MSL-1 PLAYS A CENTRAL ROLE IN ASSEMBLY OF THE MSL COMPLEX WHICH MEDIATES DOSAGE COMPENSATION IN *DROSOPHILA MELANOGASTER*

A thesis presented in partial fulfilment of the requirements
for the degree of Master of Science in Genetics
at Massey University, Palmerston North,
New Zealand

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1998

ABSTRACT

Dosage compensation in *Drosophila melanogaster* is achieved by a twofold increase of transcription of X-linked genes in males. This involves the binding of four proteins, MSL-1, MSL-2, MSL-3 and MLE (collectively known as the MSLs) which are believed to act as a multi-protein complex, to hundreds of sites along the length of the X chromosome. MOF, a putative histone acetyl transferase, is thought to be also associated with MSLs and plays a role in hypertrascription of X-linked genes. Overexpression of either a C-terminal or N-terminal domain of MSL-1 leads to male-specific lethality which is probably due to association with other MSLs to form a non-functional complex.

One aim of this study was to identify whether any known MSLs and/or unknown protein binds with the C-terminal domain of MSL-1. A second aim was to further define the domain of MSL-1 which interacts to MSL-2. Initial attempts to identify the protein which interacts the C-terminal domain of MSL-1 by either genetics analysis or co-immunoprecipitation were inconclusive. Thus, an alternative approach of affinity chromatography of epitope-tagged MSL-1/MSL-complex was followed. Transgenic flies which express either a FLAG-tagged N-terminal region of MSL-1 or FLAG tagged C-terminal domain following heat shock were generated. These lines were crossed with other transgenic lines to co-express the MSL-1 domain with Either MSL-2, MSL-3, MLE or MOF. FLAG affinity chromatography of protein extracts prepared from these flies showed that MSL-2 co-purifies with the N-terminal domain of MSL-1 (aa 85 - 263), whereas MOF and MSL-3 co-purify with the C-terminal domain of MSL-1 (aa 705 - 1039). MLE does not appear to associate with either region of MSL-1. Further, the C-terminal domain of MSL-1 also bound specifically to a glutathione S-transferase-MOF fusion protein. Co-expression of MSL-2 rescued males from the lethal effect which was caused by overexpression of the N-terminal domain of MSL-1. However, co-expression of either or both MOF and MSL-3 with the C-terminal domain of MSL-1 did not improve male viability. This suggests that additional factors may bind to the FC/MOF/MSL-3 complex. Finally, MLE also bound to GST-MOF fusion protein, suggesting a direct interaction between MLE and MOF. These findings suggest that MSL-1 plays a central in assembly of the MSL multi-protein complex that is required to achieve dosage compensation.

ACKNOWLEDGEMENTS

There is no word that can describe how much I appreciate the help I have received in last two years from my supervisor, Dr. Max Scott. Special thanks to you for your excellent guidance. You make me feel that "your door" (not just the office door!) is always opened for me, and you are ready to assist my study at any time. Thank you again!

I also want to thank my parents and my brother, Alex, the encouragement and supports you gave me have made it possible for me to concentrate in my study. So every few weekends I can go home and have a nice and relaxed weekend and try to forget about what is *Drosophila*!

To the past and present Fly Spot members - Sheralee, Xuelei, Rebecca, Jörg, Helen and Kathryn, it has been great working with you. I will never forget all the "Option" afternoon tea breaks we had for celebrating successes. Your friendship has made my time in the lab the most enjoyable and "fly" away quickly, too!

To everyone who has helped me, and friends I have made in the institute, thank you for making the working environment much easier for me. Also thanks the old Department of Microbiology and Genetics and new Institute of Molecular BioSciences for providing me with funding and equipment which have made me possible to complete my Masters degree.

I also like to thank Michiko for proofreading this manuscript which is not the easiest job in the world!

Finally, no thanks to my dearest friend *Drosophila.* After two years of battling with it, I still don't know how can one breed so fast and yet still so dumb.....

ABBREVIATION

α	alpha
aa	amino acid
N-terminal	amino terminal
β	beta
Δ	delta
C-terminal	carboxy terminal
°C	degrees Celsius
bp	base pairs
dNTP	dinucleotide triphosphate
DNase	deoxyribonuclease
DNA	deoxyribonucleic acid
EMS	ethyl methane sulfanoic acid
g	gram
GST	glutathione S-transferase
hsp	heat shock promoter
kb	kilobase pairs
kD	kilodalton
μ	micro
L (l)	liter
m	milli
M	molar
nm	nanometer
%	percent
PCR	polymerase chain reaction
RNase	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
U	units
UTR	untranslated region
V	volts
v/v	volume per volume
w/v	weight per volume

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1. INTRODUCTION

1.1 SEX DIFFERENTIATION AND DOSAGE COMPENSATION

Sex differentiation is often determined by differences in the number of copies of a single chromosome (Reviewed by Baker *et al.*, 1994). In such cases, a process of dosage compensation may evolve to allow the different number of copies of genes in the two sexes to produce the same amount of functional product. In *Drosophila*, the primary determinant of sex is the ratio of the number of X-chromosomes (X) to sets of autosomes (A). When *Drosophila* has one X-chromosome to two sets of autosomes (1X:2A = 0.5), it evolves into a male (Reviewed by Belote, 1992). An X:A ratio of 1 leads flies to develop into females. This process of somatic sex determination is largely cell autonomous, and there appears to be no hormonal component involved. Although all male *Drosophila* possess a Y-chromosome, it does not play any role in sex determination.

This leads to an interesting fact: there are many important X-linked genes on the X-chromosome. With only one X-chromosome in males and two in females, cells will produce one dose of X-linked gene product and two doses respectively. This raises a question of possible different phenotypes of these X-linked genes in male and female, as the result of different dosage. However, X-linked genes do not show signs of producing unequal amounts of gene product between male and female. In fact, these gene products are manufactured equally in both male and female (Kuroda *et al.*, 1993). This is achieved by a mechanism called dosage compensation. Different organisms have evolved what appear to be different mechanisms to equalise X-linked gene expression in the sexes. In mammals, one of the two X chromosomes in females is transcriptionally inactivated (Reviewed by Willard and Salz, 1997). In *Caenorhabditis elegans*, equalisation of X-linked gene products is achieved by decreasing the activity of genes on both X

chromosomes in hermaphrodites (XX), to reach the level of gene products produced in males (X) (Gorman and Baker, 1994). In *Drosophila melanogaster*, which has a different mode of dosage compensation, the rate of transcription of the single male X chromosome is hyperactivated twofold relative to each female X chromosome (Lucchesi and Manning, 1987).

1.2 THE DISCOVERY OF DOSAGE COMPENSATION IN *DROSOPHILA*

In 1931, Herman Huller published an observation that eye-pigment conferred by the hypomorphic X-linked allele w^a was identified in hemizygous XY males and in homozygous XX females. However, females do not produce two doses of pigment, rather they are producing an equal amount of pigment as male *Drosophila*. Similar results have been obtained for other X-linked genes, suggesting that there must be a mechanism which is able to compensate the dosage difference of X-linked genes between the two sexes.

1.3 CYTOLOGICAL OBSERVATIONS

In *Drosophila* salivary gland polytene nuclei, the male X-chromosome is distinct from autosomes and the female X-chromosome in both appearance and chromatin structure. It is puffier and paler in comparison with the female X-chromosome (Dobzhansky, 1957). This difference is not due to an increased DNA content in the male X-chromosome. Aronson *et al.* (1954) used UV microspectrophotometry to show that the paired female X-chromosome contains twice the amount of DNA material as a single male X-chromosome. Therefore, the difference between the X-chromosomes of the two sexes is either a distinct chromatin configuration or an accumulation of gene products.

1.4 TRANSCRIPTION AUTORADIOGRAPHY EXPERIMENTS

In 1965, Mukherjee and Beerman prepared polytene chromosome squashes from *Drosophila melanogaster* third instar larval salivary glands which were used as the subjects for short pulse labelling with [^{3}H]uridine (Muller, 1950). By using autoradiography, one is able to determine the level of incorporation of labelled uridine along the chromosome arms (Henikoff and Meneely, 1993). The grain counts on the male X-chromosome are greater than both the left arm of autosome 3 and the regions of paired female X-chromosomes. However, unpaired female X-chromosome still seem to have a silver grain count, and it is significantly higher than half of the value for paired regions of the same chromosome. All the observations lead to the fact that a single male X-chromosome is transcriptionally more active than a single female chromosome, and the dosage compensation is at the level of RNA synthesis. The characteristic bloated appearance could be the consequence of accumulation of nascent RNA.

1.5 SEX-LETHAL (*Sxl*)

The *Sex-lethal* gene plays a pivotal role in the processes of somatic sex determination as well as in the process of dosage compensation (Reviewed by Parkhurst and Meneely, 1994). The structure of *Sex-lethal* comprises 10 exons and two promoter regions. The use of different promoters, different exons, and different polyadenylation sites leads to the production of at least 10 different RNA species (Palmer *et al.*, 1994), with varying patterns of expression. SXL has a special feature of auto-regulation in that the protein turns on its own *Sxl* gene and produces more of its gene product (Bernstein *et al.*, 1995;

MacDougall *et al.*, 1995). When the ratio X:A is 1, as females are produced, the *Sxl* locus is making SXL protein which represses the pathway of dosage compensation and results in basal transcription of both X chromosomes (Lucchesi *et al.*, 1982). Whereas if the ratio is 0.5, male development and hypertranscrption occurs as a consequence of lacking SXL protein (Reviewed by Kelly and Kuroda, 1995). Null mutations in *Sxl* are lethal in females - presumably both X chromosomes are hypertranscribed. Conversely, constitutive expression of *Sxl* causes male lethality (Cline, 1978).

1.6 MALE-SPECIFIC LETHAL (*msl*)

There are four genes identified which are responsible for dosage compensation (Reviewed by Lucchesi, 1996). The identification of these four genes is significant for the understanding of the mechanism of dosage compensation. Genetic screens (using the chemical mutagen ethyl methane sulfanoic (EMS)) designed to isolate mutations affecting essential biochemical or physiological processes unique to males or females have uncovered several sex-specific lethal mutations that affect dosage compensation, *maleless* (*mle*) (Fukunaga *et al.*, 1975), *male-specific lethal-1* (*msl-1*) (Belote and Lucchesi, 1980), *male-specific lethal-2* (*msl-2*) (Belote and Lucchesi, 1980), and *male-specific lethal-3* (msl-*3*) (Lucchesi *et al.*, 1982). These are collectively called *msls*. All *mle*, *msl-1* and *msl-2* gene loci are located at chromosome 2, and *msl-3* is located at chromosome 3. The products of the *msl* genes are necessary to maintain an equivalent level of most X-linked gene transcripts in males relative to females. A loss of function of any *msl* through mutation has lethal effects during late larval development in males but has no detectable effect in females.

Genetic evidence suggests that the lethality produced by these *male-specific lethal* genes is directly related to the presence of only one X chromosome in males. Fukunana *et al.*

(1975) and Belote & Lucchesi (1980b) have shown that the male specific lethality is not related to the sexual differentiation. The absence of interaction between sex transforming genes (*dsx* and *tra-2*) and *male-specific lethal* genes (*msl-1* and *msl-2*), and between *dsx* and *tra-3* and *mle*, is proved by introducing sex-transforming genes into the genotype of individuals that are homozygous for *msl* genes. Also, the presence of the Y chromosome has no effect. Individuals of two sexes with zero, one or two Y chromosomes showed no effect on the sex-specific lethality and are viable. Another indication for male-specific lethality is that the difference in the number of X chromosomes has no effect on individuals as the lethality could be due to a difference in dosage of a specific X-linked gene or the number of X chromosomes as a whole. Experiments showed that the duplication of small fragments of X chromosome one at a time does not reduce the lethality caused by male-specific gene mutations in males.

It has been found that male homozygous mutations for any of the *male-specific lethal* genes exhibit a level of X-linked gene activity that is 50-65% of that seen in the wild type, whereas no reduction is observed in the amount of autosomal enzymes tested. Breen and Lucchesi (1986) showed that at the restrictive temperature, the loss of function of *mle* significantly reduced the steady state RNA levels from the X-linked gene, *Sgs-4*, as compared to the transcript levels of the autosomal gene, *Sgs-3*.

1.7 SEX-SPECIFIC LOCALISATION OF MSLs

Since the cloning of *mle* (Kuroda *et al.*, 1991), *msl-1* (Palmer *et al.*, 1993), *msl-2* (Zhou *et al.*, 1995) and *msl-3* (Gorman *et al.*, 1995), the antibodies to their encoded products reveal that all four MSL proteins bind hundreds of specific sites along the male X chromosome (Bashaw and Baker, 1995; Gorman *et al.*, 1995; Kelly *et al.*, 1995; Kuroda *et al.*, 1991; Zhou *et al.*, 1995). None of these four MSLs is associated with X chromosomes in

females, although MLE is associated with 30-40 autosomal sites (Kuroda *et al.*, 1991). MSLs also associate with autosomes in males - *mle* is associated with 30-40 autosomal sites (Kuroda *et al.*, 1991), *msl-1* and *msl-3* are associated with 10-20 autosomal sites (Kelly *et al.*, 1995; Gorman *et al.*, 1995), and *msl-2* is associated with 20-30 autosomal sites and it is co-localised with MSL-1 at these sites (Kelly *et al.*, 1995; Zhou *et al.*, 1995). All of these four *msls* are not processed sex-specifically at the RNA level, and each of the *msl* proteins is produced in both sexes except *msl-2* (Kelly *et al.*, 1995; Zhou *et al.*, 1995). Male-specific lethal binding sites on the male X chromosome are consistent with studies showing that genes transposed from an autosome onto the X chromosome frequently become hypertranscribed, while X-linked genes translocated to an autosome remain dosage compensated. As these post-translocated genes produce various amounts of dosage compensation, it is possible that X-linked *cis*-acting sequences are able to confer hypertranscription on different genes depending on their chromatin context where the genes are placed (Kuroda *et al.*, 1993). The *cis*-acting sites on the male X chromosome could be bound by *trans*-acting MSL proteins and result in dosage compensation (Bone and Kuroda, 1996).

Protein analysis of the association of MSLs with the male X chromosome has been investigated in various mutant backgrounds (Lucchesi and Manning, 1987). Each of the MSL proteins must be functional in order to observe the wild-type chromatin-pattern of the remaining three, suggesting that MSLs act in a heteromeric protein complex (Lucchesi, 1996). Western blotting showed that the MLE is expressed in male homozygous for a mutant at each of the other *msl* loci, which means the other *msls* do not regulate *mle* at the level of protein expression (Gorman *et al.*, 1993). The MSL-1 protein is present in both homozygous mutations for *mle* and *msl-3* in larvae, but not in *msl-2* homozygous mutation or female larvae. Experiments also showed the MSL-1 and MSL-2 proteins can be co-immunoprecipitated by either anti-MSL-1 or anti-MSL-2 from protein extracts of

male larvae (Kelly *et al.*, 1995). Since the MSL-1 and MSL-2 proteins are at low levels or absent in females, neither was immunoprecipitated from female extracts as expected.

Ectopic expression of MSL-2 protein in females decreases viability and delays development since MSL-2 protein is not normally present in females (Kelly *et al.*, 1995). This experiment is conducted by constructing transgenic lines expressing the *msl-2* open reading frame under control of the heat shock 83 promoter (hsp83). The hsp83 allows constitutive activity of *msl-2* in both soma and germline, and can be heat shocked for further induction. Interestingly, MSL-1 protein levels are also significantly increased in H83M2 transgenic females, suggesting that the presence of MSL-2 may be required for translation or stability of MSL-1 protein. This result supports the fact that *msl-1* transcripts are present in homozygous mutation for *msl-2* at larval stages but no functional MSL-1 protein forms at late developmental stages.

In flies which are mosaic for *Sxl* expression, MSL proteins are only associated with the X chromosome in cells which are not expressing SXL. Since there are several types of transcripts which are produced by each of the *mle*, *msl-1* and *msl-3*, and all of these appear to be equivalent in both males and females, the regulation of these transcripts by SXL protein is not direct. Therefore the only MSL protein, MSL-2, which is solely produced in males, can be the key linkage between SXL, MSL complex and dosage compensation (Bashaw and Baker, 1995) (details see Section 1.9)

In polytene chromosome squashes the male X-chromosome is more open and diffuse than that of the females (Dobzhansky, 1957). It has been proposed that this altered chromatin configuration is important in allowing hypertranscription to occur. One of the components for hypertranscription is the recognition of the acetylated isoform of histone H4 (H4Ac16) (Turner *et al.*, 1995). It is a histone H4 acetylated at lysine 16 in the N-terminal region. Using the technique of immunolabelling, a specific acetylated isoform is

not only detected predominantly on the male X chromosome, coincidently, it also has the same pattern of association with the X chromosome as that of the MSLs. H4Ac16 is not detected on the X chromosome in homozygous mutation for *msls* males, correlating with the lack of dosage compensation in these mutants. Conversely, in *Sxl* mutants, H4Ac16 is detected on the X chromosomes in females which have inappropriate hypertranscription as a consequence. All of the above suggest that synthesis or localisation of H4Ac16 is controlled by the dosage compensation regulatory hierarchy (Lee *et al.*, 1993), and may potentially be involved in dosage compensation through interaction with the products of the *msl* genes.

1.8 NON-CODING RNAS ARE INVOLVED IN DOSAGE COMPENSATION

The mutants *roX1* and *roX2* were isolated in an enhancer detector screen for mushroom body expression of the reporter gene *lacZ* (Han *et al.*, 1996). The expression of both *roX1* and *roX2* are restricted to the neuron cells of adult male flies (Amrein and Axel, 1997). The two genes are X-linked and lack significant open reading frames, suggesting they may encode non-coding RNAs (Amrein and Axel, 1997; Meller *et al.*, 1997). Mutations in any one of the *msl* genes prevent the expression of *roX1* and *roX2*, and ectopic expression in females of the normally male-specific *msl-2* gene induces expression of *roX1* and *roX2* (Amrein and Axel, 1997; Meller *et al.*, 1997). Expression of *roX1* is dependent on *Sxl* but is independent of the Y chromosome and *tra* which is a downstream effector of *Sxl* (Meller *et al.*, 1997). *In situ* hybridisation of *roX1* probes to the male third instar larval salivary gland reveals a subcellular localisation of *roX1* RNA identical to that of MSL-2 which binds to the X chromosome (Meller *et al.*, 1997). *RoX1* recognises, or paints, the X chromosome of males in a similar mechanism as the mammalian *Xist* non-coding RNA which coats the inactive X chromosome. *In situ* hybridisation of a chromosome containing

roX1 region was transposed to the Y or the 2nd chromosome and showed binding of *roX1* to the transposed X-linked chromatin as well as to the X chromosome (Meller *et al.*, 1997). This result suggests that *roX1* can act either in *trans* or in *cis* in contrast to *Xist* which can only spread in *cis* along chromatin from the X-inactivation centre.

MLE consists of RNA binding domains (Kuroda *et al.*, 1991). It would be tempting to speculate that *roX1* is required for the binding of MLE to the male X. However, the localisation of MLE to the male X appeared undisrupted by mutations in *roX1* (Meller *et al.*, 1997). Meller *et al.* (1997) suggested that there is a family of non-homologous RNAs including *roX1* and *roX2*, which are functionally redundant. They proposed that the binding of the MSL complex to the X activates the male-specific RNA which facilitates a change in chromatin structure leading to hypertranscription of the male X chromosome.

1.9 A MODEL FOR THE REGULATION OF DOSAGE COMPENSATION

Studies showed that the four histones comprise the nucleosome core and participate in the transcriptional regulation of numerous genes acting as suppressors of transcription (Turner, 1991). The core histones undergo several post-translational modifications, including acetylation at the N-termini, leading to the suggestion that the structure and function of chromatin could be altered through an enzymatic pathway. The finding that histone acetylation pre-exists transcription showed the modification is not a consequence of transcription but is most likely a prerequisite (Turner *et al.*, 1992). This suggests that histone acetylation can stabilise the binding of transcriptional factors to mucleosomal DNA, and may play a role in initiating or in maintaining the accessibility of transcriptional regulatory elements in chromatin. Acetylation of lysine at the N-terminus neutralises the positive charge of histones. Experiments illustrate that removal or acetylation of the histone H4 N-terminal tails facilitate the interaction of the

transcriptional factors USF and GAL4-AH where the highest DNA binding affinity is obtained (Turner *et al.*, 1992). As is described above, the H4Ac16 may play an important role in loosening the chromatin structure and increasing the accessibility of transcriptional factors in association with the male X chromosome in *Drosophila* (Bone *et al.*, 1994).

MLE has short sequences that identify it as a member of one of two superfamilies of nucleic acid helicases (Richter *et al.*, 1996). It is highly homologous to human RNA helicase A and bovine nuclear DNA helicase II, for which a DNA and RNA unwinding activity has been illustrated. This helicase-like MLE protein is possibly targeting a stable RNA which is the mediator for the association of MLE with the MSL complex, since RNase treatment excludes the possibilities of interaction with nascent transcripts, protein-protein interaction with the MSL complex, or direct DNA binding (Gorman *et al.*, 1993; Kuroda *et al.*, 1991; Richter *et al.*, 1996).

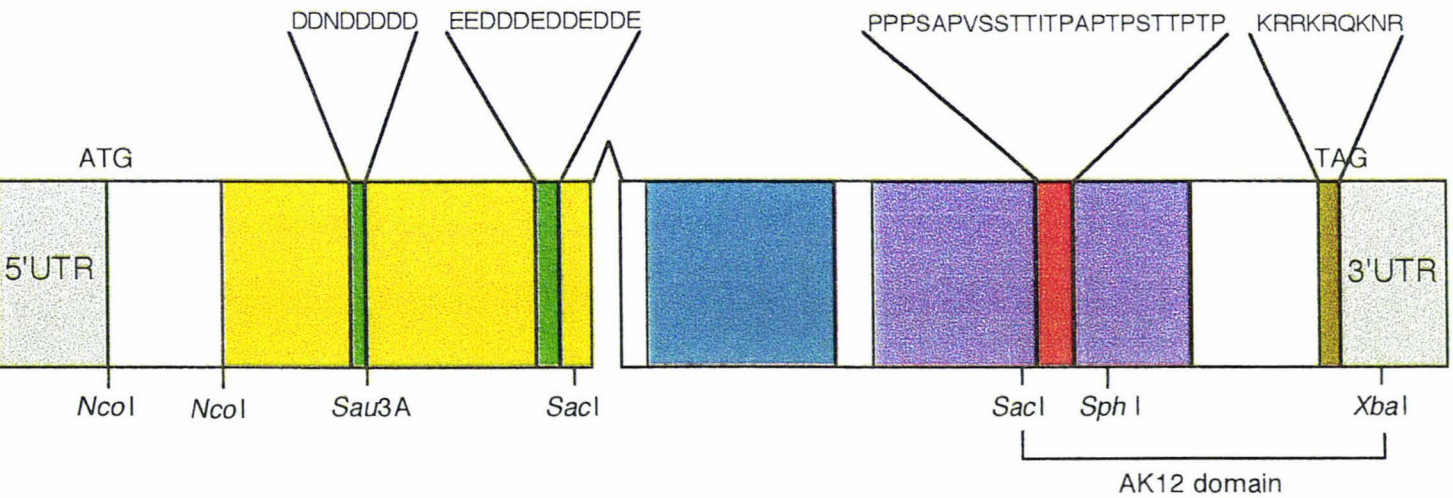
Male-specific lethal-1 encodes a 1039 aa protein with a highly acidic N terminus that includes two short stretches composed almost entirely of aspartate or aspartate and glutamate residues as well as numerous interspersed glutamate doublets (Figure 1). These characteristics are common to a large group of proteins such as nucleolin and nucleoplasmin which are thought to be involved in transcription regulation and chromatin modelling. Direct contacts may possibly be made between acidic regions of these proteins, including MSL-1, and basic chromosomal proteins such as histones. This may lead to alterations of nucleosome positioning or conformation. Also in the central region of the MSL-1, there are many serine (S), threonine (T) and proline (P), residues, which form S/T/P motifs that could be sites of phosphorylation by protein kinases, and therefore regulate the activity of the protein and perhaps the MSL complex.

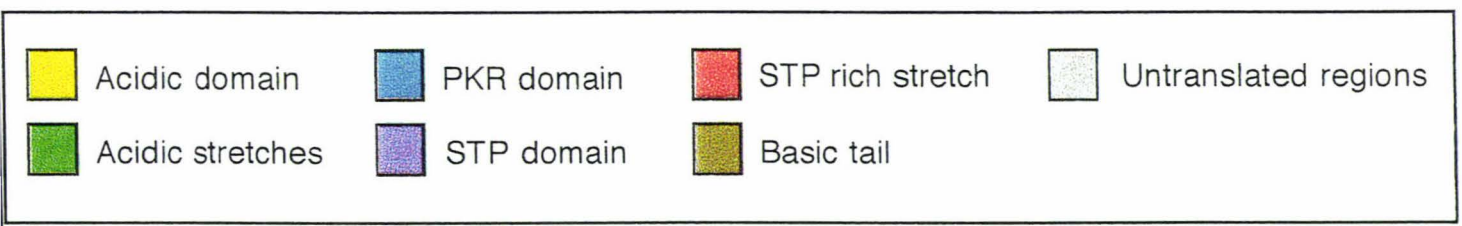
MSL-3 contains two chromo domains (Lucchesi, 1996). This feature is interesting as the secondary structure of a single domain appears to be unstable, and may require interaction

Figure 1. Diagrammatic Representation of The Open Reading Frame of *msl-1*

Protein coding sequences are drawn as boxes and the putative domains are coloured. The amino acid sequence of the asp/glu-rich stretches within the acidic domain is given using the standard one letter code as is the sequence of the basic tail and a highly ser/thr/pro-rich sequence within the STP domain.

Structure of *male specific lethal* (*msl-1*) Gene





with another chromo domain or with some globular domain in another part of the protein to yield a stable configuration. Stabilisation may also be achieved by the interaction between chromo domains of different proteins. Chromo domains share a characteristic with proteins that are responsible for repression, as several proteins are found to participate in transcriptional repression (Koonin *et al.*, 1995). However, chromo domains are also found in proteins known to function as transcriptional activators, suggesting that the function of the chromo domain is to deliver regulatory proteins to their site of action, whether they exert a positive or a negative effect on gene activity. Therefore the presence of MSL-3 indicates the potential for this type of regulation may be involved in dosage compensation.

As described above, MSL-2 is present only in males and is co-localised with the other three MSLs (Kelly *et al.*, 1995; Bashaw and Baker, 1996). With a perfect inverse correlation between the presence of SXL and the presence of MSL-2, it makes MSL-2 a likely governor of the MSL complex for transcriptional enhancement (Bashaw and Baker, 1995). Studies showed that the SXL protein, which is a RNA binding protein whose known function is to regulate pre-mRNA splicing, may target the *msl-2* gene that produces non-functional MSL-2 in females (Bashaw and Baker, 1997). Experiments suggest that there is a difference in the splicing of *msl-2* pre-mRNA between the two sexes. A 133 nucleotide intron in the 5' untranslated region (UTR) of *msl-2* is retained in females but removed in males (Bashaw and Baker, 1995; Zhou *et al.*, 1995). There are potential SXL-binding sites in the 3' UTR as well as the two potential SXL-binding sites present in the male-specific intron in the 5' UTR. That sequence in the 3' UTR plays a role in the regulation of MSL-2 expression (Bashaw and Baker 1997). It is demonstrated by removing the 3' UTR that leads to detectable binding of MSL-2 to the X chromosome in females. In addition to detecting MSL-2 associated with 50-60 sites on the X chromosomes of these transgenic females, all three of the other MSLs are also associated at these same sites. This suggests that the expression of MSL-2 due to deletion of the 3'

UTR results in the assembly of a male-type MSL complex, indicating SXL could act through these sites at some level other than splicing to control MSL-2 expression since male *msl-2* transcripts have possessed these regions. Therefore the presence of potential SXL-binding sites and the alternative splicing event make the 5' UTR a strong candidate for a region that may contain other sequences crucial to the regulation of MSL-2.

The encoded protein MSL-2 consists of 769 amino acid residues and it contains a RING finger (C_3HC_4 zinc finger) at the N-terminus of the protein, with a coiled coil at the central domain followed by positively and negatively charged segments that flank a metallothionein-like domain which has eight conserved and two non-conserved cysteines (Bashaw and Baker, 1995; Zhou *et al.*, 1995). The RING finger is related to the classical zinc finger and is found in a large group of proteins with potential for DNA or protein-protein interactions. Interactions between the zinc fingers of transcription factors, SP1 and TFIIIA, and thionein have been demonstrated *in vitro* (Zhou *et al.*, 1995). These observations suggest a possible novel mechanism, based on the intramolecular exchange of metal ions, that may modulate the activity of MSL-2. Since MSL-2 is the only MSL protein containing sequence elements that can be implicated in DNA binding, it is possible that MSL-2 interacts directly with male X-chromosomal DNA and at the same time targets the putative MSL complex to male X. When a twofold transcriptional rate is reached, MSL-2 may undergo a conformational change that allows the lowering of RING finger affinity for the zinc atoms, which may be captured by the metallothionein-like portion of the protein, restricting the transcriptional rate so that it does not reach beyond twofold. When the male X-chromosome is hypotranscribed, the condition is reversed and a twofold transcription is re-established.

Dosage compensation of the *runt* gene during embryogenesis is dependent on *Sxl* but independent of the *msl* genes (Gergen, 1987), suggesting that there is another possible kind of regulation for dosage compensation. This hypothesis is supported by the

experiment of female SXL mutants which can not be rescued by mutation of the MSLs (Kelly and Kuroda, 1995). With 20% of the genetic material located on the X-chromosome, more screening is needed in order to find other dosage-compensation-related genes.

1.10 DISCOVERY OF THE *males-absent on the first (mof)* GENE

Hilfiker *et al.* (1997) have recently discovered a possible fifth male-specific lethal gene which may be involved in the regulatory process of dosage compensation. *Males-absent on the first* (*mof*) has been identified by using the phenotype of male-specific lethality to screen the X chromosome of *Drosophila melanogaster* for EMS-induced mutations. Mutant *mof* males can develop to the third larval instar of the pre-pupal stage but fail to metamorphose and to hatch, whereas the viability of mutant female is unaffected. Staining of polytene chromosomes with anti-MSL antibodies suggests that the association of MSL-1 and MSL-2 with the male X-chromosome 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). Other evidence showed that the ectopic expression of MSL-2 in females can be rescued by expressing the MOF protein from one wild-type copy of *mof*, indicating *mof* has a functional role in dosage compensation. MOF contains the signature motif for the acetyl coenzyme A binding site found in numerous and diverse acetyl transferases (Hilfiker *et al.*, 1997). Therefore MOF could be one of the histone acetyl transferases which may be responsible for the particular histone acetylation (i.e. H4Ac16) associated with the male X chromosome, and consequently provides a functional link between nucleosomal modification and the transcriptional enhancement.

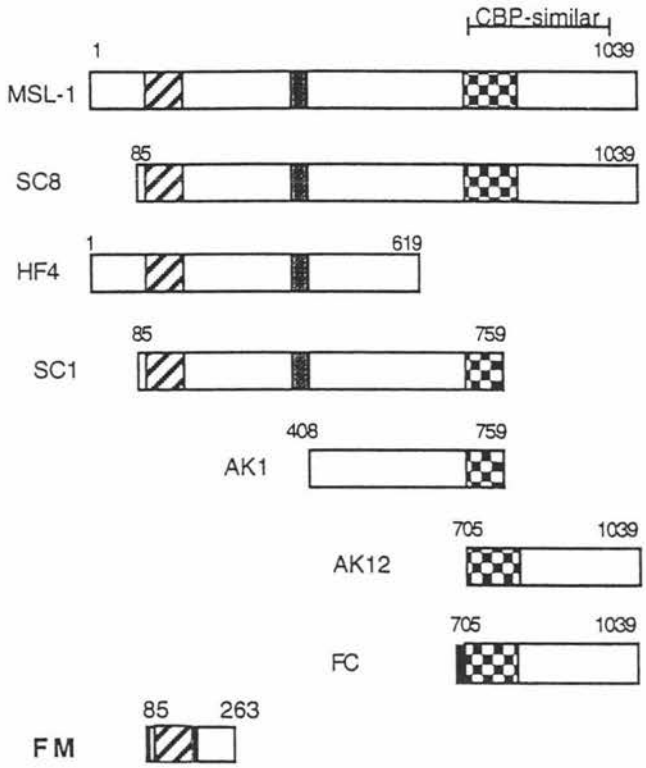
1.11 OVEREXPRESSION OF DOMAINS OF MSL-1 in *D. melanogaster*

MSL-1 (Figure 1) consists of several domains (Lucchesi, 1998). The first third of the protein (amino acid 1 to 409) contains the acidic region which is discussed in Section 1.8. The next region is a P/K/R domain which is from amino acid 410 to 634aa, which contains a predominance of proline (P), lysine (K) and arginine (R). The final region is the STP domain which is located from amino acid 702 to 877 aa. It contains several stretches rich in serine, threonine and proline. There is a basic tail which possesses seven basic amino acids out of the final nine amino acids (Figure 1).

Plasmid pAK12 contains the 1.17 kb region of *msl-1* 3' end bounded by *Sac*I and *Xba*I restriction sites inserted into the expression vector pCaSpeR-hs (Table 1). In this construct, expression of the C terminal domain of MSL-1 is under the control of heat shock promoter 70 (*hsp70*) (A. Knox, unpublished results). The restriction fragment codes for the basic tail and approximately half of the STP domain. This construct was microinjected (Section 2.18) into recipient *yw* stocks (Table 3) and homozygous flies were obtained (A. Knox, unpublished results). In these transgenics (AK12 flies), overexpression of the C-terminal domain (amino acid 705 - 1039, Figure 2) was achieved when raised at 30°C and heat shocked daily for one hour at 37°C (Section 2.21). Under these conditions, males showed a significant decrease in viability compared to either the injection stocks or transgenic with full-length MSL-1 (Dr M. Scott, unpublished results). It is proposed that the lethality is caused by the competition with functional MSL-1 for binding to a protein required for dosage compensation. Overexpression of MLE, MSL-2 or MSL-3 one at the time with the C-terminal domain shows no significant improvement in the viability of heterozygous AK12 males (M. Scott, unpublished results). This suggests that either the C-terminal domain is bound to more than one MSL protein or the domain binds to an unknown factor which is required to achieve twofold dosage

Figure 2. Dominant-Negative Versions of MSL-1.

The numbers at the beginning and end of each construct indicate the region of MSL-1 which is expressed in transgenic flies. The FLAG tag (DYKDDDDK - shaded black) is at the amino terminus of the protein encoded by the FC and FM constructs. Cross-hatched region is a predicted amphipathic α-helix (aa 96-172). The shaded region is a highly acidic stretch (aa 708-801). The region between aa 712 to 988 shows similarity to amino acids 863 to 1117 of mouse CBP.


CBP-similar
1
1039
MSL-1
85
1039
SC8
1
619
HF4
85
759
SC1
408
759
AK1
705
1039
AK12
705
1039
FC
85
263
FM

compensation. The recent finding of the MOF protein suggests it could be a possible target for the C-terminal region of MSL-1.

Similar experiments were carried out for transformants carrying the FMS construct (Figure 2) and results showed there is a significant reduction in male viability when heat shocked daily (Section 2.21) (Dr. M. Scott, unpublished results). The FMS protein lacks the first 84 amino acids of MSL-1, and contains a FLAG octapeptide which is tagged at the amino terminus. A FMS line was crossed with lines carrying null mutation for either *msl-1, msl-2, msl-3* or *mle*. These crosses would produce progeny with reduced concentration of 50 % of normal *msl-1, msl-2, msl-3* and *mle*, and overexpressed FMS when heat shocked daily (Section 2.21). Male viability was significantly reduced if the males of FMS were heterozygous for *msl-2*, however, there was no significant difference in the relative viability of heterozygous *msl-1, msl-3* and *mle* males compared to their respective wild-type siblings (Dr. M. Scott, unpublished results). These results indicate that in FMS males the concentration of MSL-2 available for dosage compensation is limiting. Further, the viability of males which overexpressed both FMS and MSL-2 was significantly improved compared to males which expressed only the FMS (Dr. M. Scott, unpublished results), suggesting that FMS interacts with MSL-2. Similarly experiments were carried out with transformants lines carrying the ANT which express aa 85-759 of MSL-1 with a FLAG tag at N terminus (Figure 2). Overexpression of the ANT protein caused a decrease in male viability which however was significantly improved if MSL-2 was co-expressed. This indicates that the domain which interacts with MSL-2 should be in the first two-thirds of MSL-1.

1.12 RESEARCH AIMS AND SPECIFIC OBJECTIVES

This study has two aims. The main aim is to determine whether MOF, or other factor(s), associate with the C-terminal domain of MSL-1. The second aim is to define the MSL-2 binding domain of MSL-1.

1.12.1 Factor(s) Associated With The C-Terminal Domain of MSL-1

The first aim of this project is to determine what factor, either one of the MSLs, MOF or an unidentified factor, associates with the C-terminal domain of MSL-1. Both biochemical and genetic approaches were used to identify the interacting factor(s).

The specific objectives are as follows:

1. Screen for EMS induced mutation which enhance or suppress male lethality caused by overexpression of the C-terminal domain.
2. 2. To generate transgenic flies which express a (FLAG) tagged C-terminal domain of MSL-1.
3. To clone the *mof* gene and make transgenic *Drosophila* lines which express MOF.
4. To make and transform *E. coli* with a plasmid designed to express a glutathione S-transferase MOF fusion protein.
5. To determine which protein(s) co-purify with the FLAG-tagged C-terminal domain of MSL-1 over a FLAG affinity column.
6. To determine which protein (s) co-purify with the GST-MOF fusion protein over a glutathione affinity column.
7. To determine if males can be rescued from the dominant-negative effects of overexpression of the C-terminal domain of MSL-1 by co-expression of any of the MSLs (or combination).

1.12.2 Define MSL-2 Binding Domain of MSL-1

The second aim is to further define the domain of MSL-1 which interacts with MSL-2.

The specific objectives are as follows:

1. To generate transgenic flies which express amino-terminal region of MSL-1 (aa 85 - 263) with a FLAG - tag FM (Figure 2).
2. To determine if overexpression of the FM protein causes male-specific lethality. If so, determine if FM can be rescued by co-expression of MSL-2.
3. To determine if MSL-2 co-purifies with the FMS, ANT, and FM proteins over a FLAG affinity column.

2. MATERIALS AND METHODS

2.1 DNA PLASMIDS

DNA plasmids used in this study are described in Table 1.

2.2 BACTERIAL STRAINS

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

2.3 BACTERIAL MEDIA

2.3.1 Luria Borth (LB)

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

2.3.2 SOB Medium

SOB medium contained (g/L): tryptone, 20; yeast extract, 5; NaCl, 0.5 and KCl, 0.2. The medium was supplemented with (g/L): $MgCl_2$, 0.2 and $MgSO_4$, 2.5 after autoclaving.

2.3.3 SOC Medium

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

Table 1. DNA Plasmids and Bacterial Strains

Plasmids or Bacterial Strains	Relevant Characteristics	Source
Bacterial Strains		
Escherichia coli		
DH5α	ϕ80d*lac*ZΔM15Δ(*lac*ZYA-*arg*F)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44λ$^-$ *thi*-1 *gyr*A96 *rel*A1	Hanahan (1983)
BL21(DE3)	F$^-$ *omp*T *hsd*S_B(r_B^-, r_B^-) *gal dcm* (DE3)	
Plasmids		
pUC118	rep_{pMB1} Apr *lacZ*	Messing (1983)
pCaSpeR-hs	8.9 kb, pUC containing *hsp70* promoter, *hsp70* 3' flanking, mini-white, P-element 5' and 3' ends.	C. Thummel[a]
pUChsΔ2,3	7.3 kb, pUC18, 3.6 kb P element coding sequences, *hsp70* promoter and *ry* 3' flanking	Rio and Rubin (1985)
pBluescript KS(-)	3.0 kb derived from pUC19 Amp^R	Stratagene
pMSL1-5.9	pBluescript KS, *Acc*I *msl-1* genomic fragment	J. Lucchesi[b]
pGEX	4.97 kb containing pBR322 *ori*, *tac* promoter, Amp^R, *lacIq*, glutathione S-transferase coding sequence	Pharmacia
pETM1[d]	8.71 kb, pET-21d (+) containing T7 *lac* promoter, *ori* Amp^R, f1 *ori*, *lac*I, with *msl-1* coding sequence	J. Lucchesi
pETM2[d]	7.76 kb, pET-21d (+) containing T7 *lac* promoter, *ori* Amp^R, f1 *ori*, *lac*I, with *msl-2* coding sequence	J. Lucchesi
pBS/FLAG-C-term[d]	4.2 kb, pBsII KS$^-$ containing 1.2 kb *Sac*I/*Xba*I C-terminal coding sequence of *msl-1* (aa 705 - 1039) and a *Eco*RI/*Sac*I FALG linker	This study
pFC[d]	10.11 kb, pCaSpeR-hs containing 1.2 kb *Sac*I/*Xba*I C-terminal coding sequence (aa 705 - 1039) and a *Eco*RI/*Sac*I FALG linker	This study
pFM5'Δ[d]	9.72 kb, pCaSpeR-hs containing 0.6 kb N-terminal coding sequence of *msl-1* (aa 85 - 263) and a FALG linker	F. Miller[c]
pBS/MOF[d]	5.5 kb pBS KS$^-$ containing 2.5 kb *mof* soding sequence	This study
pLP1[d]	11.36 kb, pCaSpeR-hs containing 2.5 kb *mof* coding sequence	This study
pGEX4T-1/MOF[d]	7.45 kb, pGEX4T-1 containing 2.5 kb *mof* coding sequence	This study
pGEX4T-3/MOF[d]	7.45 kb, pGEX4T-3 containing 2.5 kb *mof* coding sequence	This study
pETM1-N-term[d]	6.15 kb, pET-21d (+) containing 0.8 kb *msl-1* N-terminal coding sequence (aa 1 - 263)	This study
pETM2-N-term[d]	6.08 kb, pET-21d(+) containing 0.7 kb *msl-2* N-terminal coding sequence (aa 1 - 240)	This study

[a] C. Thummel, University of Utah, USA
[b] J. Lucchesi, Department of Biology and Graduate Program in Genetics and Molecular Biology, Emory University, USA
[c] M. Scott, Institute of Molecular BioSciences, Massey University, New Zealand
[d] Plasmid maps are listed in Appendices Section

2.3.4 2 x YTA Medium (pH 7.0)

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

2.3.5 Antibiotics and Medium Additives

170 μg/ml of ampicillin was added to LB, LB agar (section 2.3.1), and 2 x YTA (section 2.3.4) when required. 25 μg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) in dimethylformamide and 25 μg/ml of Isopropylthio-β-D-galactoside (IPTG) were added to LB agar when required. 120 μg/ml of IPTG was added to 2 x YTA when required.

2.4 MAINTENANCE OF BACTERIAL CULTURES

3 ml of LB (section 2.3.1) was inoculated from a single colony using a loop and grown overnight at 37°C. 700 μl of this culture and 300 μl of sterile glycerol were combined in a sterile cryotube and stored at -70°C.

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

2.5 SYNTHETIC OLIGONUCLEOTIDES

Synthetic oligonucleotides used in this study are described in Table 2.

Table 2. Synthetic Oligonucleotides

Oligonucleotide	Sequence 5' to 3'	Use
Sequencing Primers		
pUC forward	GGAAACAGCTATGACCATG	Sequencing of pBS/MOF N-terminus
pUC reverse	GTAAAACGACGGCCAGT	Sequencing of pBS/MOF C-terminus
VE3	ACTACTGAAATCTGCC	Sequencing of pFC N-terminus
Oligonucleotide Linkers		
flag/cterm/top	AATTCACCATGGACTACAAGGACGAC GATGACAAGGAGCT	Forms linker with flag/cterm/bot
flag/cterm/bot	CCTTGTCATCGTCGTCCTTGTAGTCC ATGGTG	Forms linker with flag/cterm/top
PCR Primers		
mof5	GTTAGAATTCACAATGTCTGAAGCGG AGCTGG	PCR amplification of 2.5 kb *mof*
mof3	GTTGGATATCTAGCCGGAATTTCCCG GAG	PCR amplification of 2.5 kb *mof*

2.6 BUFFERS AND SOLUTIONS

All solutions were made up to the appropriate volume with MilliQ H_2O, autoclaved and stored at room temperature unless otherwise stated.

2.6.1 Solutions for Agarose Gel Electrophoresis

2.6.1.1 10 x Gel Loading Dye

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

2.6.1.2 1 x TAE Buffer (Tris Acetate EDTA Buffer)

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

2.6.1.3 1 x TBE Buffer (Tris Borate EDTA Buffer)

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

2.6.2 Solutions for Preparation of DNA Plasmids

2.6.2.1 GTE (Glucose/Tris/EDTA)

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

2.6.2.2 TE Buffer (Tris EDTA Buffer, pH 8.0)

TE buffer contained 10 mM Tris-HCl and 1 mM Na_2EDTA.

2.6.2.3 Phenol (Tris Equilibrated)

Phenol crystals were heated at 65°C until melted. The bottle was filled with deionised H_2O, shaken to form a fine emulsion and placed at 4°C until the phases separated. The upper phase was removed and hydroxyquinoline was added to a final concentration of 0.1 %. An equal volume of 0.5 M Tris-HCl (pH8.0) was added and mixed. After phase separation, the upper aqueous phase was removed. An equal volume of 0.1 M Tris-HCl was added, the solutions were mixed and the upper aqueous phase was removed. This step was repeated until the aqueous Tris-HCl phase had a pH of 8.0. The phenol was overlaid with an equal volume of 0.1 M Tris-HCl (pH 8.0) and stored at 4°C in a foil covered bottle. Phenol was not autoclaved.

2.6.2.4 Potassium Acetate (pH 4.8)

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

2.6.3 Solutions for Transformation

2.6.3.1 DnD

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

2.6.3.2 K-MES

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

2.6.3.3 Transformation Buffer (TFB)

TFB contained 10 mM K-MES; 45 mM $MnCl_2 \bullet 4H_2O$; 10 mM $CaCl_2 \bullet 2H_2O$; 3 mM $HCoCl_3$ and 100 mM KCl. TFB was filter sterilised and stored at 4°C.

2.6.4 Solutions for Quantitation of DNA

2.6.4.1 1 x TNE Buffer

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

2.6.4.2 Working Dye Solution B

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

2.6.5 Solutions for Microinjection

2.6.5.1 Injection Buffer (pH 6.8)

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

2.6.6 Solutions for Fly Protein Extraction

2.6.6.1 2 x Laemmli Buffer (pH 6.8)

Laemmli buffer contained (g/L): Tris base, 15.2; SDS, 1.0; 20 % glycerol (v/v) and 2 % β-mercaptoethanol (v/v). Laemmli buffer was filter sterilised and stored at 4°C.

2.6.6.2 RIPA Buffer

RIPA buffer contained 50 mM Tris-HCl (pH 8.5); 300 mM NaCl; 1% Nonidet P40 (v/v); 0.5 % sodium deoxycholate (w/v) and 0.1 % SDS (v/v). RIPA buffer was not autoclaved.

2.6.6.3 Modified RIPA Buffer (ΔRIPA)

Modified RIPA buffer contained 50 mM Tris-HCl (pH 8.5); 300 mM NaCl and 1% Nonidet P40 (v/v). Modified RIPA buffer was not autoclaved.

2.6.6.4 10 x NHB Buffer

10 x NHB buffer contained 150 mM HEPES (pH 7.6); 50 mM $MgCl_2$; 5 mM EGTA; 100 mM KCl; 1mM EDTA and 10 mM DTT.

2.6.7 Solutions for Immunoprecipitation

2.6.7.1 10 x Immunoprecipitation Buffer (IP Buffer)

10 x Immunoprecipitation Buffer contained 250 mM HEPES; 1 M NaCl; 50 mM $MgCl_2$; 1mM EDTA and 1 % Nonidet P40 (v/v).

2.6.8 Solutions for Affinity Chromatography

2.6.8.1 10 x PBS (Phosphate-Buffered Saline)

10 x PBS contained 1.4 M NaCl; 27 mM KCl; 101 mM Na_2HPO_4 and 18 mM NaH_2PO_4 (pH 7.3).

2.6.8.2 1 x TBS (Tris-Buffered Saline, pH 7.4)

1 x TBS contained 50 mM Tris-HCl and 150 mM NaCl.

2.6.8.3 Glutathione Elution Buffer

Glutathione elution buffer contained 10 mM Glutathione and 50 mM Tris-HCl (pH 8.0). Glutathione elution buffer was not autoclaved and was stored in 3 ml aliquots at -20°C.

2.6.8.4 FLAG Elution Buffer

FLAG elution buffer contained 1.25 µM FLAG peptide; 50 mM Tris-HCl and 150 mM NaCl. FLAG elution buffer was not autoclaved and was stored in 1 ml aliquots at -20°C.

2.6.9 Solutions for Running SDS-PAGE

2.6.9.1 6 x Sample Buffer

6 x Sample buffer contained 0.35 M Tris-HCl; 30 % glycerol (v/v); 0.35 M SDS; 0.6 M DTT and 0.012 % bromophenol blue (w/v). 6 x sample buffer was not autoclaved and was stored in 1 ml aliquots at -20°C.

2.6.9.2 5 x Electrode Buffer

5 x Electrode buffer contained (g/L): Tris base, 15; glycine, 72 and SDS, 5. 5 x Electrode buffer was not autoclaved and was stored at 4°C.

2.6.10 Solutions for Western Blots

2.6.10.1 Transfer Buffer

Transfer buffer contained 25 mM Tris base; 192 mM glycine and 20 % methanol (v/v). Transfer buffer was not autoclaved.

2.6.10.2 Blocking Buffer

Blocking buffer contained 10 % blocking reagent (w/v); 20 mM Tris base; 500 mM NaCl and 0.05 % Tween-20 (v/v). Blocking buffer was made immediately before required.

2.6.10.3 Antibody Buffer

Antibody buffer contained 1 % blocking reagent (w/v); 20 mM Tris base; 500 mM NaCl and 0.05 % Tween-20 (v/v). Antibody buffer was made immediately before required.

2.6.11 Solutions for Coomassie Blue Staining

2.6.11.1 Coomassie Blue Staining Solution

Coomassie blue staining solution contained 0.25 % Coomassie brilliant blue (w/v); 45 % methanol (v/v) and 9 % acetic acid (v/v). Coomassie blue staining solution was not autoclaved.

2.6.11.2 Destaining Solution

Destaining solution contained 45 % methanol (v/v) and 9 % acetic acid (v/v). Destaining solution was not autoclaved.

2.6.12 Solutions for Silver Staining

2.6.12.1 Farmer's Reagent

Farmer's reagent contained 0.3 % $Na_2S_2O_3 \bullet 5H_2O$ (w/v); 0.15 % $K_3Fe(CN)_6$ (w/v) and 0.05 % Na_2CO_3. Farmer's reagent was not autoclaved.

2.6.12.2 Silver Nitrate Solution

Silver nitrate solution contained 0.1 % $AgNO_3$ (w/v). Silver nitrate solution was made immediately before required.

2.6.12.3 Developing Solution

Developing solution contained 2.5 % Na_2CO_3 (w/v) and 0.05 % formaldehyde. Developing solution was made immediately before required.

2.7 MAINTENANCE OF FLY STOCKS

Two sets of stocks were kept separately at 18°C and 22°. Every four weeks (for 22°C stocks) and every six weeks (for 18°C stocks) flies were transferred into new cornmeal agar vials. When required, flies were expanded in 100 ml bottles and kept at 25°C.

2.7.1 Fly Media

2.7.1.1 Cornmeal Agar

6.4 g agar (Davis), 56 g cornmeal, 19 g yeast and 600 ml H_2O were combined and heated. The porridge was constantly stirred until boiling. 77 g sugar and 2 g methyl paraben dissolved in 20 ml of ethanol were added. Stirred further and poured into 30 ml vials (Labserve), 100 ml Schott bottles or 60 x 15 mm tissue culture dishes (Corning).

2.7.1.2 Formula 4-24 (Carolina Biological Supply Company)

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

2.7.2 Collection of Virgin Females

Virgin females were collected from bottles in which flies were beginning to emerge. Bottles were cleared of emerged flies and incubated at 25°C for no more than 8 hours. Female flies were collected after the incubation and kept in vials until required.

2.7.3 Setting Fly Crosses

Five virgin females and five males were mated in a cornmeal agar vial. Adult flies were either discarded or transferred to a new vial after five days. Progeny emerged 5 to 7 days later.

2.7.4 Fly Stocks and Transgenic Fly Stocks

Fly stocks used in this study are described in Table 3.

2.8 PREPARATION OF PLASMID DNA

2.8.1 Small Scale Preparation of Plasmid DNA

Small scale preparation of bacterial plasmids were carried out according to the method of Birnboim and Doly (1979) and Birnboim (1983). 3 ml sterile LB was inoculated with a single colony and grown to saturation with moderate shaking at 37°C overnight. 1.5 ml of cells were centrifuged 1 minute at 13,000 rpm in a Heraeus Biofuge 13. The supernatant was removed and the pellet was resuspended in 100 µl GTE. 200 µl NaOH (0.2M)/SDS (1%) solution was added and mixed, and the solution was placed on ice for 5 minutes. 150 µl KAc was then added to the solution and vortexed at maximum speed for 2 seconds. Samples were allowed to sit on ice for a further 5 minutes and were centrifuged at 13,000 rpm for 4 minutes to pellet cell debris and chromosomal DNA. The supernatant was

Table 3. Drosophila melanogaster Fly Stocks

Fly Strains	Use	Source
yw	Microinjection recipient strain	M. Ashburner[a]
w^{1118}	Microinjection recipient strain	V. Pirrotta[b]
yw; L^2/CyO, Cy pr cn^2 y^+	Determination of chromosomal linkage	M. Ashburner
w; In(3LR)TM3, Sb ry^{rk}e/In(3LR)TM6, Tb e	Determination of chromosomal linkage	M. Ashburner

Transgenic Fly Stocks

Fly stock	Chromosomal linkage	Source
AK1: {*w+*}*yw*	?	A. Knox[c]
AK12^{41}: {*w+*}*yw*	?	A. Knox
AK12^{36}: {*w+*}*yw*	?	A. Knox
AK12^{38}: {*w+*}*yw*	?	A. Knox
SC1: {*w+*}*yw*	?	S. Cleland[d]
SC8: {*w+*}*yw*	3rd	S. Cleland
HF4: {*w+*}*yw*	3rd	H. Fitzsimons[e]
HF1 (MSL-3): {*w+*}*yw*	3rd	H. Fitzsimons
FMS: {*w+*}*w*	2nd	J. Lucchessi[f]
ANT: {*w+*}*yw*	2nd	A. Poole[d]
hsp70-msl-2; yw	X	M. Kuroda[g]
hsp70-mle; yw	3rd	M. Kuroda
FC8: {*w+*}*yw*	2nd	This study
FMΔR (FM): {*w+*}*yw*	3rd	This study
LP1 (MOF): {*w+*}*yw*	2nd	This study
SF3 (Rec. AK12): {*w+*}*yw*	3rd	This study
Rec. MSL-3; MLE: {*w+*}*yw*	3rd	This study
Rec. FC8; MOF: {*w+*}*yw*	2nd	This study
Rec. MLE; MOF: {*w+*}*yw*	3rd	This study

[a] M. Ashburner, Department of Genetics, Cambridge University, Cambridge, U.K.
[b] V. Pirrotta, Baylor College of Medicine
[c] A. Knox, Wellcom/CRC Institute, Cambridge, U.K.
[d] Massey University, Palmerston North, New Zealand
[e] H. Fitzsimons, Auckland Medical School, Auckland, New Zealand
[f] J. Lucchesi, Department of Biology and Graduate Program in Genetic and Molecular Biology, Emory University, USA
[g] M. Kuroda, Howard Hughes Medical Institute, Baylor College of Medicine, USA

carefully transferred to a new microcentrifuge tube and mixed with 800 µl absolute ethanol. Plasmid DNA was pelleted by centrifugation and washed with 1 ml 70 % ethanol. DNA samples were dried under vacuum in a SpeedVac concentrator (Savant), and then were resuspended in 30 µl TE and stored at -20°C.

Bio-Rad Quantum Prep Plasmid Miniprep Kit was also used following the manufacturer's instructions. This yielded high quality plasmid DNA which was subsequently used for automatic sequencing, microinjection and cloning.

2.8.2 Large Scale Preparation of Plasmid DNA

Large scale preparations were carried when large quantities of plasmid DNA were required for sequencing, microinjection, cloning and plasmid DNA stock. Qiagen Plasmid Maxi Kit was used according to the manufacturer's instructions for the above purposes.

2.9 AMPLIFICATION OF DNA BY POLYMERASE CHAIN REACTION

Two mixes were prepared on ice, each containing the following ingredients. Mix 1: 1µl of 10 µM reverse primer, 1µl of 10 µM forward primer, 2 µl of 2 mM dNTPs, 2 µl of genomic DNA and 11.5 µl of sterile H_2O. Mix 2: 2µl of 10 x buffer (Mg^{2+}) and 0.5 µl of *Pwo* DNA polymerase (Boehringer Mannheim). The two mixes were combined immediately before placing in a thermal cycler. The following cycles were used for amplifying DNA:

Cycle 1, denaturation at 94°C for 2 minutes; cycle 2-4, denaturation at 94°C for 30 seconds, annealing at 48°C for 45 seconds and extension at 72°C for 3 minutes; cycle 5-31, denaturation at 94°C for 30 seconds, annealing at 58°C for 45 seconds and extension at 72°C for 3 minutes; cycle 32, extension at 72°C for 7 minutes; hold at 4°C.

2.10 RESTRICTION ENDONUCLEASE DIGESTION OF DNA

2.10.1 Restriction Endonucleases

All enzymes were from New England Biolabs (NEB) with the exception of *Asp*718 which was from Boehringer Mannheim.

20 Units/µl: *Bam*HI, *Eco*RI, *Eco*RV, *Pst*I, *Sac*II, *Xba*I, *Xho*I

15 Units/µl: *Bsi*WI

10 Units/µl: *Asp*718, *Hin*dIII, *Not*I, *Sac*I, *Stu*I

5 Units/µl: *Sph*I

4 Units/µl: *Nco*I

2.10.2 Restriction Endonuclease Digestion Analysis

Restriction endonuclease digestions were performed in commercially prepared buffer specifically matched to the restriction enzymes used following a transformation and a small scale plasmid DNA preparation. 2 µl of DNA, or 4 µl of DNA if transformed in BL21 *E. coli* cells (Table 1), was mixed with 10 units of the appropriate restriction enzyme(s) which constituted less than 1/10 of the final volume of the digestion. 1µl of 500 µg/ml RNase was added to each digest. BSA was also added to a final concentration of 0.1 mg/ml when required by a specific restriction enzyme. Digestions were incubated at 37°C (with the exception of *Bsi*WI, at 55°C) for 2-3 hours. 10 x gel loading dye was added to each digest which were size fractionated on an agarose gel. DNA sizes were determined by comparing to λ *Hin*dIII/*Sac*II ladder.

2.10.3 Preparative Restriction Digestion

5 to 40 µg DNA was digested using 3-10 Units of the appropriate enzyme(s) per µg of DNA. 200 - 500 ng of digested DNA was run on an agarose gel to determine whether complete digestion had been achieved. If DNA was only partially digested, more enzyme was added and digestion was continued overnight. When digestion was completed, the enzyme was heat inactivated at 65°C for 20 minutes. If the enzyme could not be heat inactivated, a Phenol/Chloroform extraction was carried out (Section 2.12.2).

2.10.4 Partial Restriction Digestion

To determine optimal conditions for partial restriction digestion, plasmid DNA was digested with 20 units of *Bam*HI per 0.5 µg DNA for 10, 20 and 30 minutes. Using optimal conditions, a larger quantity of DNA was digested and run on an agarose gel. The appropriate DNA fragment was then excised and gel purified (Section 2.12.4).

2.11 AGAROSE GEL ELECTROPHORESIS

DNA was size fractionated by electrophoresis on a 1 % agarose gel (w/v) in 1 x TBE buffer or on a seaplaque agarose gel in 1 x TAE buffer. DNA samples containing 1 x loading dye were loaded into the appropriate wells alongside 10 µl of λ *Hin*dIII and *Sst*II ladder. Gel electrophoresis was carried out in a Horizon or BioRad minigel apparatus for 1 hour at 90 Volts. The gel was then stained for 15 minutes in 2 µg/ml ethidium bromide and destained in water. The gel was visualised under short wave UV light and photographed using a Gel Documentation System (Alpha Innotech).

2.12 DNA PURIFICATION

2.12.1 Purification of DNA after PCR

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

2.12.2 Phenol/Chloroform Extraction of DNA after Restriction Digestion

An equal volume of a 1:1 mixture of Tris equilibrated phenol and chloroform were added to the same volume of DNA sample and shaken for five minutes. The sample was centrifuged at 13000 rpm for five minutes. The upper aqueous phase was transferred to a fresh microcentrifuge tube and ethanol precipitated.

2.12.3 Ethanol Precipitation of DNA

To precipitate DNA, 1/10th volume of 3 M NaAc and 2 volumes of absolute ethanol were added to the DNA sample and mixed. The sample was kept at -70°C for 1 hour or -20°C overnight, and was pelleted at 13000 rpm for 15 minutes. The pellet was washed with 70 % ethanol, dried under vacuum for five minutes and resuspended in the appropriate amount of TE.

2.12.4 DNA Extraction from Seaplaque Agarose

After the appropriate restriction digest, DNA was fractionated on a 1 % 1 x TAE seaplaque agarose gel. The detection was similar to the description in section 2.11. The desired fragment was excised with a scalpel under UV light. The Qiagen QIAquick Gel Extraction Kit was then used according to the manufacturer's instructions.

2.13 DETERMINATION OF DNA CONCENTRATION

2.13.1 Determination by Comparison to DNA Ladder

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

2.13.2 Spectrophotometric Determination of DNA Concentration

DNA concentration was determined by measuring the UV absorbance of a diluted sample at 260 nm. An O.D. of 1 corresponds to 50 µg/ml of double stranded DNA. The absorbance reading was multiplied by 50 and the dilution factor to give the DNA concentration in µg/ml.

2.13.3 Fluorometric Determination of DNA Concentration

The Hoefer Scientific TKO 100 Fluorometer was adjusted to zero against 2 ml of Working Dye Solution B, and then calibrated by adding 2 µl of 100 µg/ml calf thymus DNA to give an adjusted reading of 100. 2 µl of sample DNA was added to 2 ml Working Dye Solution B and measured. The reading indicated was the concentration of the DNA in ng/µl.

2.14 SUBCLONING

2.14.1 Filling in 5' Overhangs with Klenow

Following restriction enzyme digestion and heat inactivation (or purification, Section 2.12), 1 U/µg Klenow (NEB) with 10 x Klenow buffer and 33 µM of dNTPs were added

to 50 µg/ml DNA. The solution was incubated at 25°C for 15 minutes and then heat inactivated at 75°C for 10 minutes.

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

Following restriction enzyme digestion and heat inactivation (or purification, Section 2.12), 1 U/µg Mung Bean Nuclease (NEB) and 1 mM $ZnSO_4$ were added to 100 µg/ml DNA. The solution was incubated at 30°C for 30 minutes and heat inactivated at 75°C for 10 minutes.

2.14.3 Removal of 5' Phosphate Groups from Vector DNA

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

2.14.4 Annealing of Oligonucleotides

Oligonucleotides were resuspended at 100 µM in sterile TE. 25 µg of the two complementary oligonucleotides was combined with 5 M NaCl (final 75 µM) and the volume was made up to 500 µl with sterile H_2O. The solution was incubated at 90°C for 10 minutes then removed from the heat and left until cooled to room temperature.

2.15 DNA LIGATION

Ligations of DNA fragments were carried out in 1 x ligase buffer with 0.5 µl of T4 DNA ligase (New England Biolabs) in a final volume of 10 - 25 µl. The mixture contained 200 ng vector and insert DNA at a 2 - 3 fold molar excess of insert to vector. A 200 - 400 fold

molar excess was used when annealed oligonucleotides were ligated to vectors. Vector DNA was treated with Calf Intestinal Alkaline Phosphatase to prevent self religation unless ligated with annealed oligonucleotides. Ligations were incubated at 18°C overnight and could be used directly for transformation.

2.16 TRANSFORMATION OF PLASMID DNA

2.16.1 Transformation Using *Esherischia coli* DH5a Cells

Plasmids were transformed into *Esherischia coli* DH5α cells by the method of Hanahan (1983). DH5α cells were streaked out for single colonies from a glycerol stock onto an LB plate and incubated at 37°C overnight. Several 2 mm diameter colonies were picked off the plate and dispersed into 1 ml SOB medium by vortexing. The cells were inoculated into a 1 L Erlenmeryer flask containing 30 ml of SOB and incubated at 37°C until $O.D._{550}$ reached 0.45 - 0.55. The culture was poured into an SS34 tube and chilled on ice for 15 minutes. The cells were pelleted by centrifugation in a Sorvall RC5C centrifuge at 3000 rpm for 15 minutes at 4°C. The supernatant was removed and the cells were resuspended in TFB to 1/3 of the original culture volume. After sitting on ice for a further 15 minutes, cells were pelleted as previously described and resuspended in TFB to 1/12.5 of the original culture volume. 7 µl of DnD was added per 200 µl of cell suspension and mixed by swirling the tube for several seconds. The cells were incubated on ice for 10 minutes and the second aliquot of DnD was added. After 10 more minutes incubation, 1 µl of ligation sample was added to a 210 µl aliquot of cells in a chilled 15 ml polypropylene tube (Falcon) and swirled to mix. 1 µl of pUC118 monomer was also added to 210 µl of cells as a positive control. All the mixtures were incubated on ice for 30 minutes and heat shocked by immersing in a 42°C water bath for 90 seconds. After chilling on ice, 800 µl of SOC was added to each tube and the cells were incubated at 37°C with moderate shaking

for 1 hour. 100 µl of each culture was spread onto an LB plate with appropriate antibiotics and medium for blue/white selection. The remaining culture was concentrated 10-fold and spread onto another plate with appropriate medium. The plates were left to dry and then incubated at 37°C overnight. A transformation frequency of 2 x 10^8 amp^R colonies/µg pUC was usually achieved. This frequency was an indication of how efficiently plasmids were transformed into *E. coli* cells.

2.16.2 Transformation Using *Esherischia coli* BL21 Competent Cells

Plasmids were transformed into *Esherischia coli* BL21 when reduction of proteolysis was required for protein induction, extraction and purification. 100 µl of competent BL21 cells were taken from a frozen stock provided by Dr. C. Day, then mixed with 5 -10 µl of appropriate plasmids and incubated on ice for 5 minutes. The culture was spread onto a pre-warmed (37°C) LBamp plate and left to dry before incubating at 37°C overnight.

2.17 DNA SEQUENCING

Automated sequencing was employed for analysing DNA sequences and was provided by Massey University DNA Analysis Service. Both synthetic and 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 run on a ABI Prism 377 DNA sequencing machine.

2.18 MICROINJECTION OF *D. melanogaster* EMBRYOS

2.18.1 Co-Precipitation of Plasmid DNA

40 µg of plasmid DNA and 12 µg of pUCChsπΔ2,3 (Table 1) were mixed and make up to a volume of 100 µl with TE. The DNA was ethanol precipitated and resuspended in 100 µl of injection buffer. The DNA solution was centrifuged at 13000 rpm for 20 minutes in a bench-top microcentrifuge prior to use to remove any particles that might block the needle. Approximately 3 µl was loaded into a Femtotip (Eppendorf) using a microloader (Eppendorf) and a pipette.

2.18.2 Collection of Embryos

200 - 300 *y w* flies were placed in a plastic tripour beaker which was inverted onto a 60 x 15 mm cornmeal-agar plate with a blob of yeast paste on it. These beakers were kept in the dark at 18 - 22°C for three days to allow flies to acclimatise. A fine wet paintbrush was used to collect fresh embryos off the plates which were constantly changed every 30 minutes during microinjection. The embryos were transferred and placed onto two 2 cm rows of double sided tape (Scotch 3M) on a microscope slide.

2.18.3 Dechorionation of Embryos

The chorions of embryos were teased away by rolling the embryos with a pair of fine forceps along the double sided tape. Dechorionated embryos were aligned on one edge of the tape, then the excess tape up to 3 mm from the edge was removed at the end of dechorionation. Five minutes was allowed for as many embryos (approximately 30) as possible to be dechorionated. These embryos were then dehydrated.

2.18.4 Dehydration of Embryos

The dechorionated embryos were placed into a glass petri dish containing silica gel for dehydration. Dehydration was carried out for up to 5 minutes depending on temperature, humidity and other unknown factors that affected the softness or firmness of the

embryos. Various dehydration times were tested until dehydrated embryos were adequate for microinjection. After dehydration the embryos were immediately covered in halocarbon oil (Series 700, Halocarbon Products Corporation) or paraffin oil (Whiterex 334, Pualing Industries Ltd) to prevent further dehydration.

2.18.5 Microinjection of Embryos

Microinjection was carried out according to the method of Spardling 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 microinject the DNA into the posterior pole of the embryo where the germ cells eventually would form. After microinjection the slides carrying microinjected embryos were placed on a rack in a container with moist paper towels. The container was incubated at 18°C for 24 hours and then 22°C for a further 24 hours. The surviving larvae were transferred to a vial of Formula 4-24 food with maximum of 40 larvae per vial. The vials were incubated at 25°C until the adults hatched. These flies were termed the G0 generation.

2.18.6 Collecting and Crossing Adult Survivors

When G0 pupae started to emerge to become adults, vials were checked twice daily to ensure not only males but also virgin females were collected. G0 flies were crossed to the *y w* recipient stock in separate vials. Each G0 female was mated with two males and each G0 male was mated with five virgin females. The adults were removed after one week and the vials incubated at 25°C until the progeny (G1) hatched.

2.18.7 Identification of Transformants and Establishing a Transformant Stock

When G1 pupae started to emerge, vials were examined twice daily for transformants. Transformants (w^+) had eye colour ranging from pale yellow to wild type red.

Transformant males were crossed with five *y w* virgin females. If no transformant males were obtained, a virgin transformant female was mated with two *y w* males. Heterozygous G2 males were individually crossed with *y w* females to avoid the problem of multiple insertions as flies in the same vial might have different copies of inserts. Progeny (G3) emerged at a 1:2:1 ratio of homozygous w^+: heterozygotes: homozygous *w*. A single homozygous male and a homozygous virgin female were mated to establish a homozygous transgenic stock.

2.19 DETERMINATION OF CHROMOSOME LINKAGE OF INSERT

Crosses were carried out to determine if the DNA insertion was on either the 2nd or 3rd chromosome. Five male flies from each transformant line were mated separately with five *w; In(3LR)TM3, Sb* ry^{rk} *e/In(3LR)TM6, Tb e* and five *y w;* L^2*/CyO, Cy pr* cn^2 y^+ virgin females. *Sb* w^+ and *Cy* w^+ male progeny (F1) were further crossed with virgin *y w* females. If the insertion was on the 2nd chromosome, all the *Cy* progeny (F2) would be *w*. If the insertion was on the 3rd chromosome, all the *Sb* progeny would be *w*.

To determine the DNA insertion was on the X chromosome, five w^+ males from each transformant line were mated with five *y w* virgin females. If the insertion was on the X chromosome, all the F1 males would be *w*, and all the females would be w^+.

2.20 MAKING OF RECOMBINANT *D. melanogaster*

When two genes were required to be on the same chromosome for the purpose of protein co-expression, the following crosses were carried out to produce recombinant flies. Two different transformant lines with inserts on the same chromosome were crossed. The progeny (F1) would have one gene on the one homologue and the other gene on the other

homologue. Virgin F1 females were collected as crossing-over only occurred in female flies. If the insertions were on the 2nd chromosome, F1 females were mated with *y w; L^2/CyO, Cy pr cn^2 y* males. If the insertions were on the 3rd chromosome, F1 females were mated with *w; In(3LR)TM3, Sb ry^{rk} e/In(3LR)TM6, Tb e* males. A recombined chromosome which had both copies of genes would result in having a dark-red eye colour. These dark-red eye F2 males and virgin females with either heterozygous *Cy* or *Sb* were selected and mated. *Cy* and *Sb* were balancers and therefore further crossing-over was prevented. Non-*Cy* or non-*Sb* males and virgin females (F3) were selected and mated to establish a stock of a recombinant line.

2.21 HEAT SHOCK OF *D. melanogaster*

Appropriate homozygous or cross-mated transformants were kept in vials and incubated at 25°C overnight. These vials were moved to a 30°C room and incubated for 24 hours. Flies were then heat shocked for 1 hour daily by submersing vials in 37°C water to 0.5 mm from the top of the vials. Vials were kept at 30°C when heat shock was not applied. Parents were removed after 5 days and heat shock was continued until no more progeny emerged. Progeny were scored daily by counting the numbers of males against females, which were then discarded.

2.22 CHEMICAL MUTAGENESIS OF *D. melanogaster*

To induce mutations, flies were fed ethyl methane sulfanoic acid (EMS), a chemical mutagen which frequently induces a single base pair mutation. EMS was made up to 5 % sucrose to a final concentration of 25 mM. 200 appropriate transgenic male flies were kept in an empty Schott bottle with no food or water for 8 -12 hours before transferring to a bottle with a filter paper placed at the bottom and soaked with 800 µl of

EMS/sucrose solution. Flies were left in the fume hood for 20 - 24 hours. Flies were transferred to the cornmeal-agar bottles with 12 - 15 treated males mated with the same number of virgin *w* or *y w* females. Heat shocks might be applied depending on the conditions required for the experiment. Parent flies were discarded after five days and progeny were scored as males against females.

2.23 PROTEIN EXTRACTION

All the fly protein extractions were carried out at 4°C unless otherwise stated.

2.23.1 *E. coli* Protein Extraction

Frozen *E. coli* cells containing required plasmid DNA were streaked out on an LB plate and incubated at 37°C overnight. A single colony was inoculated in 5 - 100 ml of LB medium and incubated at 37°C for 12 - 15 hours with moderate shaking. The culture was diluted 100-fold into an appropriate flask containing fresh pre-warmed 2 x YTA medium and incubated at 22°C for 16 hours with gentle shaking. The cells were then induced by adding IPTG to a final concentration of 0.25 mM. Incubation was continued for an additional 4 - 5 hours to have maximum protein expression before the culture was transferred to GSA tubes and pelleting the cells in a Sorvall RC5C centrifuge at 7000 rpm for 10 minutes at 4°C. Supernatant was drained and cells were resuspened in 1 x PBS to 1/20 of the original culture volume. Suspended cells were transferred to SS34 tubes on ice and disrupted with three 10 seconds bursts from a probe-sonicator. The sonicate was immediately centrifuged at 10000 rpm for 10 minutes at 4°C and either the supernatant or the 1 x PBS-resuspended pellet was saved in aliquots depending on the solubility of the protein. Aliquots were frozen and stored at -20°C until use.

2.23.2 Total Fly Protein Extraction

Homozygous transformants or progeny from a transformant cross were heat shocked at 37°C for 1 hour and then incubated at 25°C for four hours for maximum protein expression. Flies were collected and instantly frozen in liquid air. To make the protein markers, 20 frozen flies were homogenised in a 1 ml glass homogeniser (Kantes) with 1 ml of Laemmli buffer. To prepare a protein extract, approximately 200 frozen flies were homogenised in a 15 ml Dounce homogeniser with either 2 ml of RIPA or ΔRIPA buffer containing 1 x complete protease inhibitor (Boehinger Mannheim) for immunoprecipitation or affinity chromatography. Laemmli fly extract was boiled with 6 x sample buffer and stored at -20°C for up to a year. RIPA or ΔRIPA homogenate was transferred to a SS34 tube and centrifuged at 9150 rpm for 10 minutes. The supernatant was used for either immunoprecipitation or affinity chromatography. RIPA fly extract was stored at -20°C and thawed immediately before immunoprecipitation. ΔRIPA fly extract was made freshly each time prior to the affinity chromatography.

2.23.3 Cytoplasmic and Nuclear Extractions

200 flies were collected, heat shocked and frozen in a similar fashion to the method described in Section 2.23.2. These flies were homogenised in a 15 ml Dounce homogeniser with 5 ml of NHB buffer containing protease inhibitors. NHB homogenate was filtered through sterilised miracloth to a SS34 tube and centrifuged at 4000 rpm for 10 minutes. The supernatant was cytoplasmic protein extract. Cytoplasmic extract was stored at -20°C in aliquots. The pellet was resuspended in 1.8 ml of NHB buffer and incubated on ice for 1 hour. 200 µl of 4 M $(NH_4)_2SO_4$ was added dropwise to a final concentration of 0.4 M. The solution was transferred to a 11 x 32 mm Quick-Seal tube (Beckman) and centrifuged in a Beckman TLV-100 rotor at 50000 rpm for 30 minutes. The supernatant of this centrifugation was nuclear protein extract. Nuclear extract was stored at - 20 °C in aliquots.

2.24 PROTEIN ASSAYS

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

2.25 IMMUNOPRECIPITATION

All the immunoprecipitation procedures were carried out at 4°C. 200 μg of total fly protein from either a RIPA, cytoplasmic or nuclear extract was diluted to a final volume of 500 μl with 1 x IP buffer containing 100 μg of BSA and protease inhibitors. Depending upon the nature of the antibody, either washed Protein A agarose (Pharmacia) or Protein G sepharose (Sigma) beads were used for cross-link with the antibody. 2 - 10 μl of an antibody and 40 μl of beads were mixed and then diluted to 100 μl with 1 x IP buffer. The solution was incubated on a rocker for 1 hour before 500 μl of diluted protein extract was added. Incubation was continued for a further hour and the beads were centrifuged at 13000 rpm for 10 seconds. The supernatant was removed and the beads were washed with 50 μl of 0.1 x IP buffer then concentrated by centrifugation. The washing procedure was repeated then beads were resuspended to 20 μl with 0.1 x IP buffer. This sample could be used for SDS-PAGE (Section 2.27).

2.26 AFFINITY CHROMATOGRAPHY

2.26.1 Glutathione S-Transferase (GST) Affinity Chromatography

GST affinity chromatography was employed for the efficient purification of glutathione S-transferase fusion proteins produced using the pGEX expression vectors (Table 1). Some proteins which might associate with the fusion protein *in vivo* could also be co-expressed and then co-precipitated. 265 µl of 75 % Glutathione Sepharose 4B bead slurry (Pharmacia) was washed according to the manufacturer's instructions using 1 x PBS buffer. The bead slurry was transferred to a 10 ml disposable column (BioRad) and mixed with the *E. coli* cell extract containing approximately 135µg of appropriate GST fusion protein. The mixture was incubated on a rocker with gentle rotation at 4°C for one hour. The column was left to drain and a matrix was formed. The matrix was washed with 10 bead volumes of 1 x PBS buffer. 500 µg of total fly protein from a ΔRIPA extract of interest was added to the column. The salt concentration was kept at 160 mM by adding the appropriate volume of 1 x PBS buffer containing protease inhibitors. The beads were resuspended by inversion and again incubated at 4°C for one hour. The column was then drained and washed four times as previously described. 600 µl of glutathione elution buffer was added to the matrix and incubated at room temperature for 10 minutes. The eluate was collected and concentrated using a microconcentrator (Pall Filtron). The eluate was concentrated approximately 10-fold and should contain the fusion protein and proteins associated with that fusion protein.

2.26.2 FLAG Affinity Chromatography

The FLAG epitope is an octapeptide (DYKDDDDK) which is recognised by its monoclonal antibody. Anti-FLAG M2 affinity gel (Kodak) was used for the purpose of purifying FLAG-tagged fusion protein and proteins associated with the fusion protein. 300 µl of 20 % Anti-FLAG M2 affinity gel was transferred to a 10 ml disposable column

and washed with Glycine-HCl and 1 x TBS buffer according to the manufacturer's instructions. 1 mg of total fly protein from a ΔRIPA extract containing FLAG tagged fusion protein was added to the column and the affinity gel was resuspended by inversion. The salt concentration was kept at 150 mM by adding appropriate amount of 1 x IP buffer contained complete tablet protease inhibitors (Boehringer Mannheim). The mixture was incubated on a rocker at 4°C for one and a half hours. The column was allowed to drain and washed with 2 ml of 1 x TBS four times. 600 µl FLAG elution buffer was then applied to the column and incubated at room temperature for 10 minutes. The eluate was collected and concentrated approximately 10-fold using a microconcentrator.

2.27 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

6 x sample buffer was added to samples containing the appropriate proteins and which were then boiled for at least 5 minutes. 20 µl of denatured protein samples were loaded onto a 7.5 % or 10 % polyacrylamide gel and were separated by electrophoresis at 200 Volts for one hour in the presence of SDS (Laemmli, 1970). Mini-PROTEAN II electrophoresis cell (BioRad) was used according to the manufacturer's instructions. A lane of Kaleidoscope standard or high range SDS-PAGE standard (Bio-Rad) was run alongside sample proteins on each gel to determine the molecular weight of each protein.

2.28 POLYACRYLAMIDE GEL STAINING

2.28.1 Coomassie Blue Staining

Coomassie Blue Staining was used either for examining the intensity and the molecular weights of a protein sample before carrying out a Western Blot, or determining if the electrophoretic transfer of proteins to nitrocellulose membrane (3M) had occurred. At the completion of SDS-PAGE, the gel was stained using Coomassie Blue staining solution for

15 minutes and destained with Destaining solution for 20 minutes. The Destaining solution was replaced with fresh solution regularly until the blue background disappeared and protein bands were visualised. If necessary, the gel was photographed and then dried onto a piece of 3 M paper at 80°C using a gel dryer (Bio-Rad, model 583).

2.28.2 Silver Staining

Silver Staining was used when the intensity of a protein sample was too low for Coomassie Blue Staining to detect. At the completion of SDS-PAGE, the gel was fixed in 30 % ethanol/10 % acetic acid for three hours. If the gel had been Coomassie Blue stained, it was fixed in 10 % ethanol instead. The gel was transferred to fresh 10 % ethanol and left on a shaker for 15 minutes before three 10-minute washes with MilliQ H_2O were applied. The gel was then incubated in Farmer's reagent for 1 minute and washed with Milli-Q H_2O three times. The process was followed by incubating the gel in silver nitrate solution for 30 minutes and a quick wash with Milli-Q H_2O before submersing in developing solution. The gel was soaked in the developing solution until the desired amount of staining was achieved. The staining was then fixed in 1 % acetic acid and Milli-Q H_2O. If necessary, the gel was dried and photographed.

2.29 WESTERN BLOTTING

At the completion of SDS-PAGE, proteins were transferred to nitrocellulose membrane by electroblotting using a Mini Trans-Blot cell (Bio-Rad) with transfer buffer for 16 hours at 22 V. The membrane was blocked in the blocking buffer for 30 minutes then incubated with a primary antibody diluted with appropriate dilution factors for one hour at room temperature. After washing with 1 x TBS buffer, the membrane was incubated with horse radish peroxidase (HRP)-conjugate rabbit (Amersham, dilution 1: 3333), mouse (Amersham, 1: 3333) or rat (Boehringer Mannheim, 1:1000) secondary antibodies.

Specific protein bands were detected using the ECL system (Amersham) according to the manufacturer's instructions.

3. RESULTS

3.1 INVESTIGATION OF AN UNKNOWN FACTOR REQUIRED FOR DOSAGE COMPENSATION

To determine if there is an unknown factor required for dosage compensation, several assumptions have been made:

1. This unknown factor is associated with one or a few of the MSL proteins that lead to dosage compensation.
2. Absence of this unknown factor is sufficient to cause male-specific lethality.

3.1.1 Searching for the New Factor Using Chemical Mutagenesis

Chemical mutagenesis (Section 2.22) was performed on flies carrying the AK12 construct which are male-specific lethal (Section 1.11). The hypothesis was that if a mutation was induced within the gene of the unknown factor, the male lethality caused by the overexpression of MSL-1 C-terminal domain would either be suppressed or enhanced depending whether the factor inhibited or was required for dosage compensation. Recipient stock males were fed with EMS and crossed with a line of $AK12^{41}$ with severe male lethality (Table 3). No male survivors were found among approximately 15,000 heterozygous AK12 progeny screened. To enhance the sensitivity of the genetic screens, heat shock conditions were found where the male to female ratio was about 0.2. A SF3 line was made by recombination of $AK12^{36}$ and $AK12^{38}$ (Section 2.20) for a screen for suppressor mutation. The male/female ratio of the heterozygous and homozygous SF3 raised at 30°C and heat shocked at 37°C daily for one hour was 0.47 and 0 respectively. It is not known why the viability of heterozygous SF3 males is significantly higher than the viability of homozygous AK12 males (described in Section 1.11), as both have two

copies of *hsp.AK12*. One possibility is that one of the *hsp.AK12* genes in either $AK12^{36}$ or $AK12^{38}$ has become inactive after the recombination. Nevertheless, this approach of mutagenesis was abandoned with the identification of MOF, a new MSL required for dosage compensation (Hilfiker *et al.*, 1997).

3.1.2 Immunoprecipitation

MSL-1 and MSL-2 co-immunoprecipitate from male nuclear extract (Kelly *et al.*, 1995). If the unknown factor also forms a complex with MSL-1, it may co-immunoprecipitate with MSL-1 which may facilitate its identification. Before searching for the unknown factor, trials were carried out to see whether the system would work and also to determine which of the MSLs bind to MSL-1. Both MSL-1 and FMS lines (Table 3) were crossed with *hsp.*MSL-2, *hsp.*MSL-3 and *hsp.*MLE lines (Table 3) to produce heterozygous progeny. RIPA (Section 2.23.2) and nuclear fly extracts (Section 2.23.3) were prepared, and immunoprecipitation and western blots were carried out. Figure 3 shows that MSL-3 seems to be the only MSL which binds to FMS. However, MSL-3 did not appear to bind to full length MSL-1 (Figure 4). MSL-2 bound to full length MSL-1 in both RIPA and nuclear extracts (Figure 4). MLE bound to MSL-1 in RIPA extract, but it seemed to lose the binding ability if the nuclear extract method was used (Figure 4). Further experiments showed inconsistent binding of MSL-3 and MLE to MSL-1 and no conclusions could be made about whether or not MSL-3 and MLE bound to MSL-1. Moreover, although MSL-2 shows consistent binding to MSL-1, the signal is rather weak for further analysis. Therefore it was concluded that it would be almost impossible to co-purify the unknown factor using the method of immunoprecipitation.

3.1.3 Cloning of FLAG Tagged C-Terminal Domain for Affinity Chromatography

FLAG affinity chromatography is a very effective technique of purifying protein complexes from crude cell extracts (Hopp *et al.*, 1988; Section 2.26.2). If the unknown

Figure 3. Protein Purification Using Immunoprecipitation (I).

RIPA extracts (Section 2.23.2) from progeny of FMS line crossed with each of MSL-2, MSL-3 and MLE which had been heat shocked to induce protein synthesis, was incubated with either Protein A which was previously bound with anti-MLE (lane 2), or anti-MSL-2 (lane 3), or Protein G which was previously bound with anti-MSL-3 (lane 4) antibodies. Lane 1 was the control for the input extract only. The amount of input in lane 2, 3 and 4 are 20 % of lane 1. Western blot was performed with anti-MSL-1 antibody. Significant co-immunoprecipitation of FMS was only found with the anti-MSL-3 antibody (lane 4).

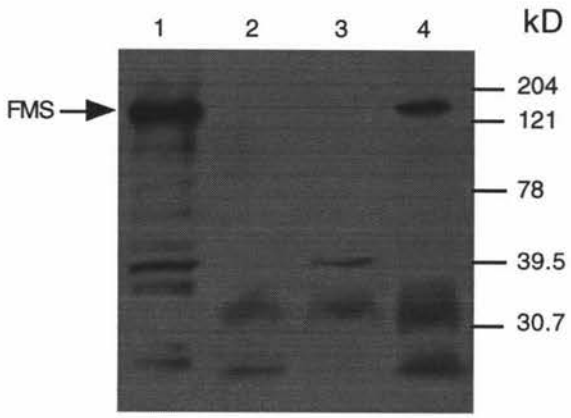
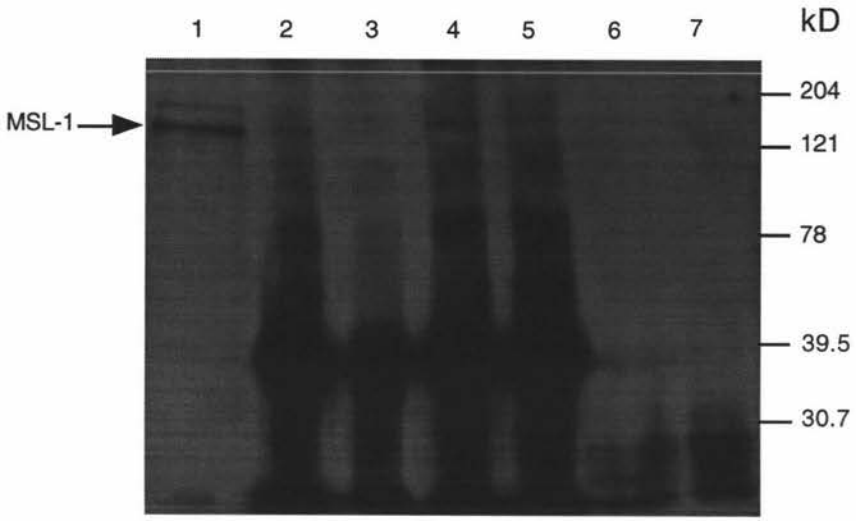

1
2
3
4
kD
FMS
204
121
78
39.5
30.7

Figure 4. Protein Purification Using Immunoprecipitation (II).

Two different types of fly extracts, RIPA (Section 2.23.2) and nuclear (Section 2.23.3), were made for this immunoprecipitation experiment. RIPA extracts from transformants of full length MSL-1 line (Section 3.3.1) which had been heat shocked to induce protein synthesis, were incubated with either Protein A which was bound with anti-MLE (lane 2); anti-MSL-2 (lane 4), or Protein G which was bound with anti-MSL-3 (lane 6). Similarly, nuclear extract was incubated with bound anti-MLE (lane 3), anti-MSL-2 (lane 5) and anti-MSL-3 (lane 7). Lane 1 was the control for the input extract only. The amounts of input in lane 2 - 7 were 20 % of lane 1. Western blots were performed with anti-MSL-1 antibody. Significant co-immunoprecipitation of MSL-1 was detected with the anti-MSL-2 and anti-MLE but not anti-MSL-3.


1
2
3
4
5
6
7
kD
MSL-1
204
121
78
39.5
30.7

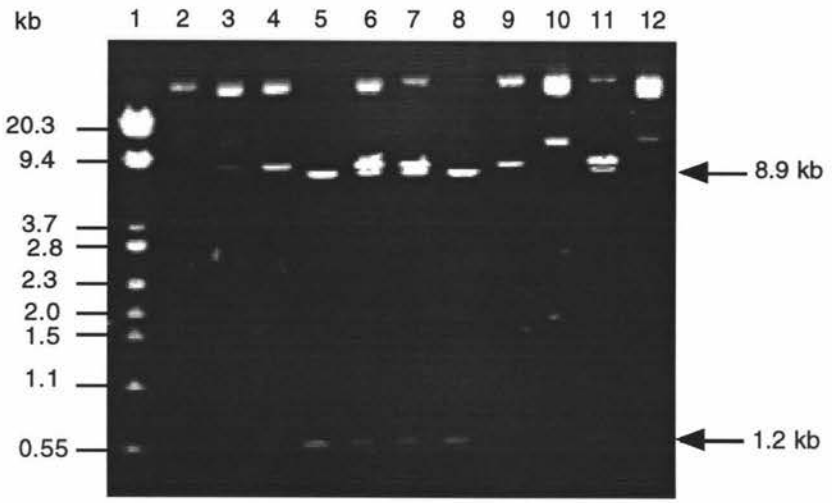
factor forms a complex with the C-terminal domain of MSL-1, the unknown factor should co-purify with FLAG.MSL-1 using FLAG affinity chromatography. Synthetic oligonucleotides which encode FLAG peptide preceded by a translation initiation codon (flag/cterm/top and flag/cterm/bot) were annealed (Section 2.14.4 & Table 2) to generate a linker with *Eco*RI/*Sac*I 5' overhang ends. pMSL1-5.9 (Table 1) was digested with *Sac*I and *Xba*I restriction enzymes (Section 2.10.2) and the 1.17 *Sac*I - *Xba*I fragment was gel purified (Section 2.12.4). The vector pCaSpeR-hs (Table 1) was also digested as described above except using *Eco*RI and *Xba*I restriction enzymes. These three DNA fragments (FLAG linker, MSL-1 3' end and pCaSpeR-hs vector) were pooled and ligated (Section 2.15). The ligated plasmids were then transformed into *E. coli* (Section 2.16), however, no transformants were obtained. To increase the chances of producing a transformant, the following changes to the cloning strategy were made:

1. A two-step cloning was used to increase the chance of a correctly ligated plasmid being selected. Instead of using pCaSpeR-hs as the vector, pBluescript (Table 1) was digested with *Eco*RI and *Xba*I, then ligated with FLAG linker and MSL-1 fragments. The blue/white selection system was performed by adding Xgal and IPTG to the agar medium (Section 2.3.5). Only five of these colonies which were white were selected, and these colonies contained the required ligation. The pBS/FLAG-AK12 (Table 1 and Appendices) was digested with *Eco*RI and *Xba*I for the next cloning step.

2. The pCaSpeR-hs vector was enzyme digested with *Eco*RI and *Xba*I then gel purified (Section 2.12.4) to reduce uncut vector background. This purified vector was ligated with FLAG-AK12 fragment containing *Eco*RI and *Xba*I ends. Eleven transformants were obtained and examined by several different restriction enzyme digestions (Section 2.10.2, Figure 5). DNA sequencing (Section 2.17) of the 5' end confirmed the orientation and the sequence of the FLAG linker MSL-1 fusion was correct. This construct was termed pFC (Table 1 and Appendices).

Figure 5. *Eco*RI/*Xba*I Double Digests of Small Scale Lysis DNA Preparations from Transformations of *hsp70*-FC.

Eleven preparations (from lane 2 to lane 12: FC 2, 4, 7, 8, 10, 11, 12, 14, 15, 16 and 17) for FC transformation were double digested with *Eco*RI/*Xba*I. Lane 4, 5, 6, 7 and 11 showed the 1.2 kb fragment which indicates successful insertion, and a 8.9 kb fragment of pCaSper-hs. Lane 1 was *Hin*dIII/*SstII* ladder. Lane 4 (FC 8) was picked for large scale preparation and then used for microinjection.


kb
1
2
3
4
5
6
7
8
9
10
11
12
20.3
9.4
3.7
2.8
2.3
2.0
1.5
1.1
0.55
8.9 kb
1.2 kb

High quality pFC DNA was prepared (Section 2.8.2) and microinjected in *y w D. melanogaster* embryos (Section 2.18). Several homozygous transgenic lines were produced and linkage crosses showed the insertion to be on either the 2nd or 3rd chromosome (Section 2.19 and Table 3). One line was raised at 30°C and heat shocked daily at 37°C (Section 2.21) to overexpress the FC protein. The FLAG-tagged C-terminal domain appeared to be fully functional since overexpression caused male-specific lethality (0 male and 284 females) as had previously been observed for the AK12 lines (Section 1.11).

At this point in time, the finding of *male absent on the first* (MOF, Hilfiker *et al.*, 1997) prompted the question of whether the unknown factor was in fact the putative histone acetyl transferase. Therefore, it was necessary to show that MOF was binding to the C-terminal domain of MSL-1 to prove that MOF is the unknown factor and is part of the MSL complex.

3.2 MOF IS THE UNKNOWN FACTOR WHICH INTERACTS WITH C-TERMINAL DOMAIN OF MSL-1

The interaction between the C-terminal domain of MSL-1 and MOF can be shown by co-purification of the two proteins using affinity chromatography. Although the paper reporting the cloning of MOF was published, neither the MOF clones nor transgenic flies could be obtained from the author.

3.2.1 Making of Heat-Shock Controlled MOF Transgenic Fly

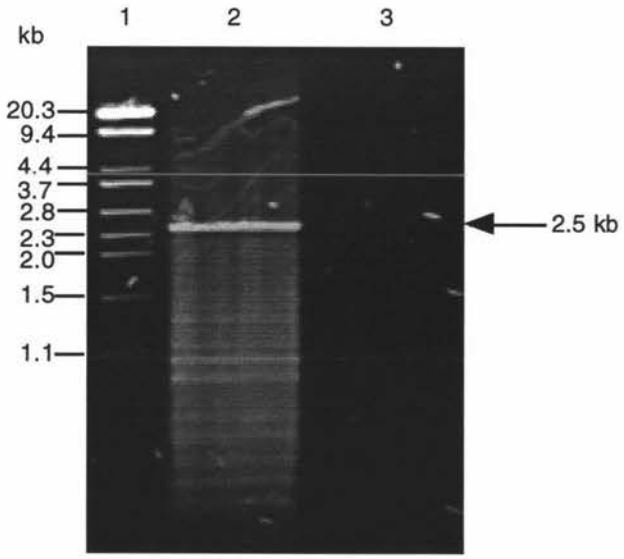
The MOF sequence was downloaded from Genebank and synthetic primers (MOF5 and MOF3, Table 2) were designed for PCR amplification of the *mof* ORF (Section 2.9). A single 2.5 kb DNA fragment was obtained by PCR amplification using *Drosophila* genomic DNA as template (Figure 6). *Pwo*I DNA polymerase was used for amplification

Figure 6. Amplification of 2.5 kb MOF Construct by Polymerase Chain Reaction.

Lane 1. *Hin*dIII/*SstII* ladder.

Lane 2. 2.5 kb *mof* PCR product.

Lane 3. Negative control in which no genomic DNA was added.


1
2
3
kb
20.3
9.4
4.4
3.7
2.8
2.3
2.0
1.5
1.1
2.5 kb

as the enzyme has a much lower error rate than Taq polymerase. The fragment was gel purified and then restriction enzyme digested with *Eco*RI and *Eco*RV to obtain *Eco*RI and blunt ends. The two-step cloning procedure described in Section 3.1.3 was used to insert *mof* into vector pCaSpeR-hs by ligating *Eco*RI and blunt ends. Two separate clones were obtained and termed pLP1A and pLP1B (Table 1 and Appendices). Both 5' and 3' ends of the *mof* insert of the two clones were sequenced and found to match exactly to the published sequence. Both pLP1A and pLP1B DNAs were microinjected in *y w* embryos separately (Section 2.18) and homozygous MOF transgenic lines were obtained (Table 3). Heat shock experiments were performed on these MOF transgenic flies (Section 2.21). Overexpression of MOF in homozygous flies appeared to be lethal for both males and females, whereas heterozygous progeny maintained a 1:1 male-to-female ratio (χ^2 test, Table 4). Flies heterozygous for the LP1 construct were obtained by crossing homozygous to the *yw* stock. This indicated that when MOF was overexpressed over a threshold, it may induce acetylation (therefore, hypertranscription) not only on sex chromosomes but also on autosomes, causing lethality to both males and females. Genetic crosses were carried out to investigate whether the lethality caused by MOF protein could be rescued with co-overexpression of FC (i.e. AK12) and/or combinations of MSLs. Heterozygous progeny of MOF transformants crossed with FC (or SF3) flies showed enhanced male-specific lethality relative to SF3 heterozygous (Table 5). This enhanced lethality suggests that the FC and MOF complex may be binding to another MSL. This MSL is unlikely to be MLE since the progeny from SF3 crossed with recombinant MOF + MLE did not show any significant reduction in male lethality (Table 5). These results indicate that MOF (and MLE) may not be sufficient to rescue the lethality caused by FC, hence some other MSLs are probably also involved in forming the complex with FC (and/or MOF).

Table 4. Overexpression of MOF Causes Lethality to Both Male and Female Flies.

Transgenic flies carrying the LP1 construct were raised at 30°C and heat shocked daily at 37°C for one hour. Few offspring were obtained as most flies died at the pupae stage. Heterozygous offspring maintained 1 : 1 male to female ratio.

Offspring	Males	Females	Ratio
Homozygous MOF	2[a]	3[a]	-
Heterozygous MOF	80	80	1.00

[a] large number of flies were lethal at the pupae stage.

Table 5. Co-expression of MOF (or MOF + MLE) and C-Terminal Domain of MSL-1 Has Enhanced Male-Specific Lethality.

Crosses were raised at 30°C and heat shocked daily at 37°C for one hour. Heterozygous offspring contained either one (FC) or two (SF3) copies of C-terminal domain of MSL-1, and either none, one copy of MOF or one copy of MOF + MLE. The viability of males carrying MOF and the C-terminal domain of MSL-1 was significantly reduced compared to males which only carried the C-terminal domain of MSL-1. Even the addition of MLE could not rescue this male-specific lethality.

Offspring from cross	Males	Females	Ratio
SF3 x *yw* (recipient stock)	120	255	0.47
FC x MOF	0	91	0
SF3 x MOF	4	510	0.01
SF3 x MOF + MLE	2	47	0.04

3.2.2 MOF and FC Co-Precipitation Using FLAG Affinity Chromatography

Homozygous LP1 and FC transgenic flies were crossed to produce heterozygous progeny which could co-overexpress both genes under the control of the heat-shock promoter. Total proteins (containing both MOF and FC) were extracted from these progeny which had been heat shocked one hour to induce protein synthesis and recovered at 25°C (Section 2.23.2). The amount of protein was measured (Section 2.24) and then FLAG affinity chromatography was carried out (Section 2.26.2). If MOF is able to bind to the MSL-1 C-terminal domain, it should co-purify with FC. Silver staining (Figure 7) of the SDS-PAGE showed there were two protein bands (130 and 46 kD) on the MOF/FC co-expressed lane, but there was only one band on the control lane which contained only purified FC protein. The purification was confirmed by Western blotting with MSL-1 antibody (Section 2.29, Figure 8) which showed that the 46 kD protein on the sample lane was the MSL-1 C-terminal domain. This result suggested that the top protein band was a possible candidate for the MOF protein. MOF antibody was later obtained and the same sample made from above was run on SDS-PAGE and Western blotted. The MOF antibody analysis showed that the 130 kD band was MOF (Figure 9) proving a direct interaction between MOF and FC proteins.

3.2.3 Purification of MOF Using the Glutathione S-Transferase (GST) Affinity Chromatography System

GST-fusion proteins can be effectively purified by chromatography over a glutathione matrix (Smith and Johnson, 1988). MOF can be fused with glutathione S-transferase which has high binding affinity with glutathione. GST/MOF fusion protein can then be purified by using the GST purification system (Section 2.26.1). To construct a plasmid which should encode a GST/MOF fusion protein, pBS/MOF (Table 1) was double digested with *Bam*HI and *Xho*I enzymes to obtain a 2.5 kb of fragment containing the *mof* ORF. However, a partial digest was required (Section 2.10.4) since *mof* contains an internal *Bam*HI site. A series of digestion trials were performed and optimal conditions

Figure 7. Protein Purification Using FLAG Affinity Chromatography (Silver Staining).

Extracts prepared from flies were purified over FLAG affinity columns. Aliquots from each extract were separated by SDS-PAGE and the gel was silver stained.

Lane 1. Protein from an FC line. The arrow indicates the major band at 46 kD which is the expected size of FC based on immunoblotting.
Lane 2. Protein from an FC x MOF line. Top arrow indicates a band at ~130 kD which may be the possible MOF protein.

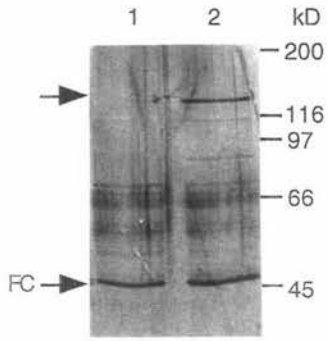

1
2
kD
200
116
97
66
FC
45

Figure 8. Western Analysis Using FLAG Antibody on FC and FC/MOF Purification Using FLAG Affinity Chromatography.

Samples described in Figure 7 were analysed using primary anti-FLAG monoclonal antibody. The 46 kD band corresponds to the purified FC protein.

Lane 1. FC ΔRIPA extract only. The amount of input is 7 % of lane 3.

Lane 2. FC x MOF ΔRIPA extract only. The amount of input is 7 % of lane 4.

Lane 3. FC ΔRIPA extract purified by affinity chromatography.

Lane 4. FC x MOF ΔRIPA extract purified by affinity chromatography.

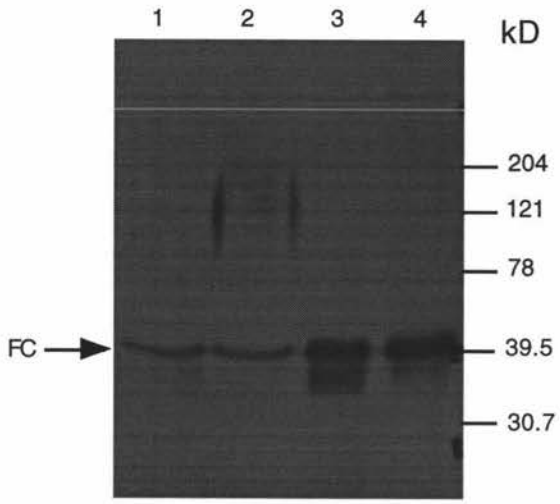
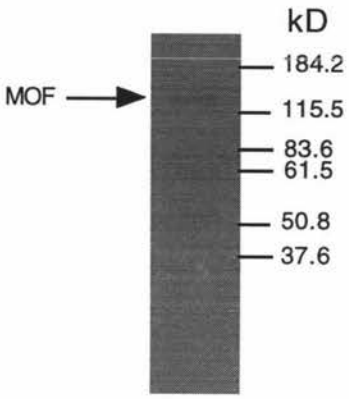

1
2
3
4
kD
204
121
78
FC
39.5
30.7

Figure 9. Anti-MOF Antibody Analysis on MOF Purification Using FC Protein and FLAG Affinity Chromatography.

FC x MOF ΔRIPA extract (same sample used in Figure 7 and 8) was analysed using primary anti-MOF antibody. The 130 kD band indicated the co-purification of MOF and FC protein by FLAG affinity chromatography.


kD
184.2
MOF
115.5
83.6
61.5
50.8
37.6

were found which yield the maximum quantity of the 2.5 kb *Bam*HI - *Xba* I fragment (30 minutes incubation, 3 U *Bam*HI/µg pBS/MOF). The digested DNA was size fractionated by electrophoresis (Section 2.11) and then gel purified (Section 2.12.4). The purified fragment was inserted into pGEX4T-1 (Table 1 and Appendices) and transformed into BL21 *E. coli* cells (Table 1). Transformed cell culture was expanded and GST fusion protein synthesis was induced by adding IPTG (Section 2.23.1). One alteration which was made from the original protocol (Pharmacia) was to lower the growing temperature of *E. coli* cells from 37°C to 22°C. The reason for this was to improve the problem of protein degradation which occurred at 37°C growing temperature. Under these conditions, degradation was reduced, but it could not be totally eliminated. Protein extract containing GST/MOF fusion protein was obtained and purified using glutathione affinity chromatography (Section 2.26.1). Western blotting carried out using GST antibody showed that the GST/MOF protein was induced and expressed in transformed *E. coli* cells, and its binding ability to glutathione had been maintained (Figure 10).

If the C-terminal domain of MSL-1 interacts with MOF, FC could co-purify with GST/MOF over a glutathione affinity column. Total fly protein extract containing overexpressed C-terminal domain of MSL-1 was made (Section 2.23.2) and mixed with Glutathione Sepharose 4B beads which had been previously incubated with *E. coli* GST/MOF fusion protein for one hour (Section 2.26.1). Glutathione affinity chromatography was performed and samples analysed by Western blot with MSL-1 antibody. This showed that the C-terminal domain co-purified with GST/MOF fusion protein but not GST (Figure 11A). Similar experiments were also carried out to compare the binding ability of MSL-1 and of other dominant-negative versions of MSL-1 (Figure 2) to the GST/MOF fusion protein. Results showed that full length MSL-1, SC8 and FC proteins (Figure 2), which all contained C-terminal domain of MSL-1, co-purified with the GST/MOF fusion protein (Figure 11A). However, the SC1, HF4 and AK1 proteins (Figure 2) did not co-purify with GST/MOF (Figure 11A). Although SC1 and AK1

Figure 10. Anti-GST Antibody Analysis on GST and GST/MOF Purification Using Glutathione S-Transferase Affinity Chromatography.

E. coli extracts of GST and GST/MOF fusion protein were made and purified using GST affinity chromatography. GST/MOF fusion protein has maintained its binding ability to Glutathione Sepharose beads.

Lane 1. Crude extract from GST expression strain. The 26 kD protein is GST. The amount of input is 67% of lane 2.

Lane 2. Glutathione affinity purified GST protein (not concentrated).

Lane 3. GST/MOF extract has shown as a 118 kD fusion protein. The amount of input is 67% of lane 4.

Lane 4. Glutathione affinity purified GST/MOF protein (not concentrated).

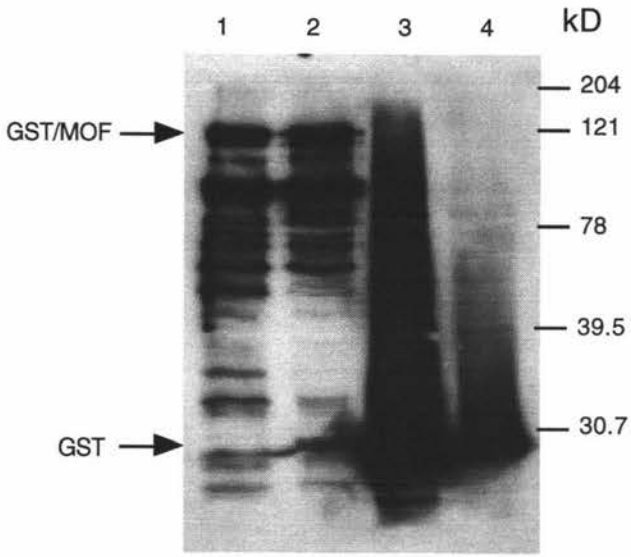
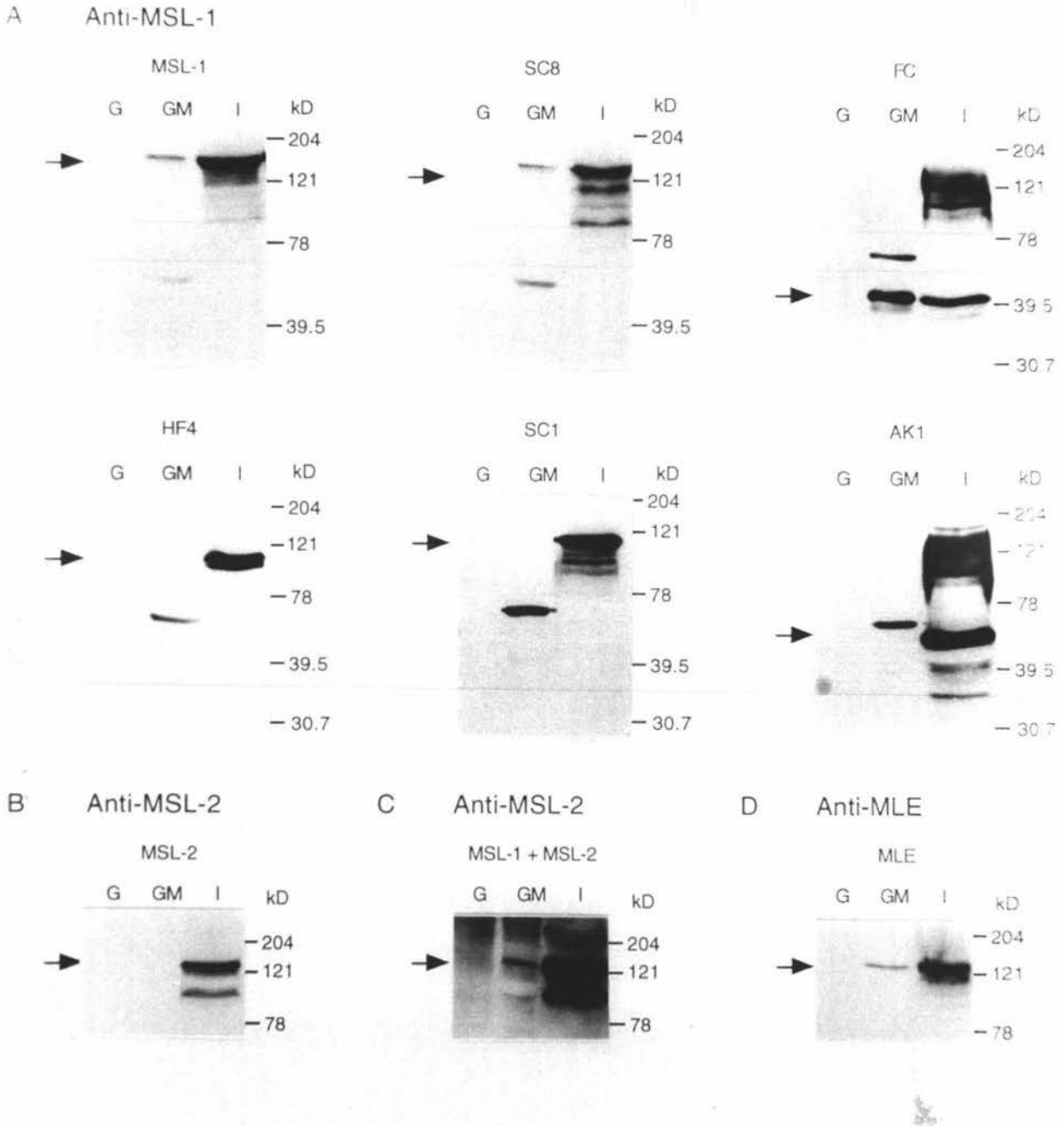

1
2
3
4
kD
204
121
78
39.5
30.7
GST/MOF
GST

Figure 11. Protein Purification Using Glutathione Affinity Chromatography

Fly extracts (input or I) from transformant lines which had been heat shocked to induce protein synthesis, were incubated with glutathione-sepharose beads containing either bound GST (G) or GST/MOF (GM). Bound proteins were eluted with glutathione. An aliquot of each sample was fractionated by SDS-PAGE then transferred to a nitrocellulose membrane. Western blots were performed with either anti-MSL-1 (A), anti-MSL-2 (B and C), or anti-MLE (D) antibodies. The amount of input in lane I is 6.7% of GM (and G) in all purifications A, B, C and D. In A, the band at 68 kD which appeared in all panels is an *E.coli* protein which co-purifies with GST/MOF and reacts with the anti-MSL-1 primary antibody.


A
Anti-MSL-1
MSL-1
SC8
FC
HF4
SC1
AK1
G
GM
I
kD
204
121
78
39.5
30.7
B
Anti-MSL-2
MSL-2
C
Anti-MSL-2
MSL-1 + MSL-2
D
Anti-MLE
MLE

contained the first 55 aa of the C-terminal domain of MSL-1, this is not sufficient enough for binding to the GST/MOF fusion protein. These results confirm that it is the C-terminal domain of MSL-1 which interacts with the MOF protein.

Similar glutathione affinity chromatography experiments were carried to determine if any other MSLs interact with GST/MOF. Results showed that MSL-2 did not co-purify with GST/MOF (Figure 11B). MSL-2 co-immunoprecipitates with MSL-1 from male nuclear extract (Kelly *et al.*, 1995). To investigate whether a formation of the MSL-1/MSL-2 complex would affect the MSL-1 interaction with MOF, extract was prepared from flies which co-overexpressed MSL-1 and MSL-2, and was then incubated with GST/MOF bound beads. Western analysis with MSL-1 and MSL-2 (Figure 11C) antibodies showed that both MSL-1 and MSL-2 co-purified specifically with GST/MOF. These results suggested that the amount of complex formed with endogenous MSL-1 when MSL-2 was overexpressed alone was too low to detect with the GST/MOF binding assay. Alternatively, the endogenous MSL-1 was associated with endogenous MOF and was thus not able to bind to GST/MOF. Similar experiments with extract from flies which overexpressed MLE showed MLE also co-purified with GST/MOF (Figure 11D), suggesting there is a direct association between MLE and MOF.

3.2.4 MSL-1 Derivatives Do Not Interact With MLE

MLE is necessary as a structural component for the recruitment of MOF to the X chromosome (Gu *et al.*, 1998). Since MOF is binding to C-terminal domain of MSL-1 (Section 3.2.2 & 3.2.3), and MOF is binding to MLE (Section 3.2.3), this suggests MLE may also directly associate with MSL-1. A similar FLAG affinity chromatography purification experiment (Section 3.2.2) was carried out to determine whether MLE interacts with truncated MSL-1. The results showed that MLE can neither bind to FC nor FMS (Figure 12), indicating that either MLE does not bind to MSL-1 or if it does, it

Figure 12. Anti-MLE Antibody Analysis on MLE Purification Using FC Protein and FLAG Affinity Chromatography.

Extracts prepared from flies were purified over a FLAG column. Aliquots from each extract were separated by SDS-PAGE. Western blots were carried out and incubated with anti-MSL-3 primary antibody. The amount of input in lane 1, 3, 5 and 7 are 6.7 % of lane 2, 4, 6 and 8 respectively. The ~130 kD band corresponds to the MLE protein in lane 1, 3, 5 and 7. No MLE protein was purified in lane 2, 4, 6 and 8.

Lane 1. MLE ΔRIPA extract only.

Lane 2. MLE ΔRIPA extract purified.

Lane 3. FMS x MLE ΔRIPA extract only.

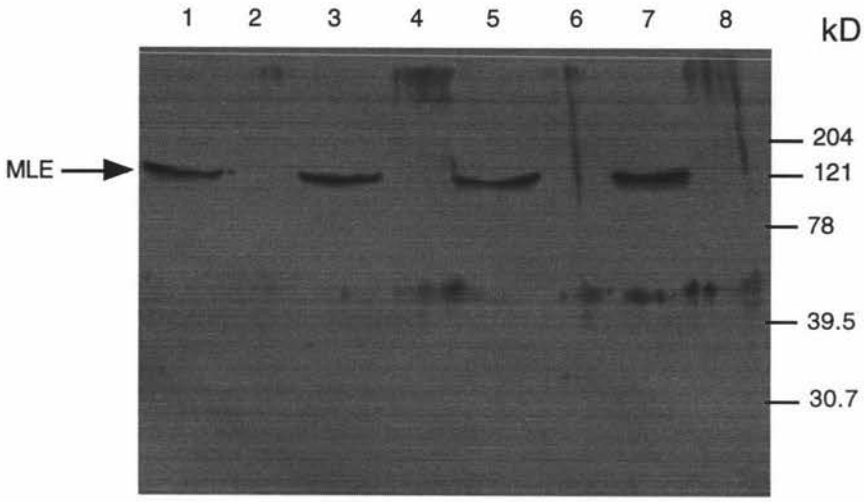
Lane 4. FMS x MLE ΔRIPA extract purified.

Lane 5. FC x MLE ΔRIPA extract only.

Lane 6. FC x MLE ΔRIPA extract purified.

Lane 7. MOF x FC x MLE ΔRIPA extract only.

Lane 8. MOF x FC x MLE ΔRIPA extract purified.


1
2
3
4
5
6
7
8
kD
MLE
204
121
78
39.5
30.7

binds to the first 84 amino acids of MSL-1. However, these experiments could not be carried out further due to time constraints.

3.2.5 C-Terminal Domain of MSL-1 Shows Association With MSL-3 Using FLAG-Affinity Chromatography

Genetic evidence (Section 3.2.1) has shown that the lethality caused by overexpressing the C-terminal domain of MSL-1 can not be rescued with co-overexpression of MOF and MLE. This indicates that there must be another factor interacting with this domain. Although the result of immunoprecipitation (Section 3.1.2) was not conclusive, it suggested that there may be some binding between MSL-1 and MSL-3, and the location of this interaction may be at the C-terminal domain of MSL-1. A similar FLAG affinity chromatography purification experiment (Section 3.2.2) was carried out to determine whether MSL-3 interacts with C-terminal domain of MSL-1. The results showed that the 58 kD MSL-3 co-purified with FMS and FC (Figure 13). A negative control which only had the input of MSL-3 extract indicated that there was no non-specific binding of MSL-3 to the FLAG column (Figure 13). Therefore MSL-3 interacts directly with C-terminal domain of MSL-1.

3.3 AN OVEREXPRESSED N-DOMAIN OF MSL-1 CAUSES MALE-SPECIFIC LETHALITY WHICH IS RESCUED BY ASSOCIATING WITH MSL-2 PROTEIN

Since MSL-1 and MSL-2 co-localise to "high Affinity" sites on the X chromosome independent of the other MSLs (Palmer *et al.*, 1994), and immunoprecipitation experiments show that MSL-1 and MSL-2 are part of a protein complex in *Drosophila* male nuclei (Kelly *et al.*, 1995), this suggests that MSL-1 and MSL-2 interact directly.

Figure 13. Anti-MSL-3 Antibody Analysis on MSL-3 Purification Using FC Protein and FLAG Affinity Chromatography.

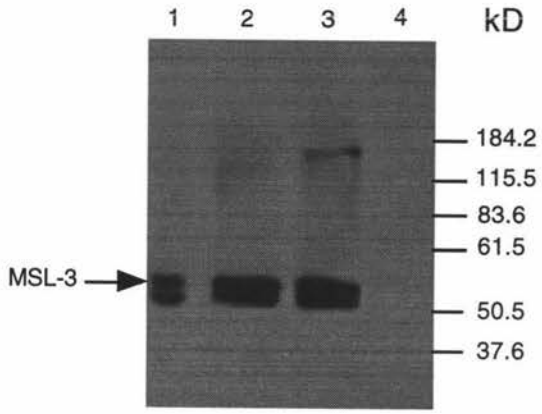
Extracts prepared from flies were purified over a FLAG column. Aliquots from each extract were separated by SDS-PAGE. Western blots were carried out and incubated with anti-MSL-3 primary antibody. It is not known why two bands are detected.

Lane 1. MSL-3 ΔRIPA extract only. The amount of input is 6.7 % of lanes 2, 3 and 4

Lane 2. FMS x MSL-3 extract was purified and showed 58 kD MSL-3 protein.

Lane 3. FC x MSL-3 extract was purified and showed 58 kD MSL-3 protein.

Lane 4. MSL-3 extract was purified and no protein reacted with anti-MSL-3 antibody.


1
2
3
4
kD
184.2
115.5
83.6
61.5
MSL-3
50.5
37.6

3.3.1 Overexpression of FLAG.ΔMSL-1 Causes Male-Specific Lethality.

To further define the region of MSL-1 which interacts with MSL-2 (Section 1.11), pFM which encodes amino acids 85 - 263 of MSL-1 (by F. Miller, Table 1 & Appendix 1) was amplified by large scale preparation (Section 2.8.2) and microinjected in *y w D. melanogaster* embryos (Section 2.18). Several homozygous transgenic lines were produced and linkage crosses showed the insertion to be on the either 2nd or 3rd chromosome (Section 2.19 & Table 3). Overexpression of the FM protein (Figure 2) (Section 2.21) causes male-specific lethality (0 male; 189 females). This indicates that the FLAG-tagged N-terminal domain is fully functional.

3.3.2 Co-expression of MSL-2 Rescues Male Lethality which is Caused by Overexpressed Dominant-Negative Mutant Forms of MSL-1 (FLAF.ΔMSL-1)

Similar co-overexpression experiments to Section 1.11 were then carried out to determine whether the FM domain was associated with MSL-2. When FM flies were crossed with recipient stock *yw* and progeny raised at 30°C and heat shocked daily at 37°C (Section 2.21), the male to female ratio was 0.21 (23 males and 110 females). When FM flies were crossed with MSL-2, the male to female ratio was 0.81 (139 males and 171 females). These results showed that the male lethality caused by overexpression of the FM domain can be significantly reduced with co-overexpression of MSL-2 proteins. This suggests that it is the FM region of MSL-1 which interacts with MSL-2.

3.3.3 Affinity Purification of FLAG.ΔMSL-1/MSL-2 Complex

FLAG affinity chromatography was performed in a similar manner to that described previously (Section 3.2.2) to determine if the FM region of MSL-1 binds directly to MSL-2. FMS, ANT, FM and FC transformants were each crossed with *hsp.msl-2* transgenic flies to produce heterozygous progeny which co-expressed MSL-1 derivatives and MSL-2 proteins when given a one-hour heat shock. Total fly protein extracts (Section

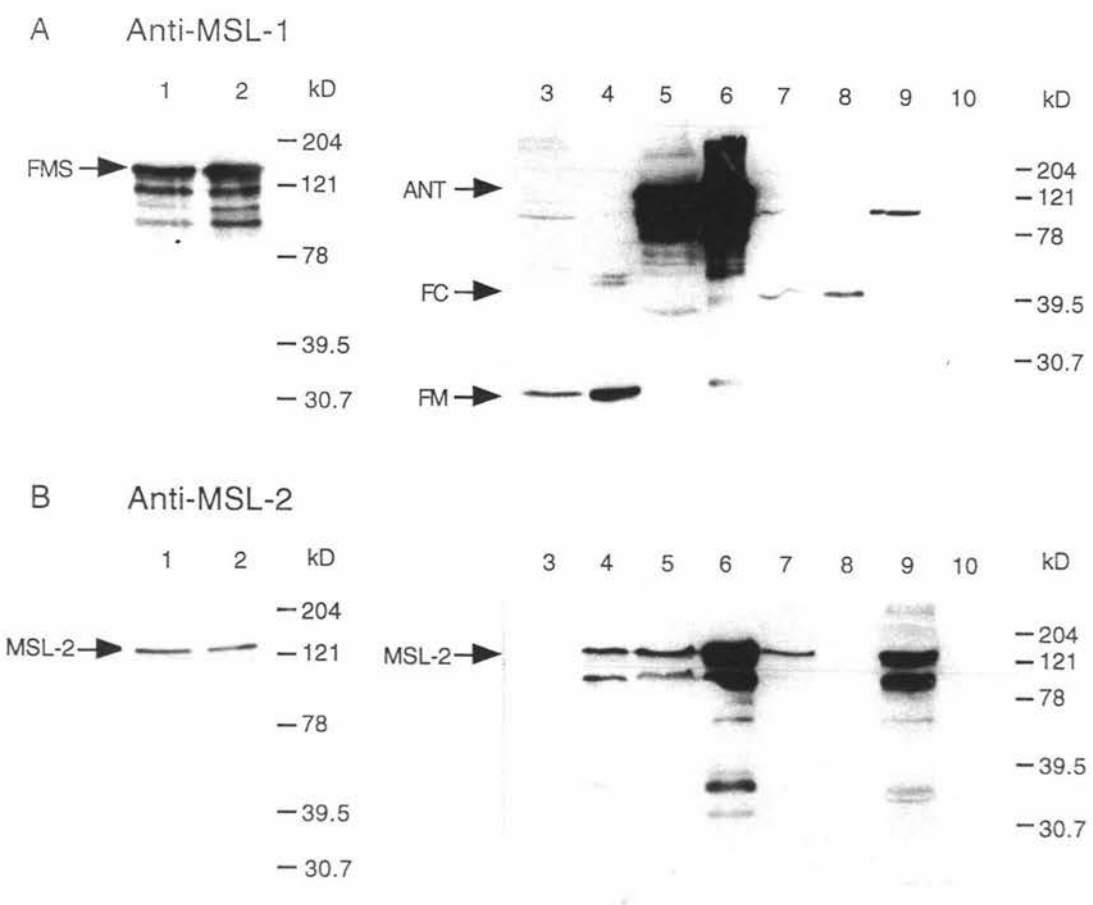
2.23.2) were made freshly each time to avoid the possibility of MSL-2 precipitation (Ausubel, Chapter 10, Current Protocol, 1995). FLAG affinity chromatography was performed, then samples were loaded onto a SDS-PAG. A sample of the extract (input) was loaded alongside each of the affinity purified samples. As expected, all of the FLAG tagged truncated version of MSL-1 (FMS, ANT, FM and FC, Figure 2) bound specifically to the FLAG affinity gel (Figure 14A). There was no significant retention of MSL-2 alone to the affinity gel (Figure 14B). MSL-2 did co-purify with FMS, ANT and FM proteins (Figure 14B). However, MSL-2 did not co-purify with FC, the region of MSL-1 which interacts with MOF and MSL-3 (Section 3.2.2 & 3.2.3). These results suggest that the dominant-negative effect of the FM region of MSL-1 is due to association with MSL-2 and forms a FM/MSL-2 non-functional protein complex.

3.3.4 Cloning of pETM1-N-Term and pETM2-N-Term For Protein Purification and Crystallisation.

The MSL-1 binds to MSL-2 through its N-terminal Domain (aa 85 - 263) (Section 3.3.3). Further, the RING finger of MSL-2 which is located near the N-terminus of MSL-2 has the function of associating with other protein through metal bindings (Zhou *et al.*, 1995), suggesting that the RING finger domain associates with the N-terminal domain of MSL-1. Both pETM1-N-Term and pETM2-N-Term (Table 1 and Appendices) were generated from pETM1 and pETM2 (Table 1 and Appendices) respectively. pETM1 was double digested with *Eco*RI and *Not*I (Section 2.10.3) and 5' overhangs were filled by Klenow (Section 2.14.1). The DNA fragment was religated (termed pETM1-N-term) and transformed into *E. coli.* BL21 (Table 1). pETM2 was double digested with *Sac*I and *Not*I and both overhangs were removed by Mung Bean nuclease (Section 2.14.2). The DNA fragment was also religated (termed pETM2-N-term) and transformed. Proteins that are expressed from these two strains can be purified using a nickel or a zinc column, and subsequently the purified proteins can be used for protein crystallisation to determine the

Figure 14. FLAG Affinity Chromatography of FLAG-Tagged MSL-1/MSL-2 Complexes.

Protein extracts were prepared from transformant flies which co-expressed either FMS and MSL-2 (lanes 1 and 2), FM and MSL-2 (lanes 3 and 4), ANT and MSL-2 (lanes 5 and 6) or FC and MSL-2 (lanes 7 and 8). Protein extract was also prepared from a line which overexpressed MSL-2 alone (lane 9 and 10). Aliquots of either unpurified extracts (lanes 1, 3, 5, 7 and 9) or FLAG affinity purified protein (lanes 2, 4, 6, 8 and 10) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blots were incubated with either anti-MSL-1 (A) or anti-MSL-2 (B) primary antibody. The amount of input in lanes 1, 3, 5, 7 and 9 is 6.7 % of lane 2, 4, 6, 8 and 10 respectively. (Note, longer exposure detected MSL-2 in lane 3 but not lane 8 and 10)


A
Anti-MSL-1
1
2
kD
FMS
204
121
78
39.5
30.7
3
4
5
6
7
8
9
10
kD
ANT
FC
FM
204
121
78
39.5
30.7
B
Anti-MSL-2
1
2
kD
MSL-2
204
121
78
39.5
30.7
3
4
5
6
7
8
9
10
kD
MSL-2
204
121
78
39.5
30.7

MSL-1 binding domain on MSL-2 and also the N-terminal domain of MSL-1 and MSL-2 protein structures.

4. DISCUSSION

4.1 EXAMINATION OF DOSAGE COMPENSATION INVOLVING MSLs

In *Drosophila*, dosage compensation is achieved by a twofold-increase in activity of genes on the single male X chromosome (Lucchesi and Manning, 1987, Section 1.1). Although the exact mechanism of how this twofold-increased activity is achieved is not fully understood, it does involve acetylation of histone H4 at lysine 16 (H4Ac16) which probably facilitates this hypertranscription (Turner *et al.*, 1992). Often multi-protein complexes containing either histone acetyl transferases (HATs) or histone deacetylases play an important role in transcription in eukaryotes (Armstrong and Emerson, 1998; Davie, 1998; Struhl, 1998). For example, the transcription co-activator CBP binds to a number of transcription factors including CREB, c-jun, c-myb, MyoD and to the HATs, P/CAF and steroid receptor co-activator-one (SRC-1) (Goldman *et al.*, 1997). CBP also has HAT activity (Bannister and Kouzarides, 1996; Ogryzko *et al.*, 1996) and binds to RNA helicase A (RHA) (Nakajima *et al.*, 1997). The coupling of these two enzymatic activities could be advantageous as the HAT could disrupt either higher order chromatin structure or histone/DNA interactions and thus facilitate procession of the helicase along the chromosome.

The MSL complex also contains a HAT (MOF) and RNA helicase (MLE). Prior to this study, the evidence that the MSLs form a complex was largely indirect. This study has shown that MSL-1 has a central role in the assembly of the MSL complex (Figure 15).

4.1.1 The C-Terminal Domain of MSL-1 Binds Directly to MOF

The C-terminal domain of MSL-1 shows similarity to mouse CBP almost entirely across the domain (24 % identity, 58 % similarity to amino acids 863 to 1117 of CBP). This region has no known function and is adjacent to the bromodomain which is found in a number of transcription co-activators (Goldman *et al.*, 1997) and which can mediate protein-protein interactions (Barlev *et al.*, 1998). Since CBP associates with HATs and one of the MSLs, MOF, shows significant similarities to the members of MYST family of HAT (Hilfiker *et al.*, 1997), suggesting that the C-terminal domain of MSL-1 could interact with MOF. This study has confirmed that these two proteins interact directly using FLAG and GST affinity chromatography (Figure 9 and Figure 11). It would be of interest to determine if the MSL-1 where region of CBP interacts with a MOF-like HAT in mammalian cells.

In this study, although the *mof* gene which was synthesised by PCR amplification (Figure 6) and has only been sequenced for 450 bases at each end of the 2.5 kb gene, there is reason to believe that the gene encodes a functional protein. If there were non-functional MOF proteins produced in those MOF flies, the lethality would be only restricted to males because the non-functional MOF would compete with endogenous MOF for binding to MSL-1. In fact, overexpression of MOF causes lethality to both homozygous males and It would be tempting to speculate that when an excess amount of MOF is synthesised over a threshold, MOF binds to the autosomes which leads to abnormal transcription and lethality. One of the future experiments to confirm this hypothesis is to carry out polytene chromosome staining to show that whether overexpressed MOF binds to autosomes.

Moreover, the activation of MOF appears to require the association with the MSL complex (Lucchesi,1998), perhaps by binding to the C-terminal domain of MSL-1. To

confirm this theory, the HAT activity of either MOF alone or MOF/FC complex can be measured by determining the amount of histone 4 being acetylated in a standard assay.

4.1.2 Two Chromo Domains of MSL-3 Could Play a Role In Stabilising the Direct Binding Between MSL-3 and the C-Terminal Domain of MSL-1

MSL-3 co-purifies with FLAG-tagged truncated MSL-1 (including FMS and FC) over a FLAG affinity column (Figure 13). This suggests that the MSL-3 binding domain is on the C-terminal domain of MSL-1, and the domain may be adjacent to the MOF binding domain. This finding is consistent with a cytoimmunoflourescence study which showed that MSL-3 and MSL-1 are co-localised on the male X chromosome (Gorman *et al.*, 1995). MSL-3 is a 512 amino acid protein which contains two chromatin organisation modifier (chromo) domains (Lucchesi, 1998). Such chromo domains are found in a group of chromatin binding proteins such as the Polycomb-group proteins (Koonin *et al.*, 1995). The common feature of the chromo domain is that the secondary structure of a single domain appears to be unstable, and may require interaction with either another chromo domain in another part of the protein or chromo domains of different proteins (Lucchesi, 1996). This suggests that the two chromo domains of MSL-3 are interacting with each other to achieve stabilisation, or alternatively, since MOF also contains a chromo domain and both MOF and MSL-3 are binding closely to the C-terminal domain of MSL-1, it is possible that MOF stabilises MSL-3 through its chromo domain and recruits MSL-3 to the MSL-1/MSL-2 complex which is already bound to the X chromosome.

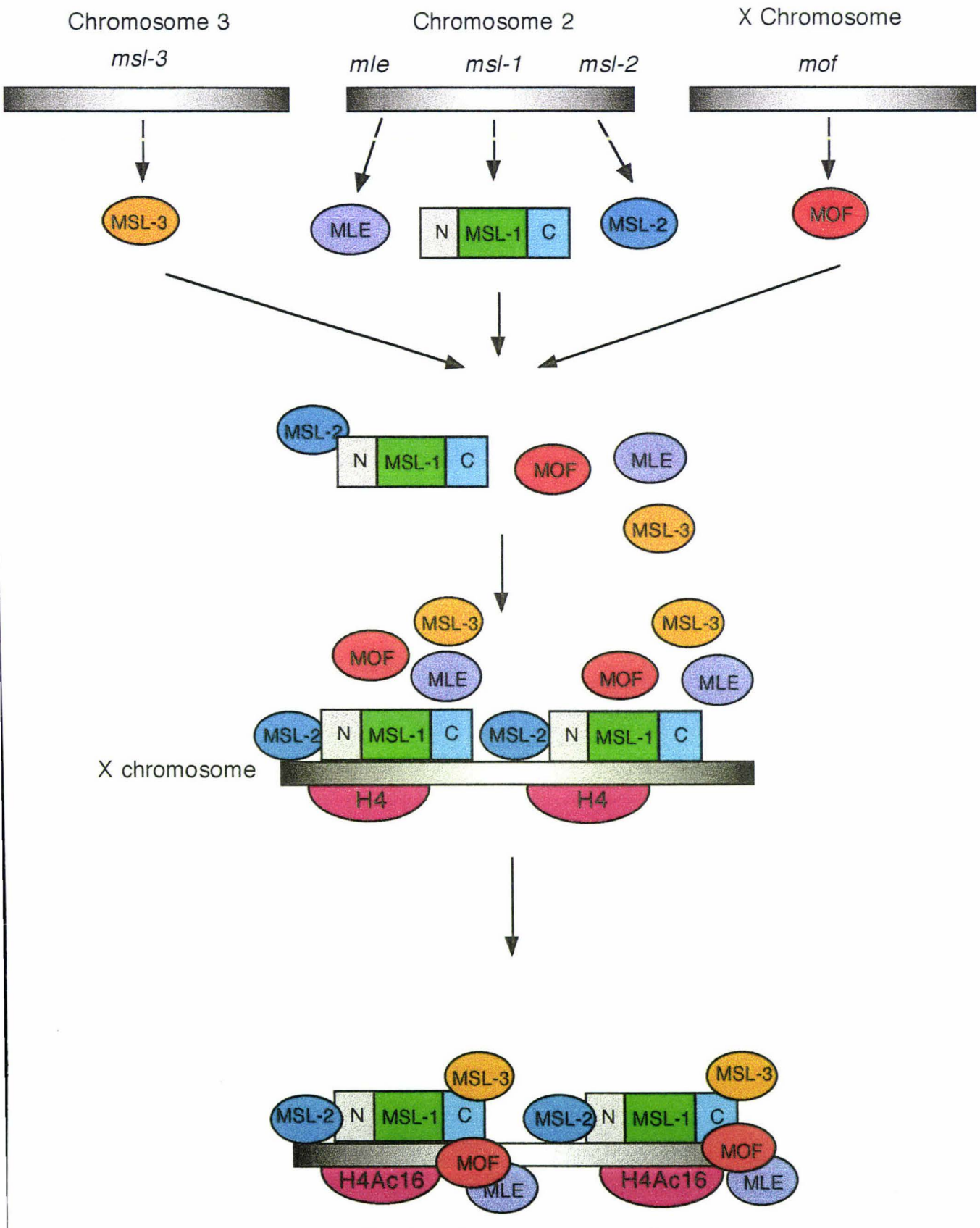
4.1.3 Localisation of MSL-2 Binding Domain On MSL-1

In this study, it has shown that FM (amino acids 85 - 263 of MSL-1) interacts directly with MSL-2 using FLAG affinity chromatography. Moreover, the dominant-negative effects caused by overexpression of the FM region can be reduced almost completely by co-expression of MSL-2 protein (Section 3.3.2). These results are consistent with previous evidence the two proteins associate, and are complementary to the recent finding

Figure 15. Model for The Assembly of The MSL Dosage Compensation Complex.

The diagram only shows the known protein components for the MSL complex. The MSL-1/MSL-2 complex initially associates with the male X chromosome, and MOF, which recruits MLE, associates with the MSL-1/MSL-2 complex by binding to the C-terminal domain of MSL-1. MSL-3 also binds to the C-terminal domain of MSL-1 to form a MSL dosage compensation complex. Histone 4 is subsequently acetylated at lysine 16 (H4Ac16).

Model of *msl* -mediated dosage compensation



Twofold dosage compensation

that part of FM region of MSL-1 (amino acids 85 - 186) associates with MSL-2 in yeast two-hybrid interaction trap experiments (K. Copps, R. Richman, L. Lyman, K. Chang, J. Rampersad-Ammons and M. Kuroda, *EMBO J.*, in press).

One notable feature of the FM region of MSL-1 is that almost half of the region (amino acids 96 - 170) is predicted to form an alpha-helical secondary structure. Further, part of the predicted alpha-helix (amino acids 96 - 131) is amphipathic, that is, these are a clustering of hydrophobic amino acids to one side of the predicted helix. Amphipathic alpha helices are an important part of the dimerisation domains of a number of transcription factors (Murre *et al.*, 1989). Interaction between amphipathic alpha helices can stabilise the structure of the dimer (Ferræ-D'amaræ *et al.*, 1993). MSL-2 contains a RING finger domain which is essential for function (Lyman *et al.*, 1997). The RING domain is found in a number of proteins including V(D)J recombination-activating protein RAG1 (Bellon *et al.*, 1997). The crystal structure of the RAG1 dimerisation domain, which includes the RING finger, reveals that dimerisation is stabilised by interaction between alpha helices which form a hydrophobic core (Bellon *et al.*, 1997). The RING finger is thought to "form the structure scaffold upon which the dimer interface is formed" (Bellon *et al.*, 1997). The RING finger of MSL-2 is followed by a region which is predicted to form an alpha-helix. It would be tempting to speculate, by analogy with RAG1, that the association of MSL-1 and MSL-2 involves the interaction of amphipathic alpha-helices which depend on the RING finger domain. This could be addressed by determining the crystal structure of the MSL-1/MSL-2 complex.

In the sequencial assembly model (Gu *et al.*, 1998), MSL-1 and MSL-2 bind to 20 - 40 "high affinity" sites on the male X chromosome without the presence of the other MSLs (Gu *et al.*, 1998; Palmer *et al.*, 1994; Bashaw and Baker, 1995), suggesting that MSL-1 and MSL-2 are the first MSLs to be associated with the X. In this study, GST-MOF fusion protein associates directly to MSL-2 bound MSL-1, and these three proteins can

be co-purified over a glutathione affinity column (Figure 11C). These results support the model that MSLs form a complex which is assembled sequencially.

4.1.4 Is MLE the Link Between the MSL Complex and Polymerase II?

Given the high similarity between MLE and RHA (Lee *et al.*, 1997), and the fact that RHA is binding directly to both CBP and RNA polymerase II *in vivo* (Nakajima *et al.*, 1997), it would seem likely that MLE also interacts with polymerase II. It would be tempting to speculate that the role of MSL-1/MSL-2 complex is to provide a CBP-like interface for recruiting a HAT and RNA helicase specifically to the male X chromosome. These two enzymatic activities are then primarily responsible for the two-fold increase in transcription of most X-linked genes. CBP interacts with RNA helicase A via a Cys, His rich domain (Nakajima *et al.*, 1997). Although none of the MSLs show any significant similarity to this domain, MSL-2 does contain regions which are Cys-rich (Zhou *et al.*, 1995) and MOF contains a CCHCH putative zinc finger (Gu *et al.*, 1998). Thus, it is possible that MLE may interact with either of these cys-rich domains.

4.1.5 Can Co-Expression of MOF and MLE be Sufficient to Reduce the Dominant-Negative Effect Caused by Overexpression of the C-Terminal Domain of MSL-1?

Overexpression of the C-terminal domain of MSL-1 has dominant-negative effects which inhibit dosage compensation and cause male-specific lethality (Section 1.11). FC males were not rescued by either co-expression of MSL-2, MSL-3, MLE (Dr. M. Scott, Section 1.11) and MOF (Table 5). Since the C-terminal domain interacts with both MOF and MSL-3, this suggests that both MSL-3 and MOF must be co-overexpressed with FC in order to rescue male lethality. However, males which express FC, MSL-3 and MOF die (Dr. M. Scott, unpublished results). Since neither MSL-2 or MLE binds to FC, this suggests that either MSL-2 or MLE was binding to either MOF or MSL-3, or an unknown factor required for dosage compensation is binding to the FC/MOF/MSL-3 complex.

MLE is required for the binding of MOF to the male X chromosome (Gu *et al.*, 1998), and the binding of MLE is significantly reduced in larvae homozygous for mof^2 which produces a severely truncated protein (Gu *et al.*, 1998). These studies are consistent with the observation that MLE binds directly to MOF (Figure 11D), suggesting that *in vivo*, MLE and MOF could bind as a complex to X chromosome bound MSL-1/MSL-2, and MLE is somehow required for either promoting or stabilising the MSL-1/MOF interaction. Therefore it is possible to speculate that MLE binds to the FC/MOF/MSL-3 complex. However, MLE did not co-purify from *Drosophila* extract which also contains FC and MOF. Since FC interacts with MOF which associates with MLE (Figure 9; Figure 11A and 11D), MLE was expected to co-purify with the FC-MOF complex. One possibility is that MOF can interact with FC and MLE, but only one at a time. Alternatively, the C-terminal domain of endogenous MSL-1 is not freely available to MOF and that the binding of MLE to MSL-1/MSL-2 complex causes a conformational change in MSL-1 such that the C-terminal domain becomes more accessible to MOF. However, it should be noted that GST pull-down assays are very sensitive but can give misleading results (Ausubel, 1995). Clearly, the MOF-MLE interaction would need to be confirmed by future studies.

4.2 CONCLUSION AND FUTURE WORK

The MSLs are believed to act as a large multi-protein complex to precisely increase the transcription of X-linked genes twofold (Reviewed in Baker *et al.*, 1994). This complex may be assembled sequentially, and it has the ability to not only recognise the male X chromosome but also modify structural proteins, i.e. histone 4, and play a role in transcription activation (Lucchesi, 1998). The results presented in this study support these theories by showing the direct interaction between MOF and MSL-1, MOF and

MLE, MSL-1 and MSL-3, and MSL-1 and MSL-2. Further, studies on the nature of the MSL complex may provide some insight into the mechanism of dosage compensation.

The first experiment that must be undertaken is to co-overexpress MOF, MLE, and MSL-3 (or possibly also MSL-2) with FC to try to identify a combination which can reduce the dominant-negative effect of the C-terminal domain of MSL-1. Thus the MSL proteins which are involved with the C-terminal domain of MSL-1 can be determined.

The MSL-2 binding domain of MSL-1 and MSL-2 RING finger domain can be purified from *E. coli* expression strains (Section 3.3.4) and used for protein crystalisation. By obtaining crystals of these two domains, it could be confirmed that the interaction of amphipathic alpha-helices may be involved in the association between MSL-1 and MSL-2.

To investigate whether there are other unknown factors involved in the MSL complex, two methods can be used. Firstly, mutagenesis screens similar to those described in Section 2.22 and 3.1.1 could be attempted. Secondly, affinity chromatography (Section 2.26) could be used to co-purify any unknown factor which binds to each of the MSL. If sufficient protein is obtained, the amino acid sequence and molecular weight of the protein could be determined.

This study has shown that affinity chromatography of epitope-tagged proteins can be used effectively to demonstrate protein-protein interactions. A direct extension of this work is to epitope-tag domains of the other MSLs. This allows not only a cross-check of the interactions between MSLs which have been previously identified, but also enables the determination of the functional protein domains of each MSL. Interactions between MSLs and other factors, such as structural protein histone 4 and X-linked non-coding *roX1* and *roX2*, can be identified using a similar method. The complete MSL complex can

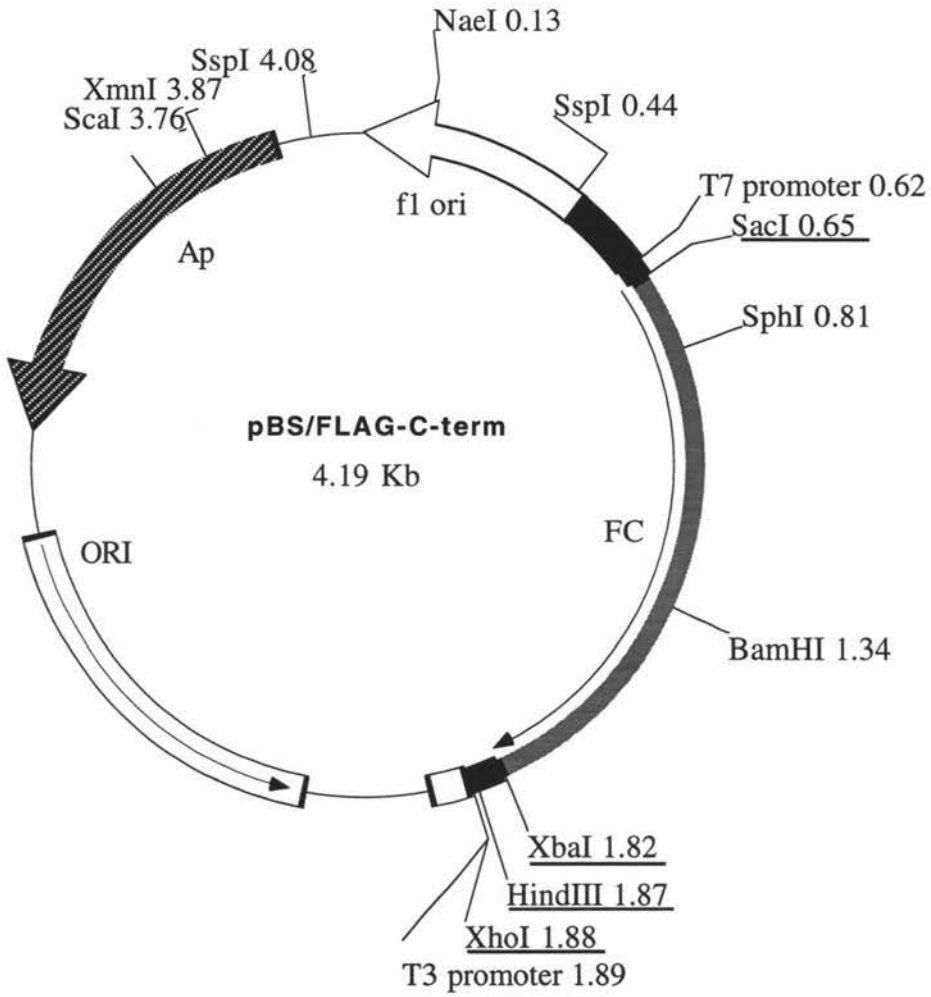
be purified from *Drosophila*, and the ability of this complex to acetylate histone *in vitro* can also be determined.

Lastly, the study of dosage compensation would not be complete without identifying the DNA sequences (dosage compensation regulatory element, DCRE) which are required to target the MSL complex to the X chromosome and distinguish the X from the remainder of the genome.

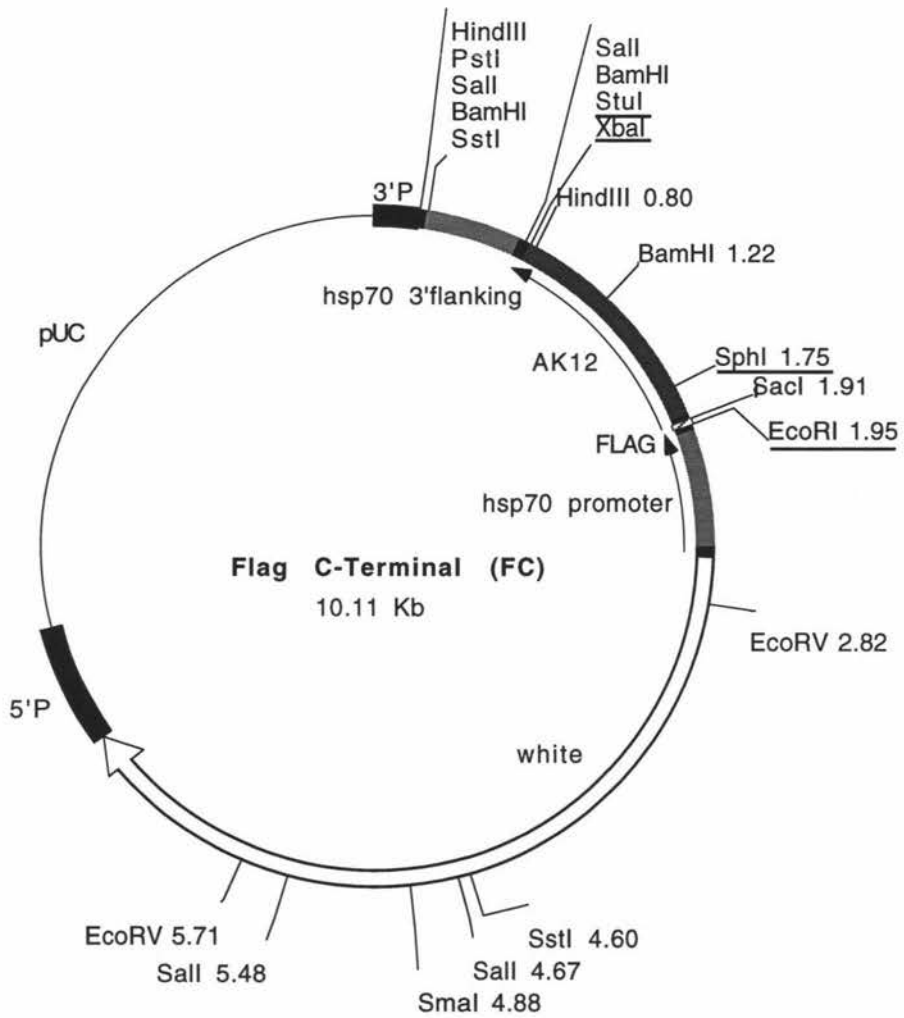
These experiments can help to understand how MSLs assemble to form a complex, and they should provide an insight into how a protein complex can regulate the expression of many genes such as dosage compensation in *Drosophila*.

5. APPENDICES

Physical maps of plasmids used in this study. Unique restriction sites are underlined

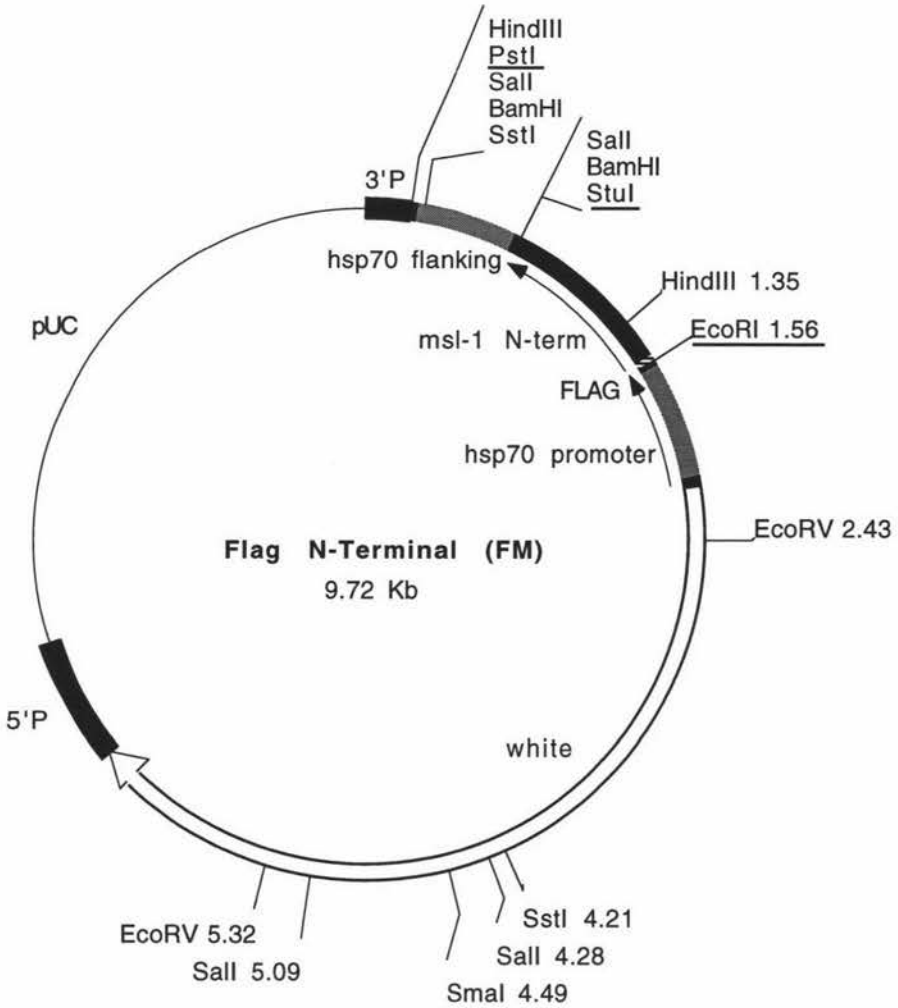


Physical Maps of pBS/FLAG-C-term
4.19 kb, pBluescript KS (-) containing 1.2 kb *Sac*I/*Xba*I C-terminal coding sequence of *msl-1* and a *Eco*RI/*Sac*I FLAG linker.



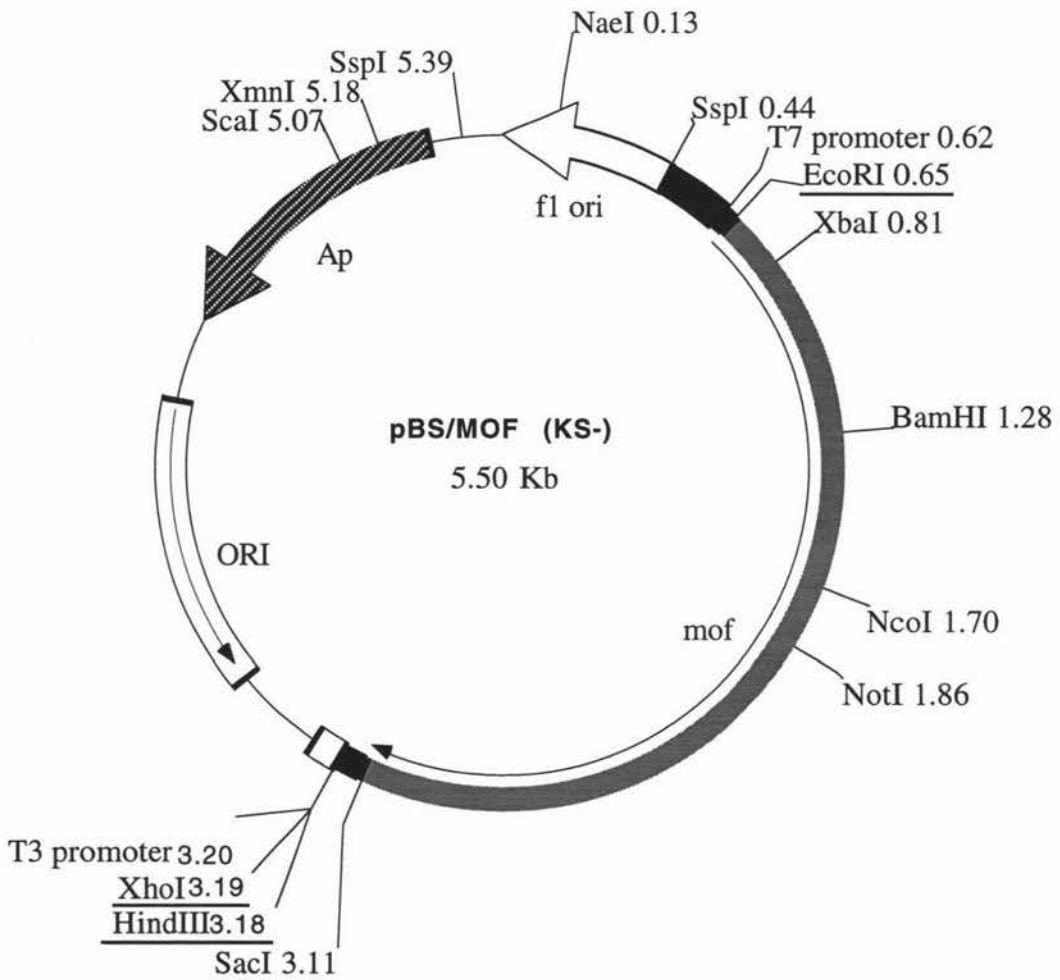
Physical Map of pFC

10.11 kb, pCaSpeR-hs (pUC, *hsp 70* promoter, mini-white, Pelement 5' and 3' ends) containing 1.2 kb *Sac*I/*Xba*I C-terminal coding sequence of *msl-1* and a *Eco*RI/*Sac*I FLAG linker.

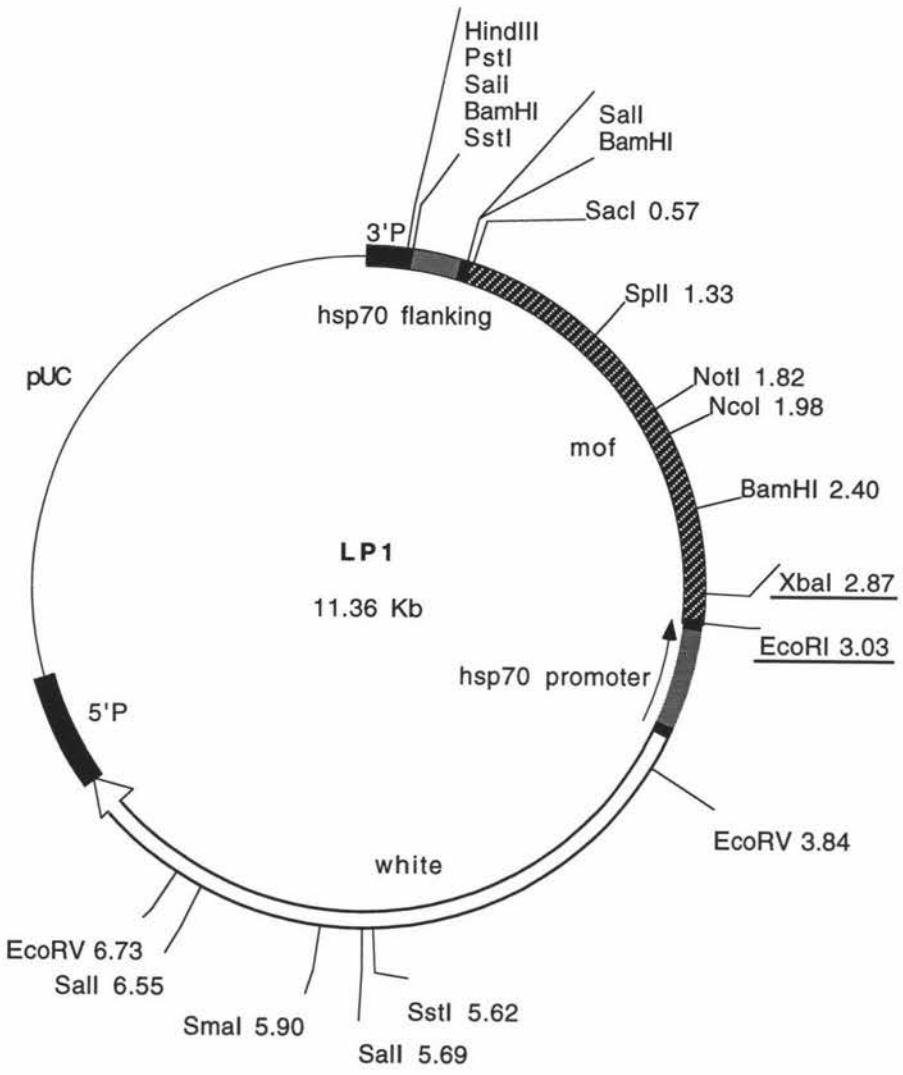


Physical Map of pFM 5'

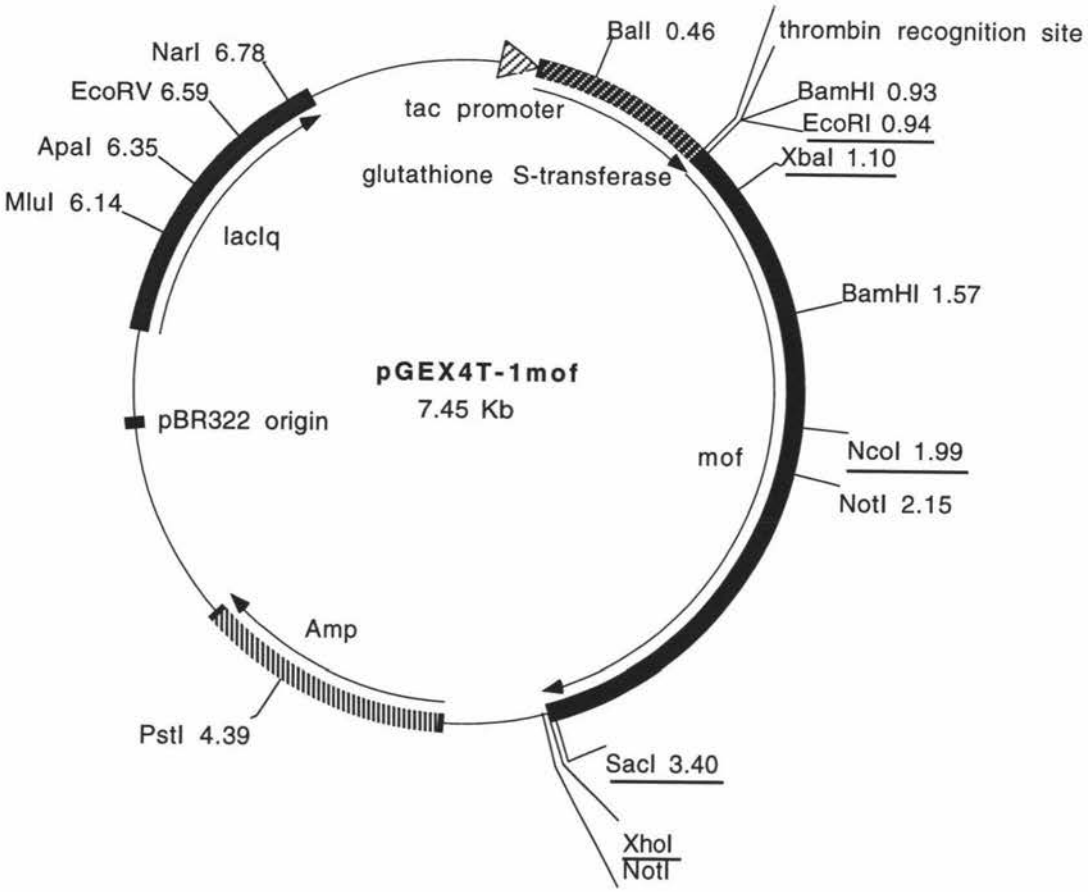
9.72 kb, pCaSpeR-hs containing 1.2 kb *Eco*RI/*Stu*I (Blunt) N-terminal coding sequence of *msl-1* and a FLAG linker.



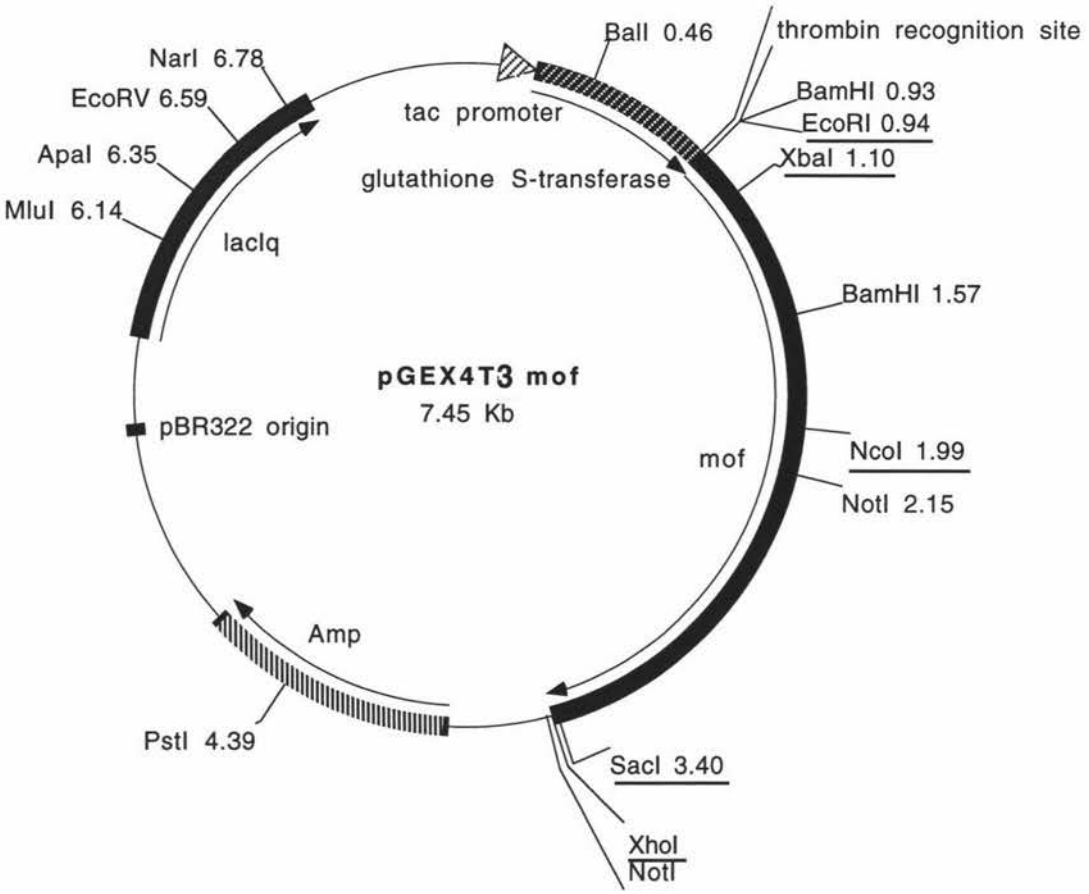
Physical Map of pBS/MOF KS(-)
5.50 kb, pBluescript KS (-) containing 2.5 kb *mof* coding sequence.



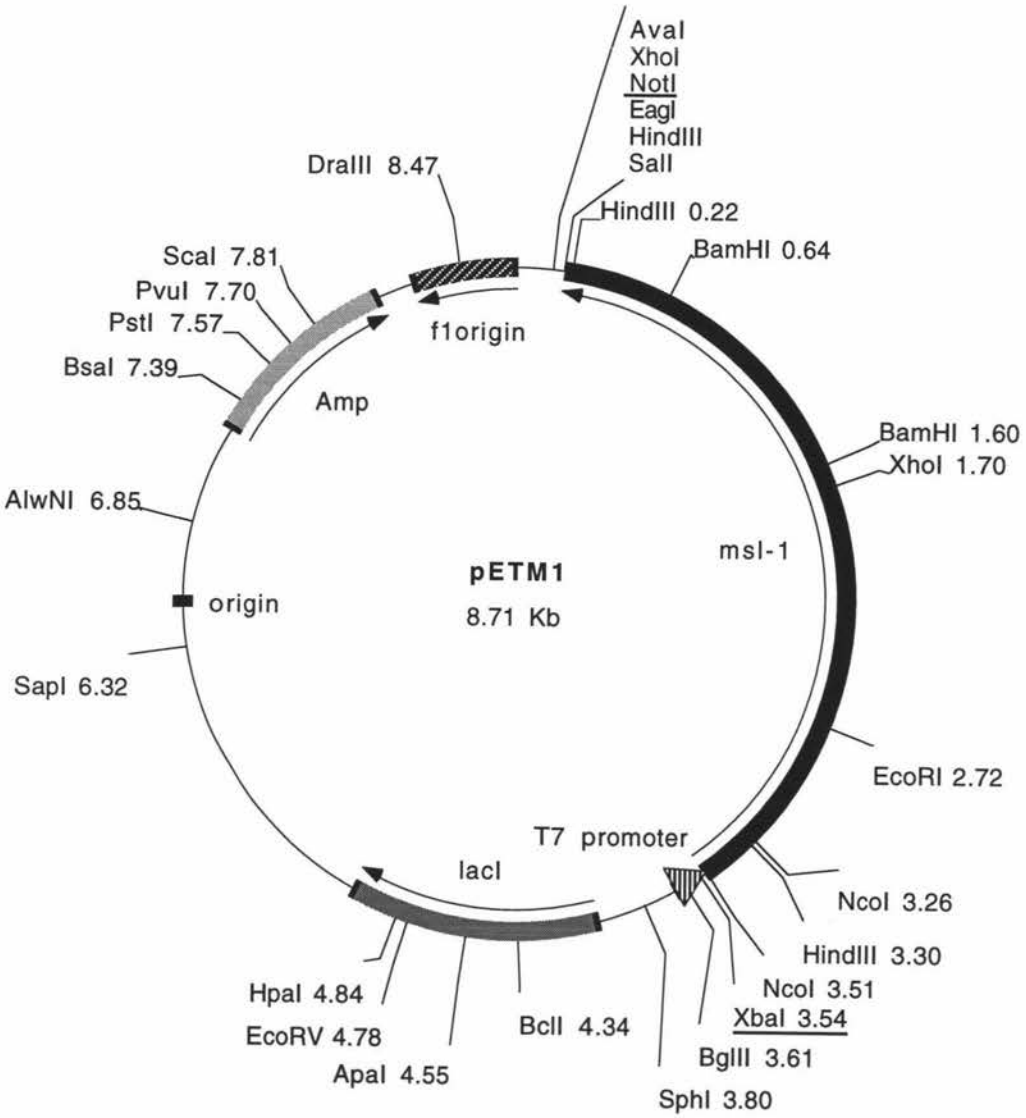
Physical Map of pLP1
11.36 kb, pCaSpeR-hs containing 2.5 kb *mof* coding sequence.



Physical Map of pGEX 4T-1/MOF
7.45 kb, pGEX4T-1 containing 2.5 kb *mof* coding sequence.

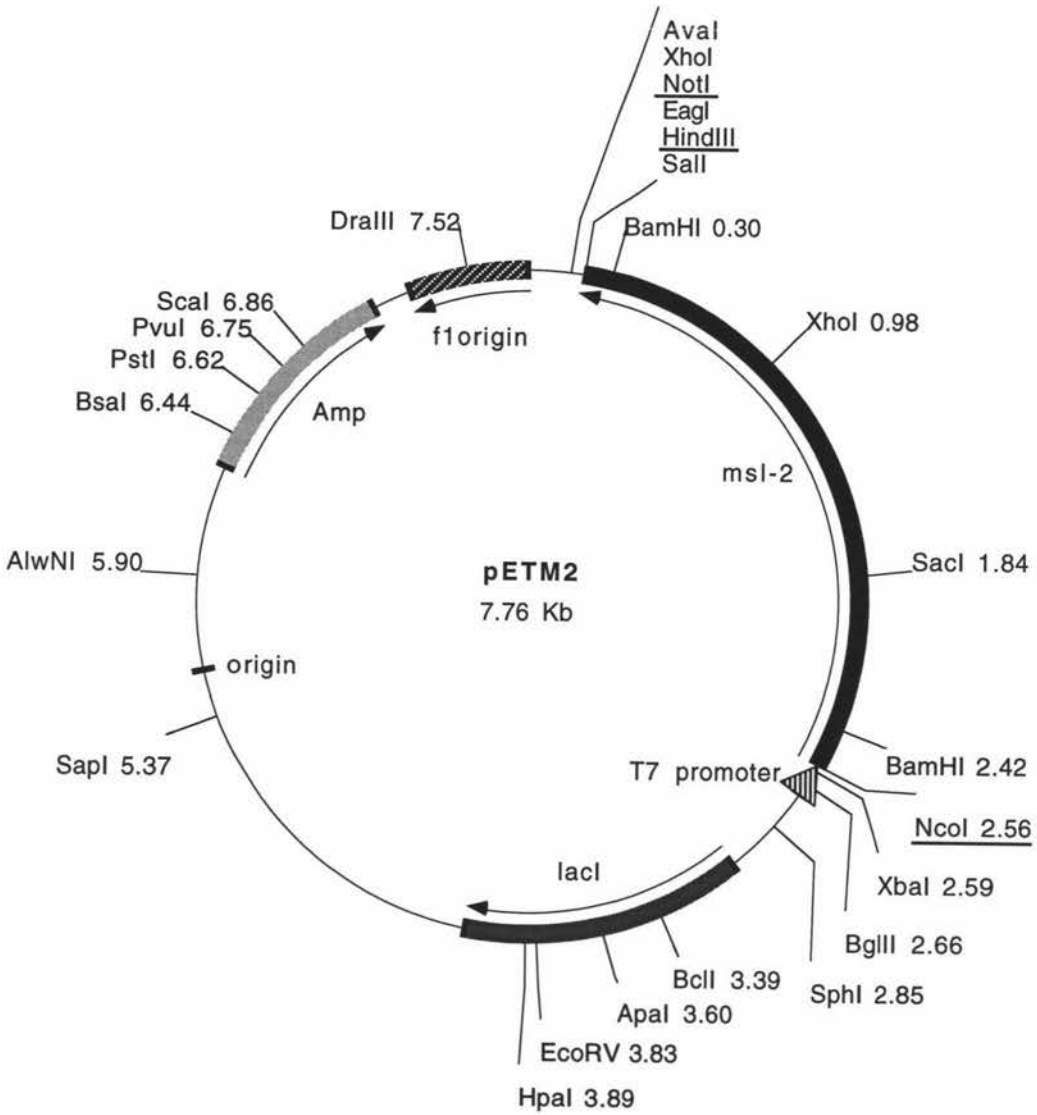


Physical Map of pGEX 4T-3/MOF
7.45 kb, pGEX4T-3 containing 2.5 kb *mof* coding sequence.

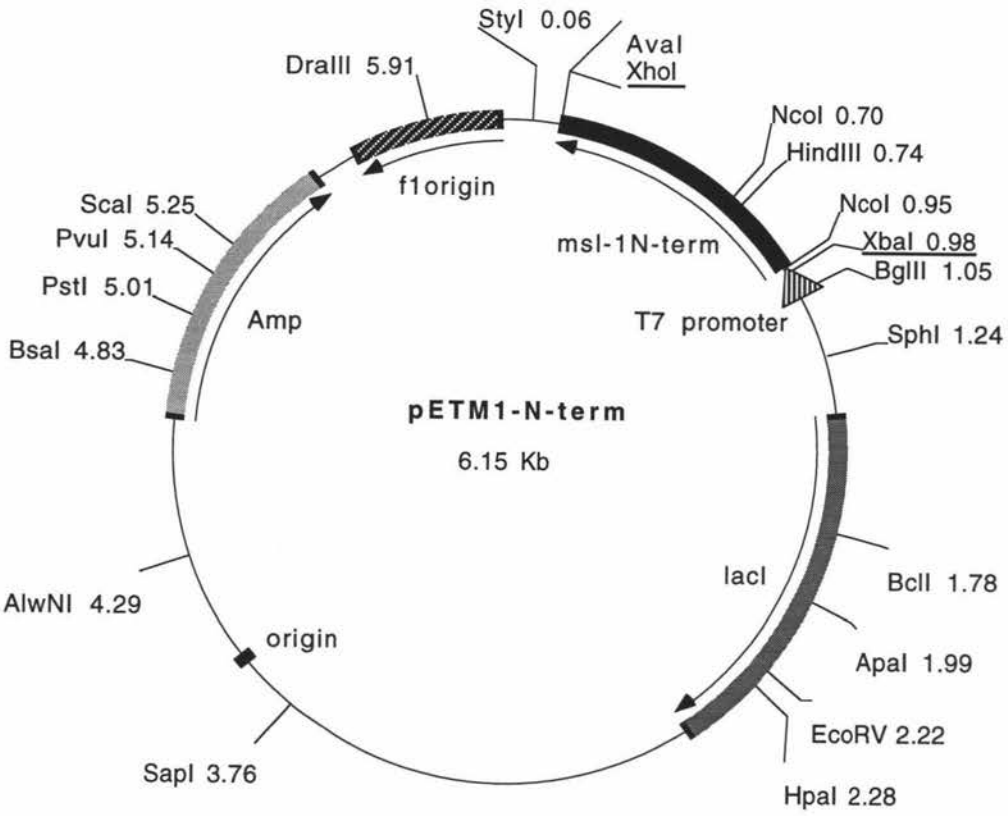


Physical Map of pETM1

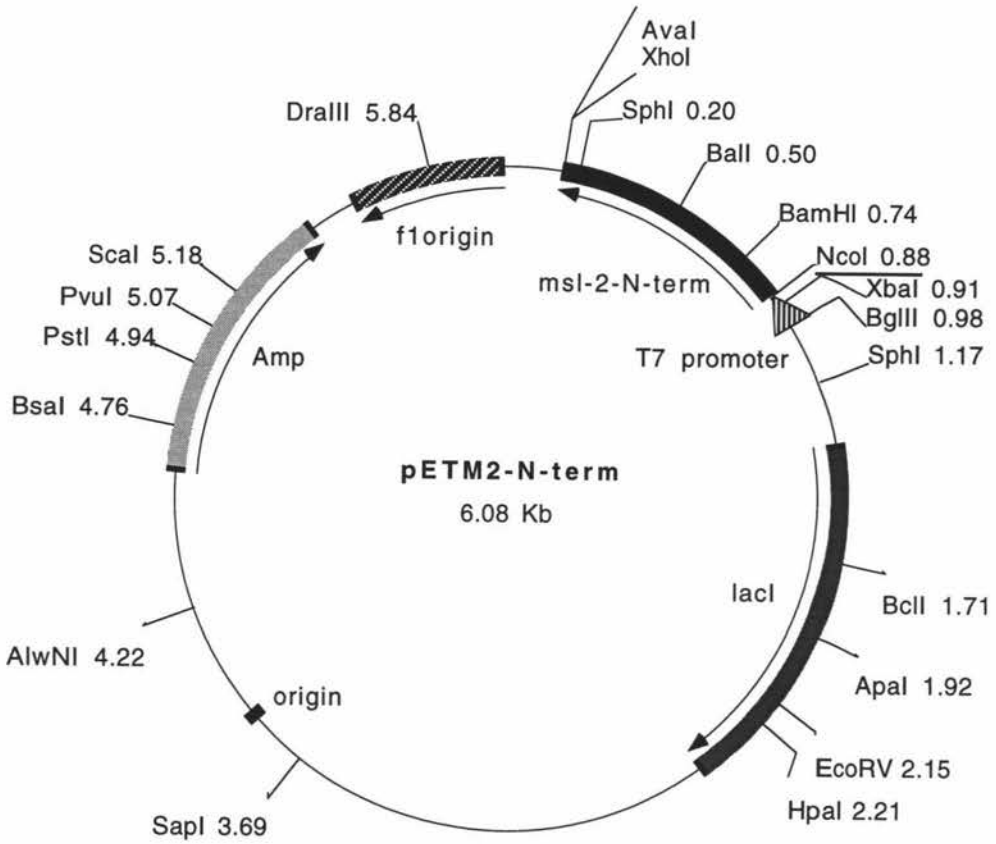
8.71 kb, pET-21d (+) containing 3.3 kb *msl-1* coding sequence.



Physical Map of pETM2
7.76 kb, pET-21d (+) containing 2.3 kb *msl-2* coding sequence.



Physical Map of pETM1-N-term
6.15 kb, pET-21d (+) containing 0.8 kb *msl-1* 5' coding sequence.



Physical Map of pETM2-N-term
6.08 kb, pET-21d (+) containing 0.7 kb *msl-2* 5' coding sequence.

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