Regulated *msl2* Expression System

Plasmid ptetO-msl2 was constructed to develop a strain carrying a tetracycline regulated *msl2* gene. It was obtained by subcloning a fragment containing the *msl2* (*Eco*R I/*Not* I) coding sequence from pXL1 (appendix II) into pWTP2 (*Eco*R I/*Not* I) (appendix II).

D. melanogaster embryos were coinjected with ptetO-msl2 and helper plasmid pTurBO (Table 2.1) to generate strains containing *tetO-msl2*. Four lines of transformants were obtained from 32 G_1 adults with a transformation rate of 12.5%. All lines were bred to homozygosity.

To evaluate the tetracycline regulated female killing system, the fly strains carrying the *tetO-msl2* construct were crossed to either the pWH-arm (line 19 or line 28) or pWH-h83 (line 3 or 18) lines respectively and raised on medium either with or without tetracycline. The expected result was that there would be no female offspring survive when tetracycline was absent in the medium and both male and female offspring survive when tetracycline was present. The results shown in Tables 5.6 and 5.7 indicate that both male and female offspring would survive in the fly medium no matter if tetracycline were present or absent.

For some of the crosses the ratio of male to female (M/F) was significantly greater than one (*eg.* WH-arm line 19 or 28 crossed *tetO-msl2* line 5 or 6) when they were raised on normal instant food lacking tetracycline. Note that for the *yw* injection stock, the ratio between male and female is around 1. Thus the lower survival of female raised on medium lacking tetracycline might be due to the expression of *msl2*. Presumably not all of the females died because the level of *msl2* expression was lower than required for 100% female lethality. The crosses of lines 5 or 6 carrying *tetO-msl2* gene with lines 19 or 28 carrying *WH-arm* genes gave the highest level of female lethality. However, there was no

significant female lethality among the offspring of the crosses between *WH-h83* and *tetO-msl2* lines (Table 5.7). These results are consistent with the earlier experiments with the *tetO-lacZ* strain which indicated that the *WH-arm* lines produce more tTA than the *WH-h83* lines.

The most direct method for confirming that females carrying both *WH-arm* and *tetO-msl2* constructs express MSL2 would be to perform Western blots with anti-MSL2 antibodies. However, our laboratory did not have a suitable antibody was available at the time these experiments were carried out. An anti-MSL3 antibody was available that had been successfully used for detecting MSL3 bound to the male chromosome by indirect immunoflurosence (Henry *et al.*, 2001). In female, MSL3 does not bind to the X chromosomes unless MSL2 protein is present (Kelly *et al,* 1995). *WH-arm* line 19 was crossed with *tetO-msl2* lines 5 or 6 and the climbing third instar larvae were collected and sexed. MSL3 binding to the female and male X chromosomes was detected by immuno-staining of polytene chromosome spreads. The results are shown in Figure 5.3. MSL3 was detected bound to female X chromosomes in larvae raised on normal medium (Figure 5.3 A-D) but not on medium contained tetracycline (Figure 5.3 E-F). In contrast, the MSL3 antibody bound to the male X chromosomes in the larvae raised on medium with or without tetracycline (Figure 5.3 G-L).

These results strongly indicate that there was significant MSL2 expression in female larvae carrying both *WH-arm* and *tetO-msl2* constructs if raised on medium lacking tetracycline. However since not all females died, this raised the possibility that the *WH-arm* lines may not be producing high levels of tTA. To address this question *Ypl-tTA* line 19 was crossed with *tetO-msl2* lines. *Ypl-tTA* line strongly expresses tTA in female fat cells (Heinrich and Scott, 2000). The results are shown in Table 5.8. For all crosses there was no significant decrease in female viability. This is in contrast to results obtained with a *tetO-hid* line (*hid* is a proapoptotic gene) were 100% of female progeny die if crosses were raised on medium lacking tetracycline (Heinrich and Scott, 2000). These

Table 5.6a Ratio of male (M) to female (F) offspring of crosses between *WH-arm* and a strain carrying *tetO-msl2* raised in the instant food (section 2.23.1.2) lacking tetracycline.

WH-arm Line	*tetO-msl2* Line	M	F	M/F Ratio	χ^2	P
19	5	136	74	1.82	18.31	<0.005
19	6	179	104	1.73	19.88	<0.005
19	13	175	136	1.26	4.89	>0.05
19	32	120	64	1.35	1.82	>0.05
28	5	148	88	1.81	15.25	<0.005
28	6	151	70	2.21	29.69	<0.005
28	13	147	137	1.09	0.35	>0.05
28	32	121	79	1.54	8.82	<0.005

Table 5.6b Ratio of male (M) to female (F) offspring of crosses between *WH-arm* and a strain carrying *tetO-msl2* raised on instant food (section 2.23.1.2) with tetracycline.

WH-arm Line	*tetO-msl2* Line	M	F	M/F Ratio	χ^2	P
19	5	34	32	1.06	0.06	>0.05
19	6	23	19	1.21	0.38	>0.05
19	13	35	35	1.00	0.00	>0.05
19	32	42	39	1.08	0.11	>0.05
28	5	35	32	1.09	0.13	>0.05
28	6	48	43	1.11	0.29	>0.05
28	13	50	46	1.19	0.17	>0.05
28	32	26	31	0.84	0.45	>0.05

Table 5.7a Ratio of male (M) and female (F) offspring of crosses between *WH-hsp83* and a strain carrying *tetO-msl2* raised on instant food (section 2.23.1.2) lacking tetracycline.

WH-hsp83 Line	*tetO-msl2* Line	M	F	M/F Ratio	χ^2	P
3	5	66	62	1.06	0.13	>0.05
3	6	113	91	1.24	2.37	>0.05
3	13	122	80	1.34	8.73	<0.005
3	32	86	67	1.28	2.36	>0.05
18	5	64	71	0.92	1.5	>0.05
18	6	75	60	1.25	1.67	>0.05
18	13	94	91	1.04	0.05	>0.05
18	32	78	61	1.25	2.08	>0.05

Table 5.7b Ratio of male (M) and female (F) offspring of crosses between *WH-hsp83* and a strain carrying *tetO-msl2* raised on instant food (section 2.23.1.2) with tetracycline.

WH-hsp83 Line	*tetO-msl2* Line	M	F	M/F Ratio	χ^2	P
3	5	36	34	1.06	0.057	>0.05
3	6	41	42	0.98	0.012	>0.05
3	13	48	51	0.94	0.091	>0.05
3	32	50	47	0.93	1.06	>0.05
18	5	38	40	0.92	0.051	>0.05
18	6	31	30	1.03	0.016	>0.05
18	13	44	42	1.05	0.047	>0.05
18	32	49	53	0.94	0.157	>0.05

Figure 5.3 Immunostaining (using an MSL3 antibody) of polytene chromosomes of female larvae carrying *WH-arm* and *tetO-msl2* constructs.

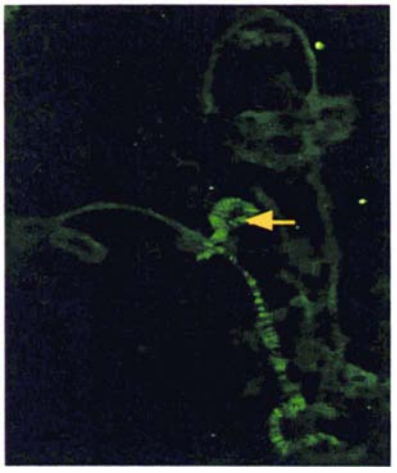
Transgenic line (#19) containing *WH-arm* genes was crossed with transgenic line (#5) containing *tetO-msl2* genes. Polytene chromosomes of the third instar larvae of the cross were sexed and stained with goat anti-MSL3 antibody and FITC-congregated anti-goat antibody (A, C, E=female; G, I, K=male).

The nuclei were also stained with DAPI to visualize all of the chromosomes (B, D, F=female; H, I, L=male).

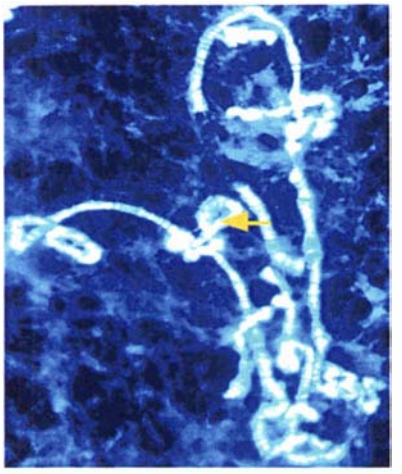
Larvae were either raised in the absence (A-D=female; G-J=male) or presence (E-F=female; K-L=male) of tetracycline. MSL3 was detected only on the female X chromosomes in larvae raised on medium lacking tetracycline (A, C).

Arrows point to the X chromosome(s).

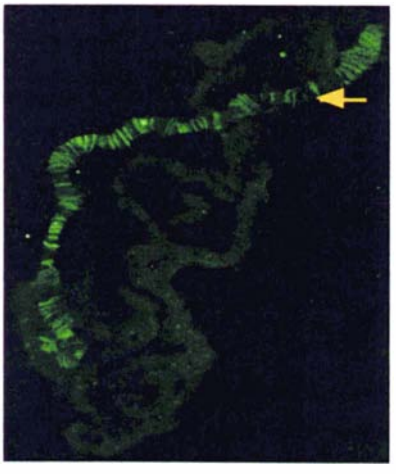
A



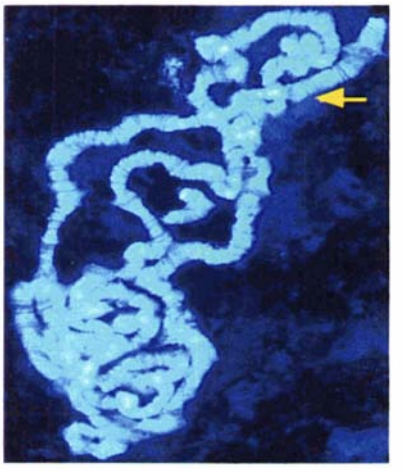
B



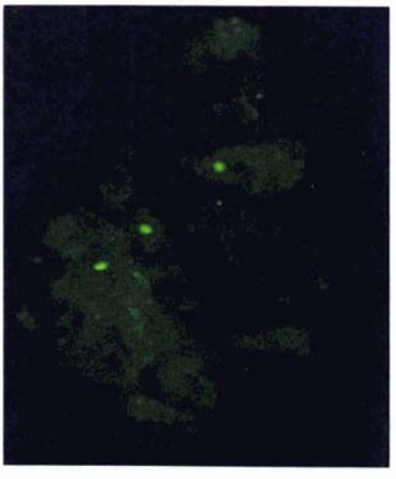
C



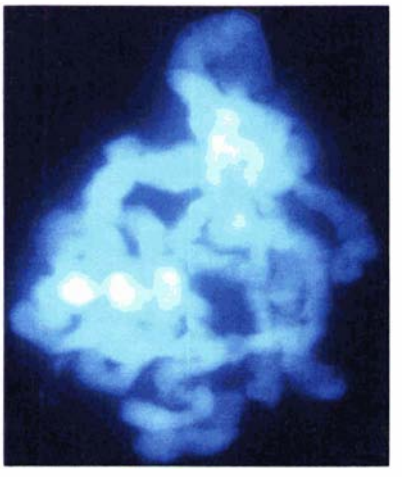
D



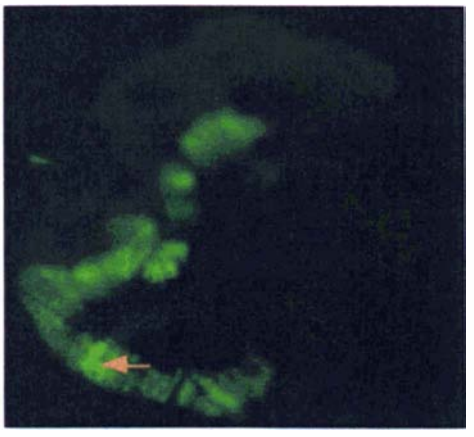
E



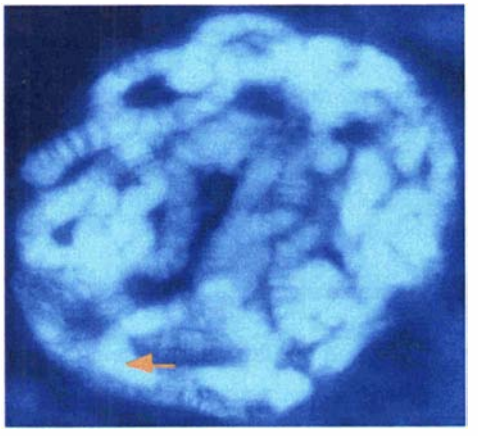
F



G

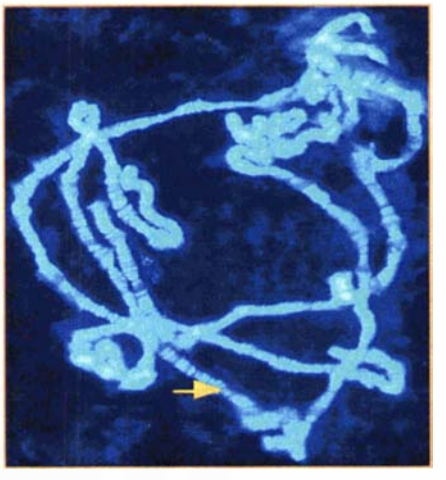


H



I

J



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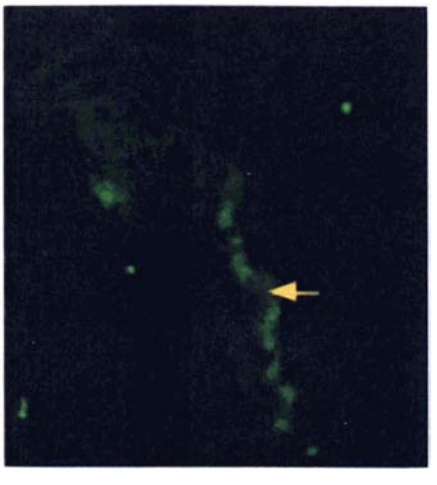

L

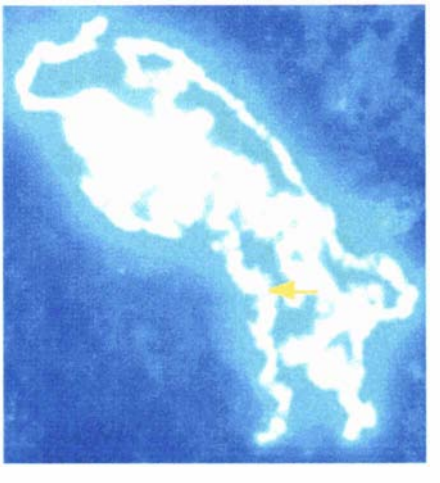

Table 5.8 Ratio of male (M) to female (F) offspring of crosses between a strain carrying *ypl-tTA* and strains carrying *tetO-msl2* raised on instant food (section 2.23.1.2) lacking tetracycline.

tet-msl2 Line	M	F	M/F Ratio	χ^2	P
5	78	82	0.94	0.20	>0.05
6	134	122	1.39	0.56	>0.05
13	109	92	1.3	1.42	>0.05
32	76	76	1.0	1.00	>0.05

results suggest that the small decrease in female viability among the offspring of *WH-arm* and *tetO-msl2* line is unlikely to be due to low tTA expression.

5.3 DISCUSSION AND FUTURE WORK

In this chapter a regulated female killing system was investigated which could be developed by controlling *msl2* expression with the tetracycline transactivator (tTA).

Initially considerable difficulty was expressed in making lines that constitutively expressed tTA. All initial constructs were based on the pWT-ΔPr vector supplied by Dr. Bruno Bello. tTA was controlled by *α1-tub, hsp83* or *arm* promoters. tTA expression in lines carrying these constructs was evaluated by crossing with a *tetO-lacZ* reporter strain. Both histochemical and spectrophotometric assays indicated there was very low if any β-galactosidase expression in the offspring of these crosses.

Since the *tetO-lacZ* strain was known to be functional (Bello *et al.,* 1998; Heinrich and Scott, 2000), then the lack of β–gal expression was most probably caused by the tTA constructs. Either the transactivitor tTA failed to bind to the *tetO* or tTA failed to be expressed or was expressed at a very low level. Each of the promoters (*α1-tub, hsp83* or *arm*) placed upstream of tTA had been used previously to control the *piggyBac* transposase gene. Sequencing of *α1–tub-tTA* construct revealed that the tTA translation start site was correct. Thus the most likely explanation was that there was some error for pW.TΔPr (appendix II), which was never confirmed by Dr. Bello to be functional.

The pWH-arm and pWH-h83 plasmids were based on the pWHT, which is a *P* based vector that contains a *hsp70* minimal promoter upstream of the *tTA* coding region. pWHT had been successfully used by Dr. Bello and as described by Heinrich and Scott (2000). The β–galactosidase staining assays showed that the *WH-arm* and *WH-hsp83* constructs could induce *lacZ* expression. There was blue color in most of the tissues in the larvae when tetracycline was absent and there was no color or weak blue in the tissue of the larvae when tetracycline was present. Both *WH-arm* lines appear to induce stronger blue

larva tissue than did the *WH-hsp83* lines indicating that the *armadillo* promoter was stronger than the *hsp83* promoter for tTA expression. The spectrophotometric β-galactosidase activity assays confirmed that the *WH-arm* lines induced higher β–galactosidase activity than that of the *WH-h83* lines. Further β–galactosidase expression was inhibited by tetracycline in the diet. It was concluded that the *WH-arm* and *WH-h83* lines constitutively express tTA and that *WH-arm* lines express higher levels of tTA than the *WH-h83* lines.

Since the *WH-arm* and *WH-h83* lines express tTA, they were crossed with lines carrying a *tetO-msl2* construct to evaluate their use in the control of female viability. Constitutive expression of MSL2 would be likely to cause female lethality due to hyperactivation of the female X chromosomes by the MSL complex (Kelley *et al.*, 1995). Indeed, in offspring of the *WH-arm/tetO-msl2* crosses there was a significant decrease in female viability. Immuno-staining of polytene chromosomes with anti-MSL3 antibody confirmed that the MSL complex was assembling in female nuclei. MSL complex assembly in females is absolutely dependent upon the presence of MSL2 (Zhou *et al.*, 1995). However this is a very sensitive assay and has been used to detect very low levels of MSL2 expression (Kelley *et al.*, 1997). To measure MSL2 levels more directly would require an anti-MSL2 antibody, which was not available for this study. However, about 50% of the females survived. The most likely explanation for this result is that the level of *msl2* expression was not high enough to cause complete doubling of transcription of X-linked genes in females. Chang and Kuroda (1998) reported ectopic expression of MSL2 protein in females is not sufficient to induce an insurmountable level of dosage compensation. Overexpression both MSL1 and MSL2 in females resulted in 100% female-specific lethality, suggesting that MSL1 is a limiting factor in females the overexpress MSL2. Thus the incomplete female lethality observed in this study could be due to low level of MSL1 in females.

Interestingly, there was no decrease in female viability among the offspring of crosses between *ypl-tTA* and *tetO-msl2* lines. The *ypl-tTA* lines are known to

strongly express tTA in the female fat body (Heinrich and Scott, 2000). This indicates that induction of MSL2 expression and consequent MSL complex assembly specifically in fat cells is not sufficient to cause female lethality. However, Thomas and Alphey (2000) reported 100% female lethality among the offspring of a cross between *yp3-tTA* and *tetO-msl2* lines. Since the *ypl-tTA* and *yp3-tTA* lines appear to make about the same levels of tTA (Thomas, personal communication) this may indicate that the *tetO-msl2* lines made by Thomas and Alphey express higher levels of MSL2. The *tetO-msl2* construct made by Thomas and Alphey (2000) was constructed by cloning the *msl2* open reading frame from the pNOPU plasmid (Kelley *et al*., 1997). The pNOPU plasmid contains point mutation in the 5′UTR of *msl2* and the 3′ UTR has been partially replaced with the SV40 polyA terminator sequence. The *tetO-msl2* construct used in this study was made by isolating the *msl2* coding region from pNETmsl2. pNETmsl2 lacks all of the *msl2* 5′ UTR and most of the 3′ UTR. Thus the *tetO*-msl2 construct used in this study and that of Thomas and Alphey (2000) have very different 5′ and 3′ UTR. This could possibly account for apparent difference in MSL2 expression.

It would have been interesting to compare the *tetO-msl2* lines made in this study with those developed by Thomas and Alphey (2000), but unfortunately their lines have been lost (Alphey, personal communication).

CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

GENERAL DISCUSSION AND FUTURE DIRECTIONS

The overall goal of this project was to use genetic engineering methods to develop strains of the Australian sheep blowfly *L. cuprina* which are ideal for male-only sterile release programs. To approach to this goal, the development of a germline transformation system and a regulatable female-killing system were investigated. This included evaluation of *piggyBac* and *Minos* mediated transpositions either in *D. melanogaster* or in *L. cuprina* (chapter 3 and 4), development of a *piggyBac* mediated germline transformation system in Australian sheep blowfly *L. cuprina* (chapter 4) and regulated expression of *msl2* by using tetracycline controlling the tetracycline dependent tTA in *D. melanogaster* (chapter 5).

Microinjection techniques were used throughout this study to make transgenic *D.melanogaster* and *L. cuprina*. As microinjection directly affects the germline transformation efficiency several key factors lead to the successful use of this technique: needle, embryo dehydration, covering oil and incubating environment.

Three different kinds of needles were used for microinjection of flies: self manually pulled needles, Femtotip I and Femtotip II (Eppendorf) needles. The self-pulled needles had a larger opening than the other needles and thus could release more DNA solution out of the needle. The survival of larvae was very low with this kind of needle for injection of both Drosophila and Lucilia embryos because a big hole in the embryo was created and this caused significant leakage of material. Femtotip I is the finest and sharpest of the three kinds of needles, but weaker than Femtotip II. There was good survival by using this kind of needle to inject Drosophila embryos, but the needle was easily bent with Lucilia embryos. Femtotip II tip is longer and stronger than Femtotip I and was the most suitable of the three kinds of needles used to inject Lucilia embryos. A disadvantage of this kind of needle is that it is easily blocked by the material from the embryos. I would recommend that for further injection of Lucilia

embryos it would be advantageous to use self-pulled needles that are both sharp enough to penetrate the chorion without causing significant leakage and strong enough so they do not easily bend and break.

Pre-blastoderm embryos were collected for microinjection in germline transformation experiments. The embryos were dechorionated for Drosophila and but were not dechorionated for most of Lucilia embryos. It was easy to manually dechorionate the Drosophila embryos and there was good survival of larvae. However, the Lucilia embryos that were dechorionated with bleach were very tough and were difficult to inject. The manually dechorionated Lucilia embryos on the other hand were easier to inject but survival was lower than non-dechorionated embryos.

The quality of the injected DNA, affected the survival and the transformation efficiency. Low quality DNA contains toxic substances leading to the death of embryos during development. High concentrations of injected DNA normally gave a high efficiency of transformation or transposition, but very high DNA concentrations resulted in poisoning of embryos as well as the formation of a pellet which resulted in broken needles.

The length of the time to dehydrate the embryos before injection was also important. It was necessary to find a suitable length of time of dehydration at the beginning of an injection day. Too short a time of dehydration leads to a lot of material leaking out of embryos and too a long time of dehydration leads to embryo death before injection. In general, in fresh silica gel, dehydration times of 1-2 minutes for dechorionated Drosophila and 8-10 minutes for non-dechorionated Lucilia embryos were suitable for injection.

Halocarbon 700 oil was used to cover the Drosophila embryos after injection However with Lucilia embryos, larva survival was very low (about 25%) with this oil. To increase the survival, paraffin oil was used instead of Halocarbon oil and resulted in a high survival rate (over 80%) in both *Drosophila* and *Lucilia*

embryo injection. Halocarbon is more viscous than the Paraffin oil and this may have prevented the larvae from absorbing oxygen from the air and caused low survival.

For *Lucilia* the number of viable larvae significantly increased if the developing embryos were incubated in a sealed oxygen container before larvae hatched. For Drosophila this also appeared to help the survival of larvae. Larvae normally hatched 42 hrs after injection for *Drosophila* or 27 hrs for *Lucilia*.

Two transgenic lines of *L. cuprina* were obtained with the *PUbnlsEGFP* marker gene and the *piggyBac* transposase helper controlled by the *D. melanogaster hsp70* promoter. Although no transformants were obtained by using *piggyBac* helper controlled by the *Drosophila hsp83* or *α1-tubulin* promoter, this does not necessarily indicate that these helpers are significantly less effective than the *hsp70.piggyBac* helper as the number of fertile G_0s obtained was much less than with the *hsp70* helper. In order to find a high efficiency transposase for germline transformation of *L. cuprina*, it would be worthwhile in the future to repeat the experiments using the *piggyBac* transposable element with *hsp83.piggyBac* or *α1-tubulin.piggyBac* as a helper or *Minos* RNA helper. It may also be worthwhile to isolate the promoter for strongly constitutively expressed *L. cuprina* genes to control piggyBac transposase expression. Further, it may be worthwhile to try other transposable elements that have a wide host range, such as *mariner* and *Hermes* (section 1.4.2.3).

A female killing system for Diptera was developed based on the *D. melanogaster msl2* gene, controlled by the tetracycline dependent transactivator (tTA). However, only the *WH-arm/tetO-msl2* strains showed a significant decrease in female viability but lethality was not 100%. The problem might be due to the deletion of 5′ and/or 3′ UTR of *msl2* in the *tetO-msl2* construct in this study (discussed in section 5.3). To increase the efficiency of a female-killing system in the future, it would be interesting to construct a new *tetO-msl2* in which the *msl2* gene has a 5′ and/or 3′ UTR sequence without the

SXL binding sites. Alternatively, it may be advantageous to flank *tetO-msl2* with insulator elements to block any negative chromosomal effects (Bell and Felsenfeld, 1999). Because overexpression both MSL1 and MSL2 in females resulted in 100% female-specific lethality (Chang and Kuroda, 1998), it may be worthwhile to construct a strain carrying both *msl1* and *msl2* genes controlled by suitable promoters (such as *tetO* or *hsp70* or *hsp83*). It may also be worthwhile to develop female-killing systems using different regulators. For example, a doxycycline-induced transgene expression system ("tetracycline-on" system). In this system the regulator rtTA is a mutant tetR: VP16 fusion protein which has the reverse property of only binding to the *tetO* and activating transcription in the presence of the tetracycline derivatives, doxycycline or hydrotetracycline. Alternatively, a progesterone antagonist (RU486) regulated inducible gene expression system could be used to control *msl2* expression (Wang *et al.,* 1994, 1997). The regulator (GLVP) contains the fusion of a ligand-binding domain of the mutant of human progesterone receptor (hPRB891) to the yeast transcriptional activator Gal4 DNA-binding domain and the Herpes Simplex Virus protein VP16 activation domain (Wang *et al.,* 1994). The target gene is controlled by four copies of the GAL4 consensus binding sequence linked to a *thymidine kinase* (*tk*) promoter (*17*4-tk-reporter gene*). The transcription of the target gene is induced by addition of RU486.

CHAPTER 7

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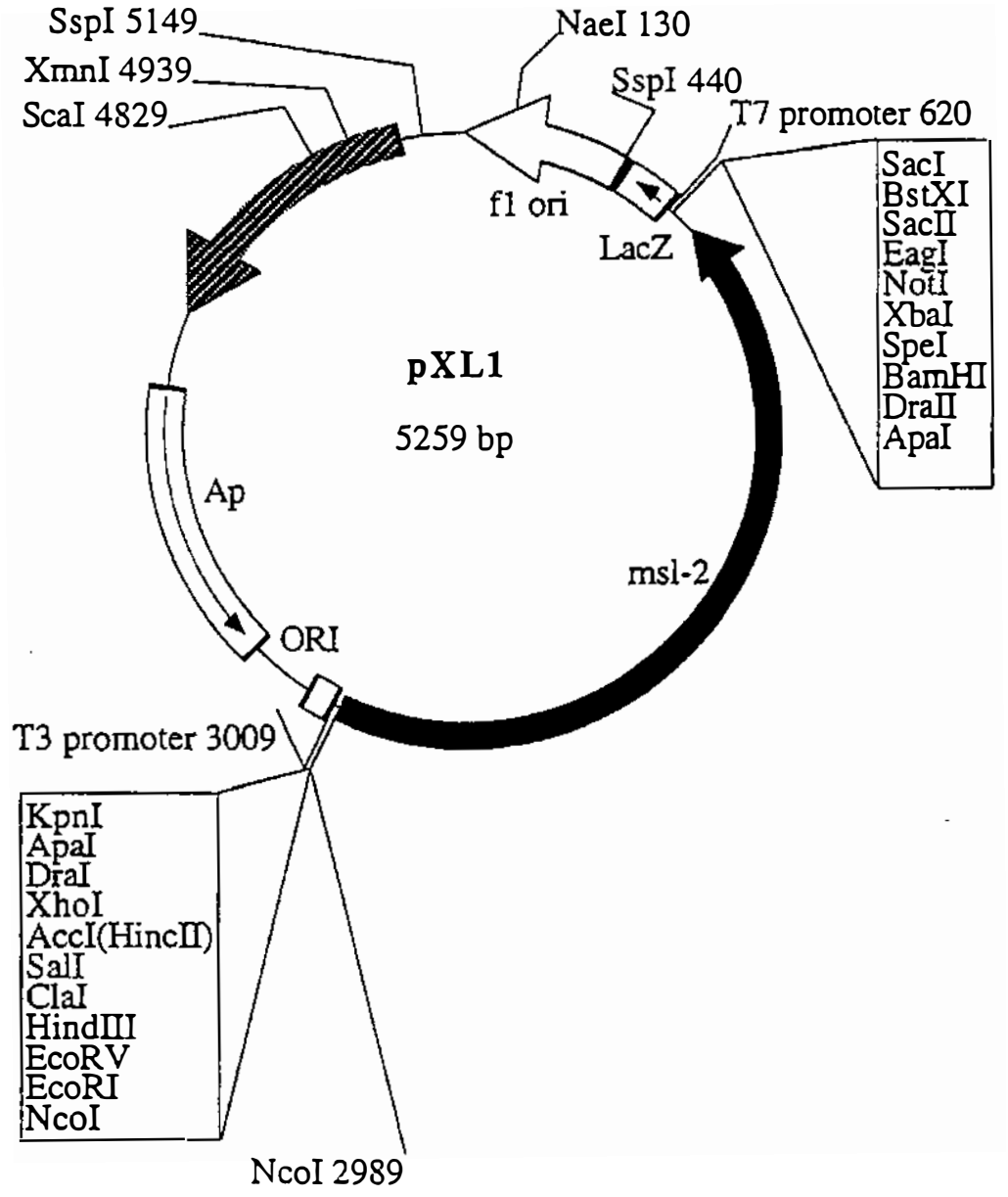
APPENDIX 1

PUBLICATION

APPENDIX 2

VECTOR MAPS



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Plasmid size: 5.239 kb
Comments/References: From Strategies. Only an approximate map.
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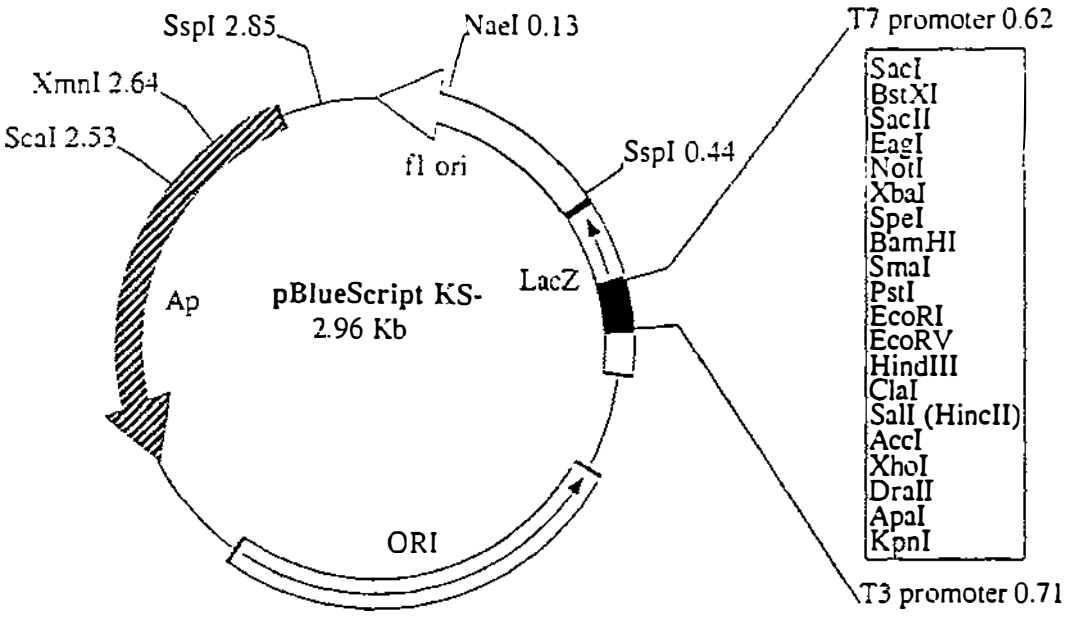
Bot: (CATGGTTGTTTTAGATCTG).



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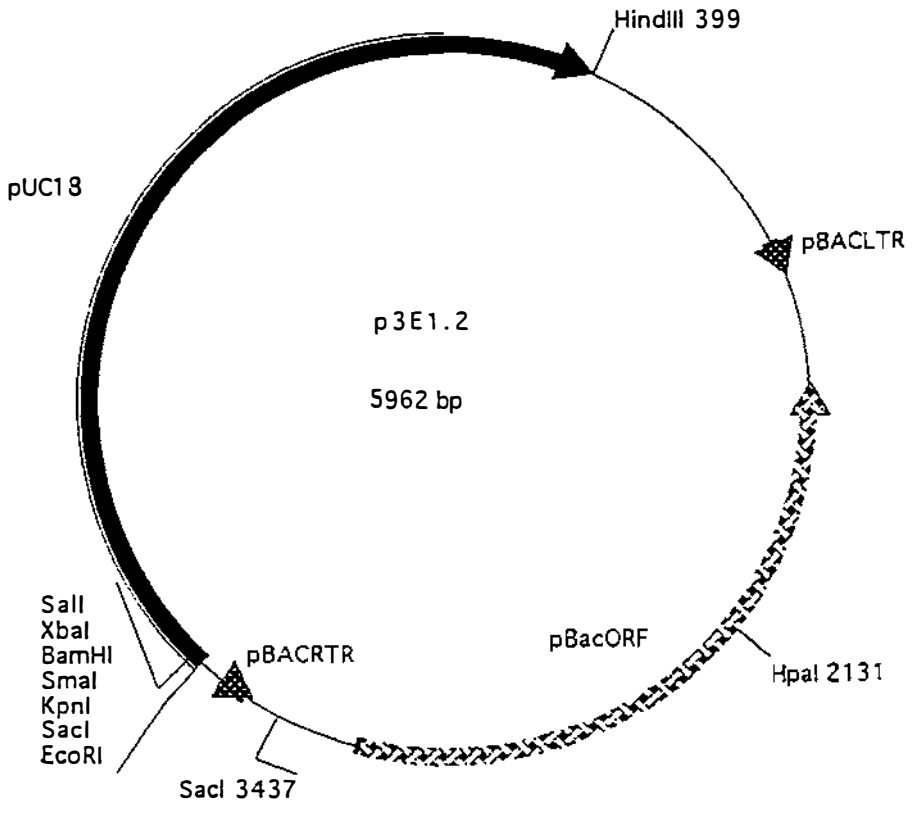

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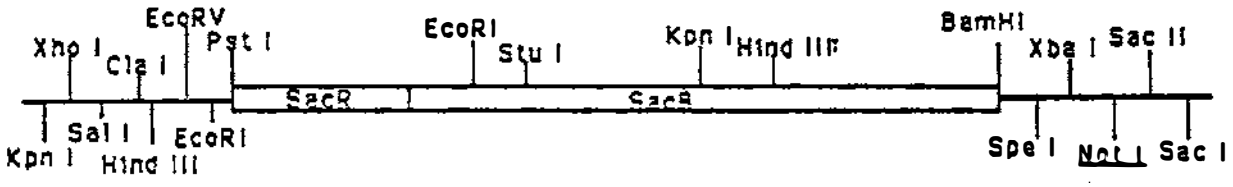

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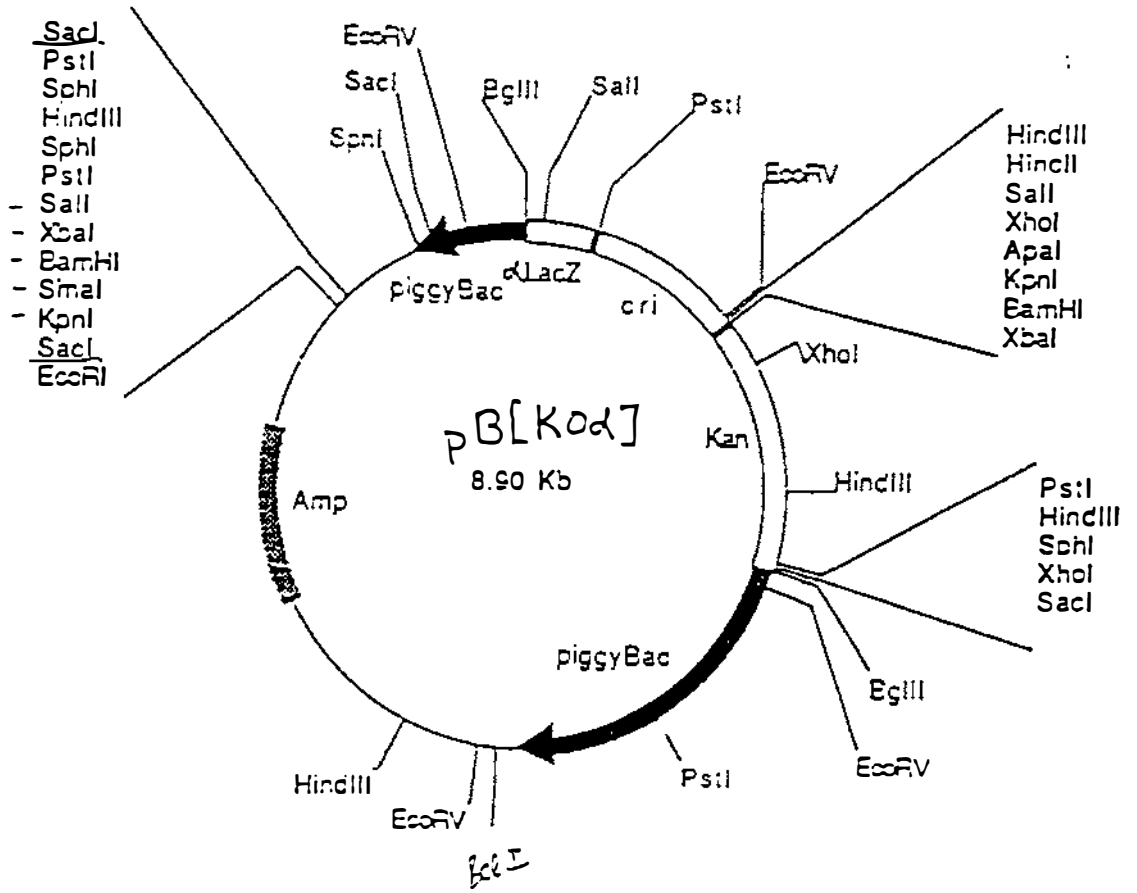

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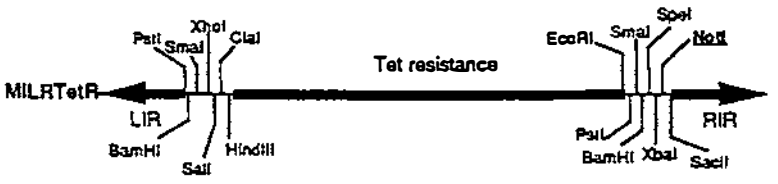
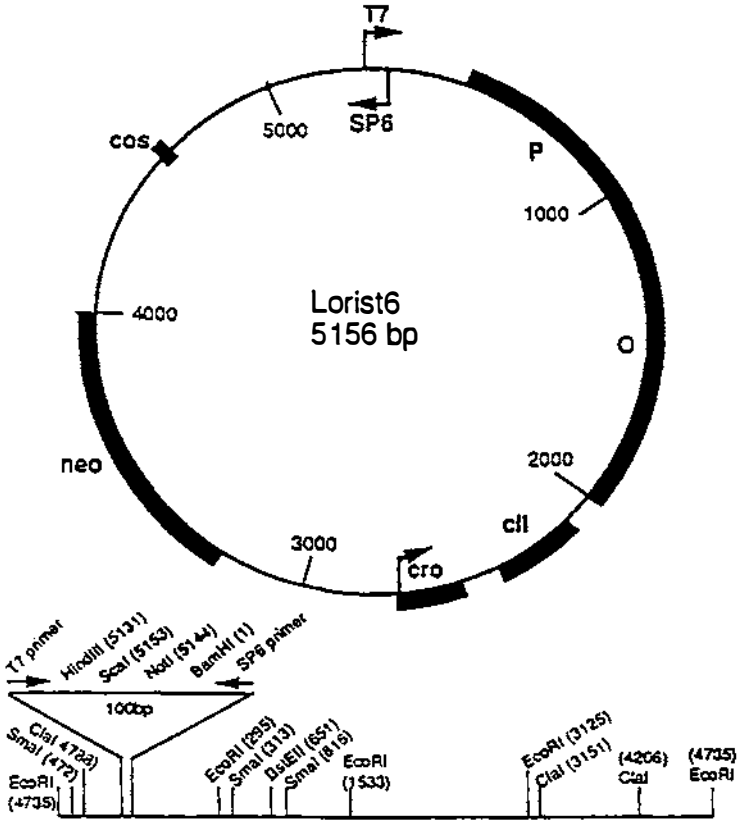
Plasmid size: 8.9 kb



MiLRTetR casette inserted as a blunt fragment into the ScaI site of Lorist6



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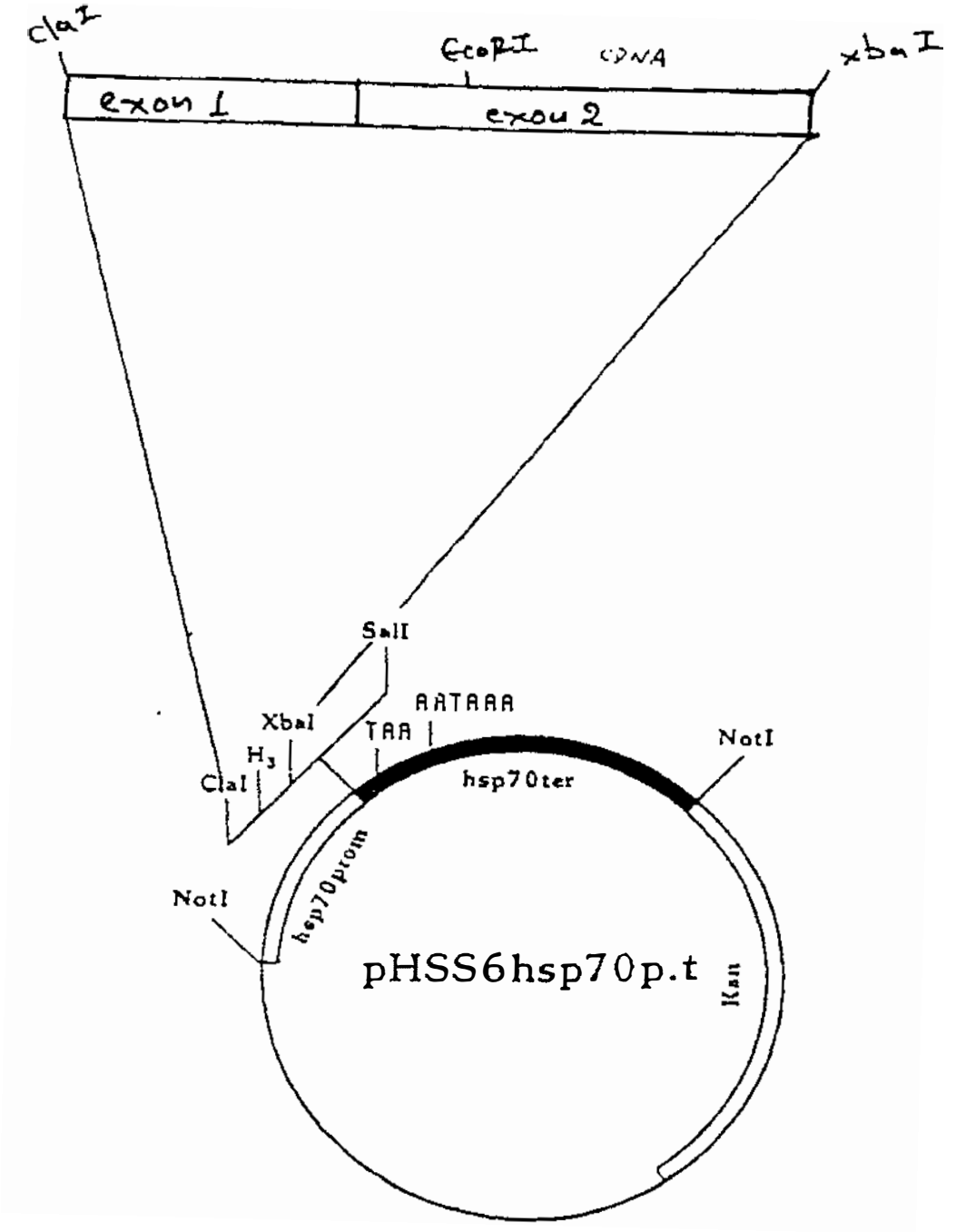

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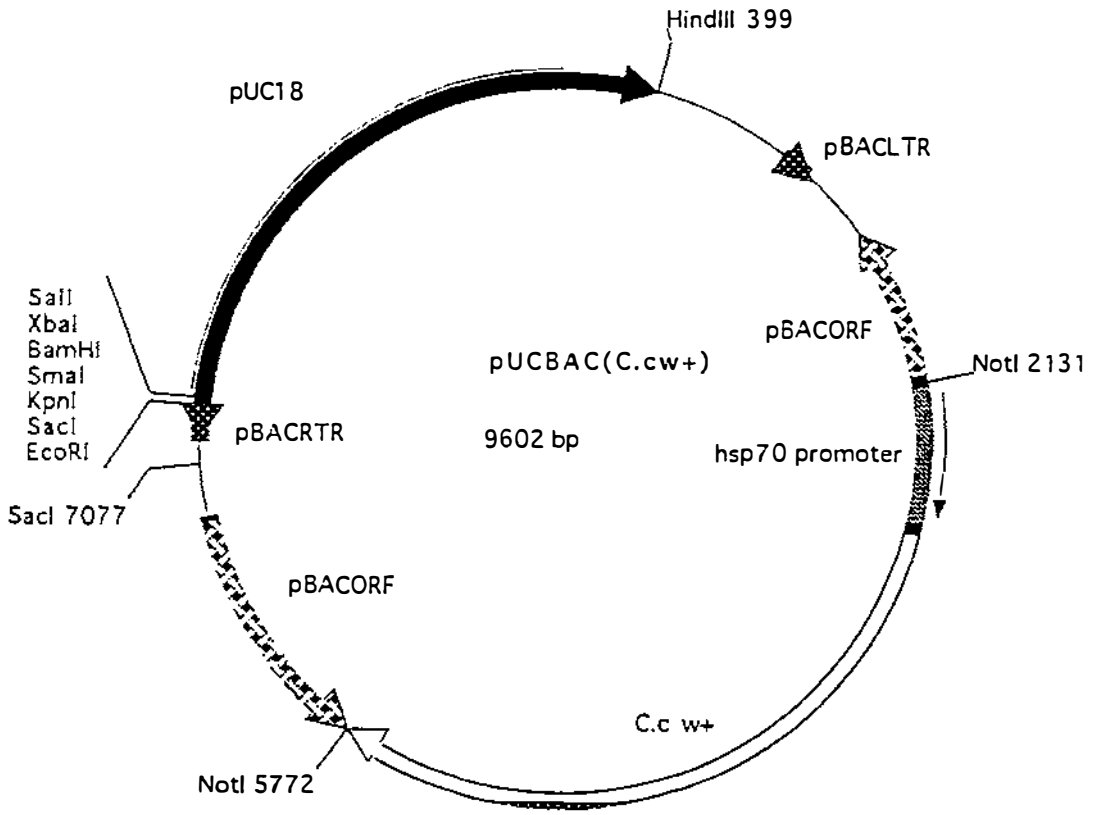

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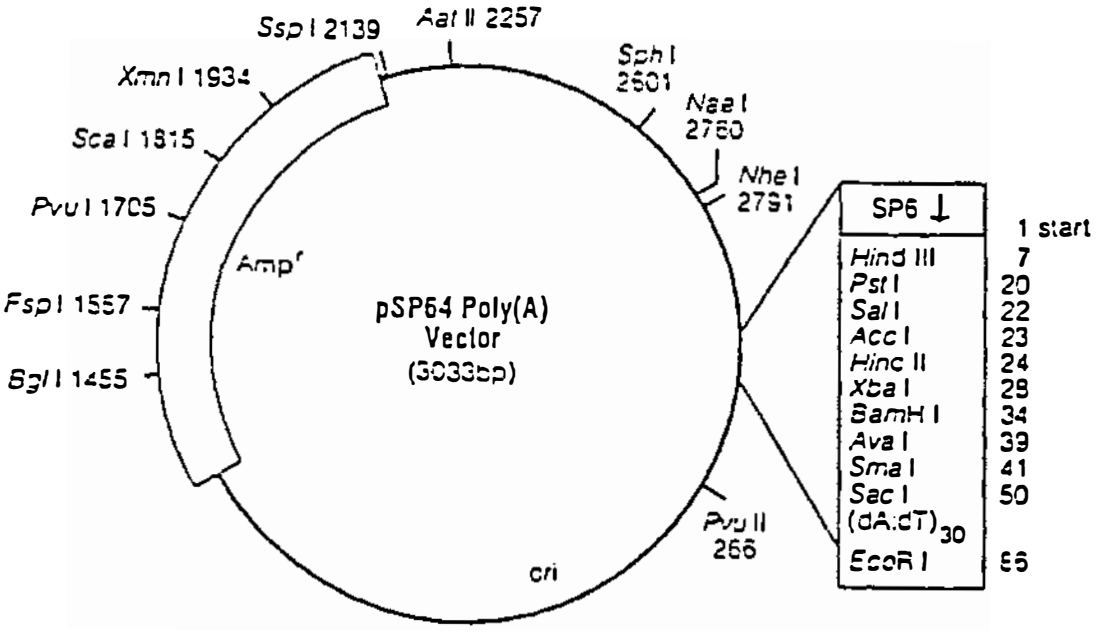

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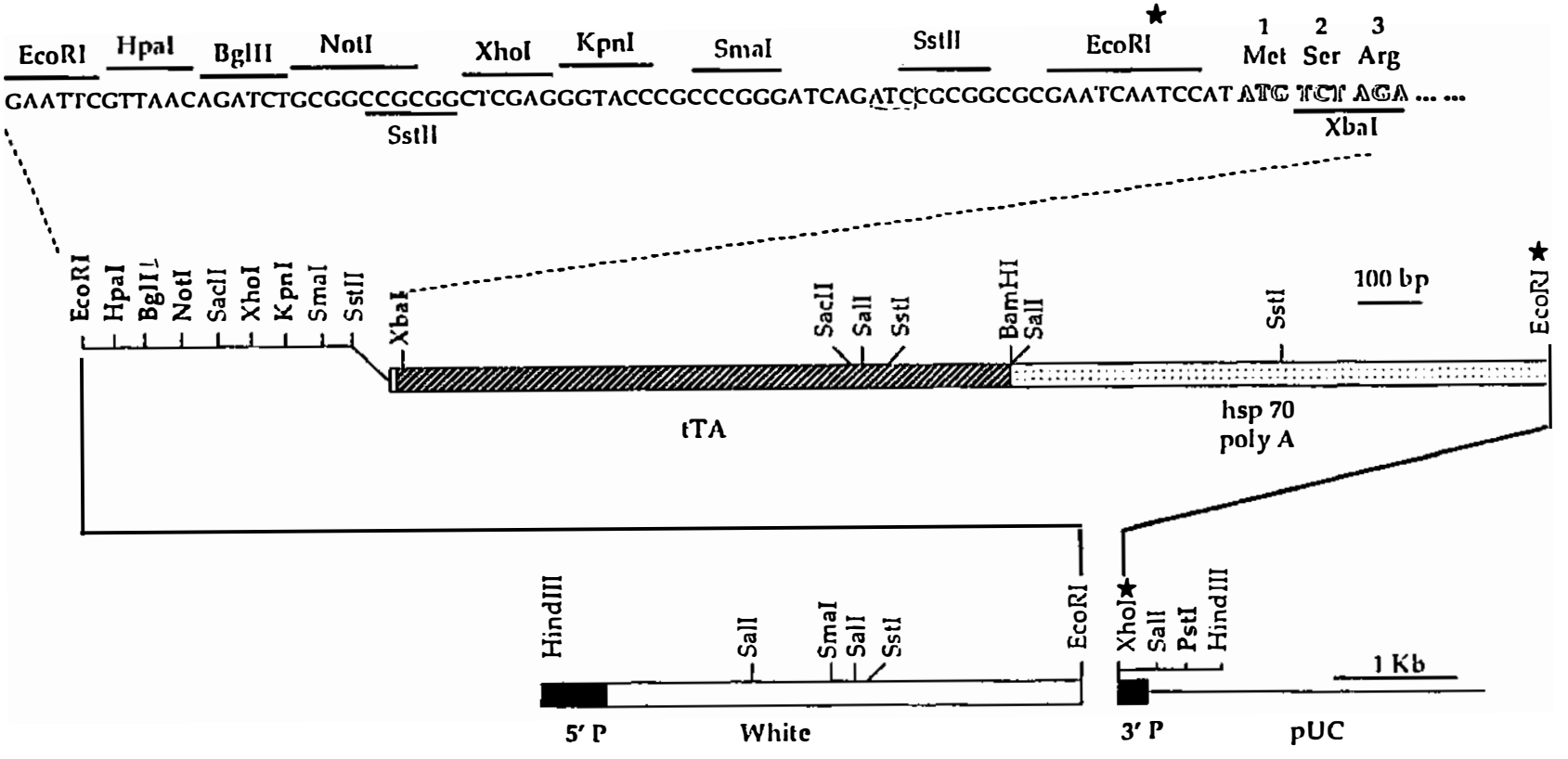

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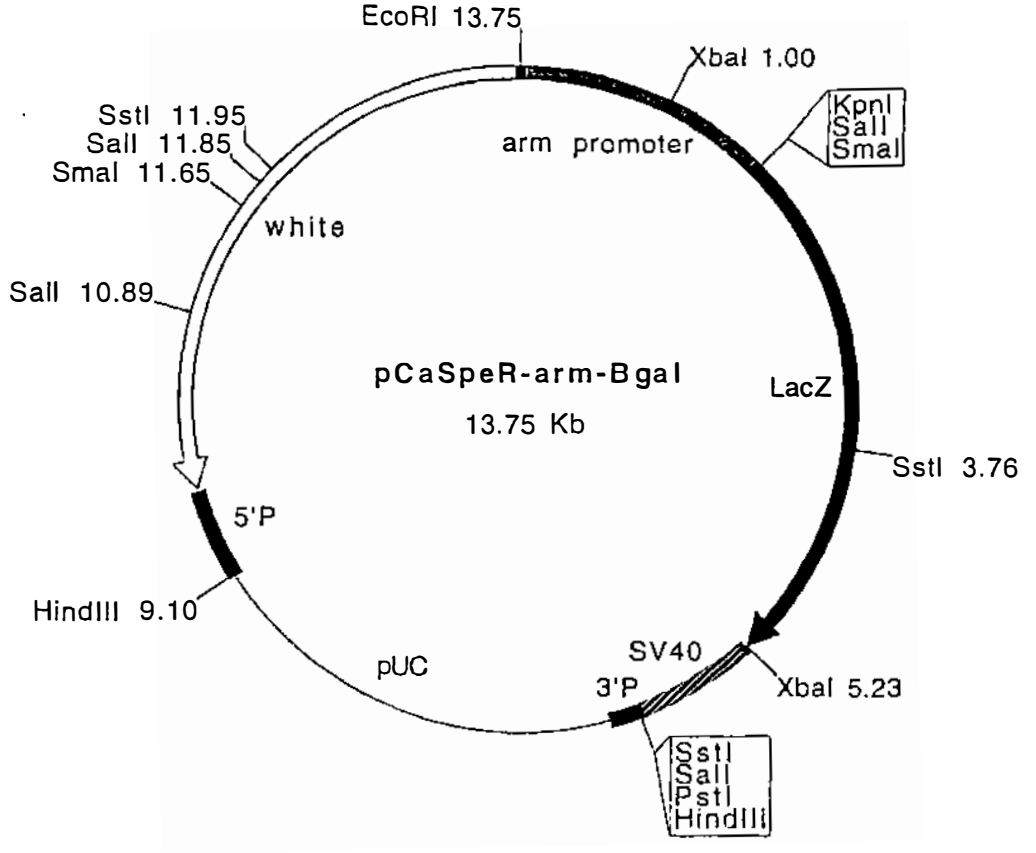

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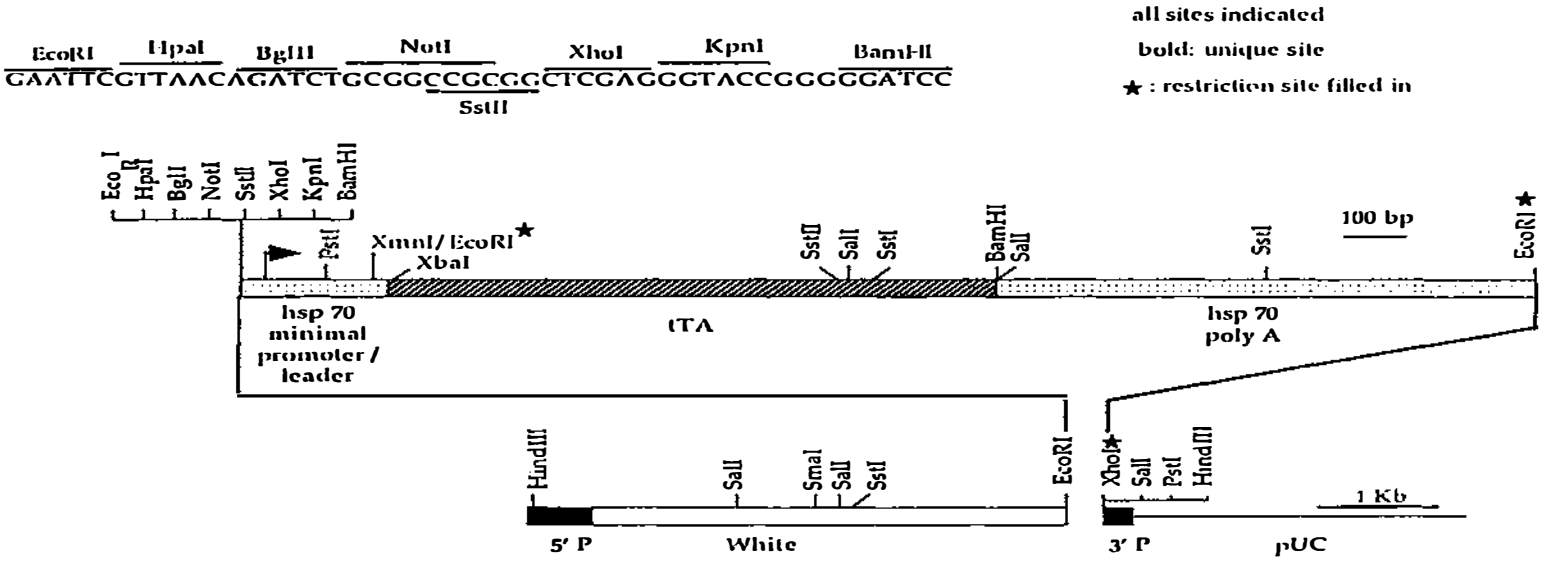

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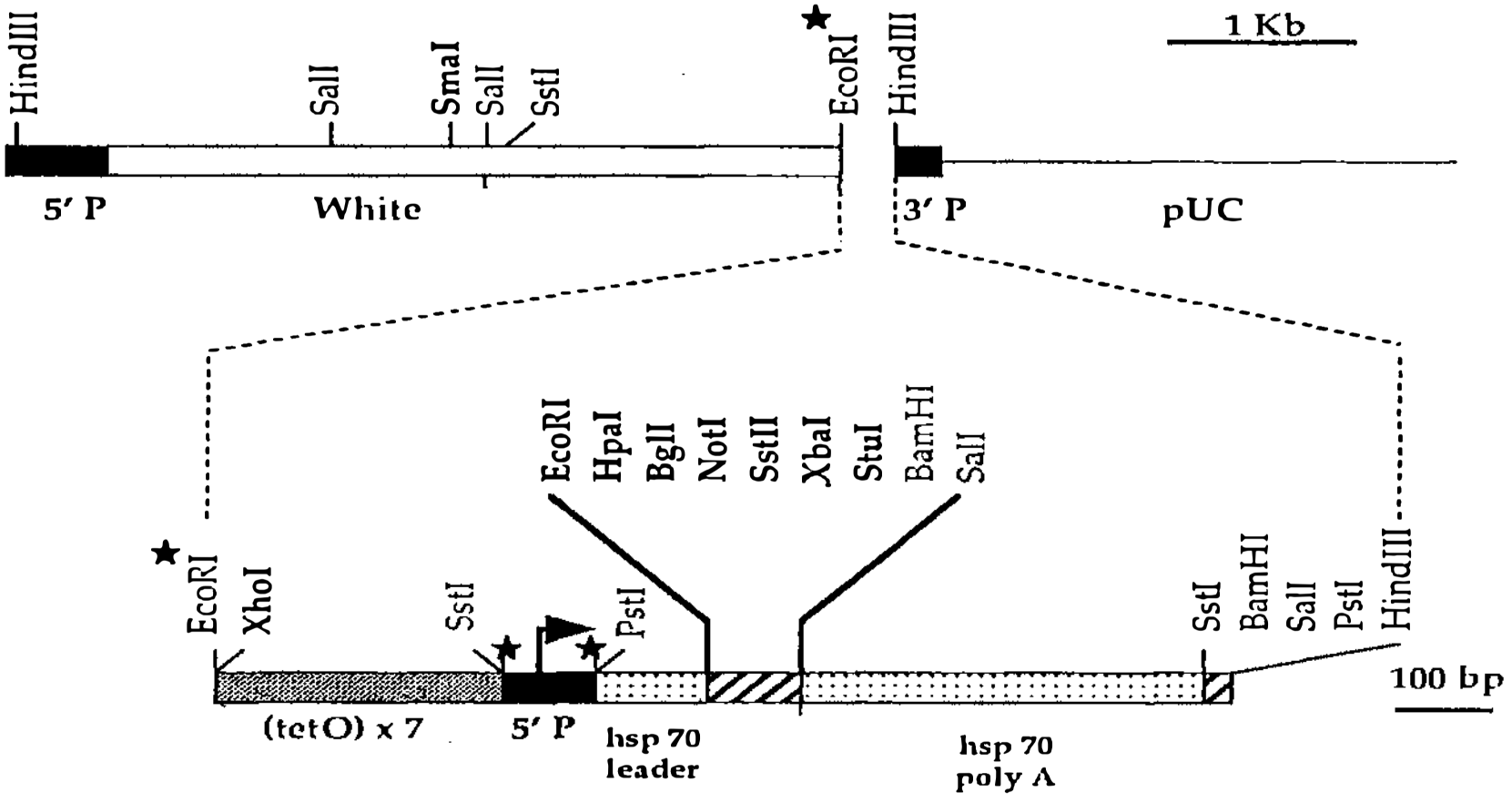

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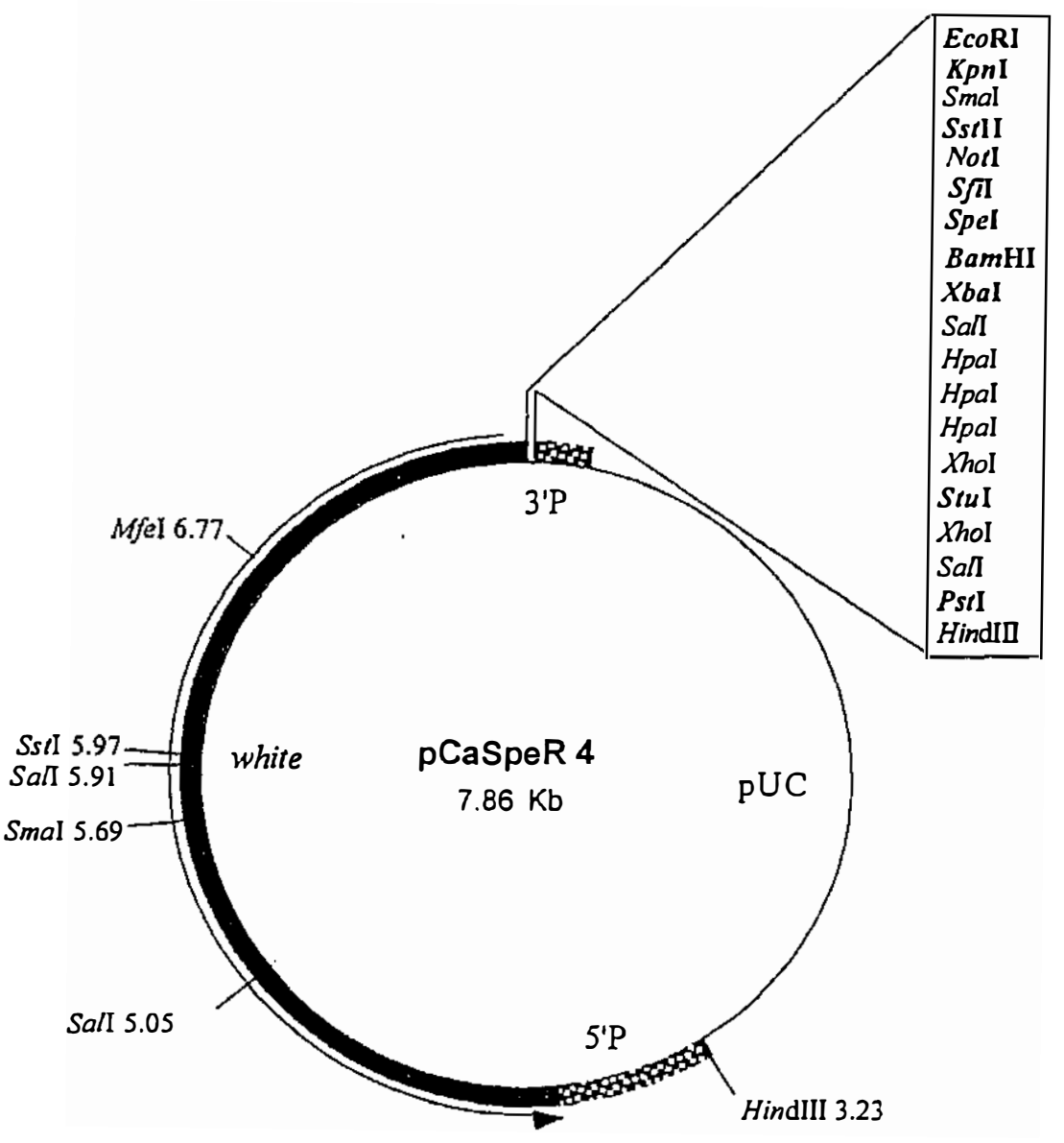

Plasmid name: pCaSpeR4
Plasmid size: 7.86 kb

APPENDIX 3

TABLES OF STATISTICAL DATA

Differences of Squares Means of *piggyBac* helpers According to Table 3.3 Data:

Helper name	Df	p
α1-tub and *hsp70*	1	0.0008
α1-tub and *hsp83*	1	0.0154
α1-tub and *hsp70hs*	1	0.0654
hsp70 and *hsp70*hs	1	0.1669
hsp70 and *hsp83*	1	0.1522
hsp83 and *hsp70hs*	1	0.9110
α1-tub, hsp83, hsp70 and *hsp70hs*	3	<0.0001

Differences of Squares Means According to Table 4.1 data:

Helper name	Df	p
Minoshsp70 and *RNA*	1	< 0.0001
RNA and *α1-tub*	1	0.4084
RNA and *hsp70*	1	0.0247
RNA and *hsp70hs*	1	<0.0001
RNA and *hsp83*	1	0.0019
α1-tub and *hsp70*	1	0.0028
α1-tub and *hsp70hs*	1	<0.0001
α1-tub and *hsp83*	1	0.0139
hsp70 and *hsp70hs*	1	0.0002
hsp70 and *hsp83*	1	<0.0001
hsp70hs and *hsp83*	1	<0.0001
α1-tub, hsp83, hsp70, hsp70hs and *RNA*	4	< 0.0001