A cDNA Subtraction Approach to Isolate Male-Specific Genes From *Ceratitis capitata*

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Simon Francis Kahu Hills
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Abstract

The Mediterranean fruit fly, *Ceratitis capitata* (medfly), is a significant world wide agricultural pest. Sterile Insect Technique (SIT) is a biological method that has been used to control medfly successfully in several parts of the world for around two decades. SIT involves the release of sterile insects into wild populations which, due to sterile matings, lead to a reduction in the size of the wild population. The effectiveness of this technique is significantly increased when only sterile males are released. This can be achieved by using sexing strains, but these strains are prone to breakdown as a result of recombination leading to the disassociation of the selection gene from the Y chromosome. An alternative system, that could be more robust, would involve the control of the expression of the male determining gene.

The aim of this thesis was to identify the male determining gene of medfly by creating subtracted cDNA libraries enriched for male-specific transcripts. Subtracted libraries were made by subtractive suppression PCR, using the ClonTech cDNA subtraction kit. The libraries were screened by Southern hybridisation analysis using male and female total cDNA probes. Only one clone appeared to display a bias toward male-specific hybridisation, but this was found to be a result of unequal transfer of DNA. A selection of clones were individually used to probe membrane bound genomic DNA. These hybridisation analyses indicated a general lack of male–specific enrichment. In addition to this, sequence analysis of a selection of clones revealed a number of mitochondrial gene fragments, showing that there had been insufficient subtraction. As results indicated that the creation of subtracted, male-specifically enriched libraries had been unsuccessful another approach to the identification of the male determining gene was attempted. Genomic DNA was screened with an *Sxl* probe, under low stringency hybridisation conditions, to identify Y-linked encoding RNA binding proteins distantly related *Sxl*, which could represent the male determining gene. This screen showed that there were no male-specific RNA binding proteins, of the SXL family in medfly.

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Finally, special thanks to my family, who were unsure if this would ever be finished. Without your support (and occasional funding) this would never have been possible.

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Abbreviations

α	alpha
β	beta
λ	lambda
°C	degrees Celsius
ADP	adenine diphosphate
ATP	adenine triphosphate
BLAST	basic logic Alignment Search Tool
bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
DEPC	diethyl pyrocarbonate
DNA	deoxyribose nucleic acid
DNase	deoxyribonuclease
dNTPs	dinucleotide triphosphates
DTT	dithioreitol
EDTA	ethylenediamide tetraacetic acid
g	gram
IPTG	isopropyl-ß-D-thiogalactopyranoside
kb	kilo base pairs
L	litres
M	molar
MOPS	4-morpholine-propanesulphonic acid
mg	milligrams
mL	millilitres
mM	millimolar
µCi	micro Curies
µg	micrograms
µL	microlitres
mRNA	messenger RNA
ng	nanograms
nm	nanometres

OD	optical density
Ω	ohms
PCR	polymerase chain reaction
Poly A RNA	polyadenylated RNA
RFLP	restriction fragment length ploymorphisim
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
RT-PCR	reverse transcriptase PCR
SIT	sterile insect technique
UTR	untranslated region
UV	Ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
X-gal	5-Bromo-4-Chloro-3indolyl β-D-galactopyranoside

1 Introduction

1.1 The Mediterranean Fruit Fly

The Mediterranean fruit fly (medfly) *Ceratitis capitata* is one of the world's most significant agricultural pests. Medflies lay their eggs on ripe, tree bound fruit, where the eggs hatch, and the larvae feed. In addition to this the Medfly has a very broad host range of over 200 host plant species (Carry 1991, Sheppard *et al.* 1992, McPheron *et al.* 1994), around 100 of which are of economical importance. This coupled with a wide distribution of the pest (White and Elson-Harris 1992), leads to large annual income loss due to medfly infestation. World wide losses due to medfly have been estimated to be in the range of hundreds of millions of dollars annually (Ashburner 1995).

The medfly is thought to have originated in tropical Africa, and the Mediterranean region. From there it spread through southern Africa, Australia, Hawaii, Central and South America (Davies *et al.* 1999). It continues to threaten agriculture in the USA, Mexico, and Asia. Outbreaks and infestations occasionally occur in other regions such as New Zealand. These small scale outbreaks are usually a result of the importation of contaminated fruit.

There has been a large amount of study into the population genetics and bionomics of medfly, because of its economic impact. A reasonable proportion of this work has been devoted to developing genetic identification methods that would allow an infestation to be traced back to its source population. It is thought that this will aid in the understanding of infestations, and lead to a better ability to control outbreaks at an early stage, before major damage is caused by the pest.

1.2 Control of Medfly

Due to the large economic impact of medfly, there have been many different eradication and pest management strategies developed to deal with the pest. More traditional control methods consist of insecticide bait sprays. These usually have to be applied 2 to 8 times per year, and depend greatly on weather conditions. These poison drops also represent important ecological and health issues. Large scale use of pesticide sprays can have major detrimental ecological effects by eradicating potentially beneficial, non-target species (Oakeshott *et al.* 1993). Studies into the effects of these spraying programs on human health, have shown that the wide spread use of pesticides can cause illness among residents of the target area (Oakeshott *et al.* 1993). Furthermore the effectiveness of these sprays is questionable. Elimination of a large population in a single spray is virtually impossible, so continual use of the spray is required to control the pest. Continual use of the spray can lead to selection for resistance to the pesticide in the pest species. After resistance has developed the use of the pesticide will provide selection pressure that will allow the resistant population to thrive. Once resistance has developed the spraying processes must be carried out with a new pesticide. In addition to this the extremely wide distribution of medfly means that effective spraying operations are large and expensive.

A system which does not involve large scale use of potentially dangerous chemicals is needed to effectively control the medfly, and other pests. The most effective system that fits these parameters, that has been implemented for the control of several insect species, is known as Sterile Insect Technique.

1.3 Sterile Insect Technique

The sterile insect technique (SIT) was first developed by Dr E. Knipling to control insect populations on a large scale (Knipling 1960). The technique involves the mass rearing and release of large numbers of sterile insects. The sterile insects released compete with the wild population for mates. Every mating between a sterile individual and a wild individual will not produce progeny. In this way large numbers of sterile insects competing for mating will cause a marked decrease in the numbers, of the wild population, in the subsequent generation. When this type of release

program is repeated over several generations of the target species, the wild population can be effectively wiped out completely.

SIT was first used to control populations of the New World Screwworm in the South-Eastern USA, in 1957.

The technique has possible applications for many species of agricultural pests, and some species that carry disease. Indeed, sterile insect technique has been used successfully to control several insect species including; tsetse flies, codling moth, melon fly, Mexican fruit fly, and the Mediterranean fruit fly. Several species of mosquitoes have also been trialled for SIT programs. In addition to these, a number of species of insect are currently under development for use in SIT, including the Australian sheep blow fly *Lucilia cuprina*.

1.3.1 Medfly in SIT

C. capitata has been the target of several sterile insect control programs. The largest control effort was the MOSCAMED program in Central America (Linares and Valenzula 1993, Liedo *et al.* 1993, Orozco *et al.* 1994). This program was developed by the Mexican government, in conjunction with the USA and Guatemala, after the detection of medfly in Guatemala in 1976. This followed the rapid infestation of other parts of Central America by medfly. By 1980 a mass rearing facility had been established, and was producing its goal of 500 million sterile flies per week. Combined with small scale chemical suppression of the wild population, the sterile insect release program led to the eradication of wild medfly from Mexico by 1982. After this a 'sterile fly barrier' was set up at the Mexico-Guatemala border to prevent the northward spread of the fruit fly into Mexico, and the USA beyond. This 'sterile fly barrier is still maintained, and it protects the fruit crop industry in the lower USA such as in the California region.

1.3.2 Sexing Systems

SIT is much more efficient when only sterile males are released (Economopoulos 1996). It is advantageous to release males only, as females even though they are sterile and cannot lay fertile eggs, will still attempt to lay and cause damage to the fruit. The damage to the fruit from sterile females can then be invaded by other pathogens, such as fungi and bacteria (Ashburner 1995). This would undermine the

effectiveness of the system in areas where fruit crops are in immediate threat. Also the effectiveness of the technique can be increased with the release of males only, as this will eliminate the occurrence of matings between sterile flies (Franz and Kerremans 1994). Another advantage of releasing only sterile males is that a single male fly will mate with many females, unlike the female which will mate only once.

Sex separation systems have been developed to allow the separation of male and female flies. These systems are based on morphological, or biochemical differences that allow mass separation of the sexes. Where no adequate natural differences are found, genetically engineered sexing strains need to be developed. Genetic sexing strains provide a system whereby the different sexes can be easily separated so only males are released.

Sexing strains are based on translocations between a chromosome containing one allele, usually the wild-type, of a chosen selection gene, and the sex determining Y chromosome. Females are homozygous recessive for the mutant allele of the selection gene. All males will express the wild-type phenotype for the mutant allele of the selection gene as it is attached to the male determining chromosome. The selection system distinguishes between mutant and wild-type expression, and selects for only wild-type there by producing only males.

The downfall of this translocation based system is that it is prone to breakdown after several generations. This breakdown is the result of recombination in the males which uncouples the wild-type selection marker gene from the sex determining chromosome (Willhoeft *et al.* 1996, Franz *et al.* 1996). In the subsequent generations this produces wild-type females and mutant males, thus rendering the selection system ineffective.

Sexing strains can be divided into two general categories, sex sorting and sex killing (Robinson 1990, Rossler *et al.* 1994).

Sex sorting involves physical separation of males from females based on observable differences. These strains are based on genes where different morphologies can be seen in the sexes before adulthood. This makes males easily distinguishable from females. Females can then be physically removed. Sex sorting systems are hampered by a need for specialised sorting equipment, and that this equipment is not totally accurate and can damage pupae.

Sex killing strains are based on selection genes which have biological function. Mutant genes used for such strains cause an inability to complete a metabolic

function, or provide resistance to an environmental condition. Selection is achieved by applying the correct environmental condition to induce female death, during the rearing process. Genes that have been used for this type of sexing strain include; pesticide resistance (Wood 1990), alcohol dehydrogenase (Riva Francocs 1990), and temperature sensitive lethals (Franz *et al.* 1996). The problem with these sexing strains is that they require the addition of expensive chemical agents to induce female specific death.

1.3.3 Medfly Sexing Systems

Research has established that there are no natural sexually dimorphic traits or mutations that can be used for separation of male and female medflies. This has lead to the development of sexing strains produced by genetic modification. Two widely used sexing strains use pupal colour, or temperature sensitive lethals as selection genes.

Pupal colour was used in the first medfly sexing strain that was applied in the field. Males express the wild-type phenotype, and have brown pupae. Females express the mutant phenotype, which is black (Walder 1990, Zapter 1990), but white pupal mutants have been utilised as well (Economopoulos 1990). The sexes are separated using optical sorting machines. There are a number of genes which have been identified as having a role in pupal colour. Several of these have been used for the creation of sexing strains.

More recently sexing stains based on temperature sensitive lethal genes have been developed (Franz *et al.* 1996, Rendon *et al.* 1996). These sex killing strains result in female specific death after heat shock. Temperature sensitive lethal sexing strains are replacing the pupal colour based strains in medfly rearing plants. This is due to the cost reduction of not needing sorting equipment and, because selection is applied in late embryogenesis, savings are made in larval feed costs.

1.4 Sex Determination

1.4.1 Drosophila

The most well studied Dipteran sex determination system is that of *Drosophila melanogaster*. In *D. melanogaster* sex is determined by the ratio of X chromosomes to autosomes (A). Male development is determined by a ratio of 1X:2A, and female development is determined by 2X:2A (for review see Cline 1993). In females the chromosomal ratio switches on the key gene *Sex-lethal* (*Sxl*). SXL is an RNA binding protein that leads to the splicing of its targets. SXL binds both *transformer* (*tra*) pre-mRNA, and its own RNA. Splicing of its own RNA leads to the autoregulation of *Sxl*, continuing female specific expression. Unspliced *Sxl* mRNA does not produce functional protein due to the presence of multiple in-frame stop codons.

The splicing of *tra* is the next step in the sex determination pathway, and is mediated by SXL. Without splicing *tra* mRNA, like *Sxl*, will not produce functional protein. TRA protein resulting from correct splicing of *tra* mRNA promotes the splicing of *double-sex* (*dsx*). DSX is a transcription factor, which controls the global expression of further sex specific genes. The splicing of *dsx* mRNA is dependent on the presence of both TRA and the constitutively expressed TRA-2. In the presence of both TRA and TRA-2, *dsx* is spliced female-specifically. When functional TRA is not present *dsx* is spliced male-specifically. This leads to two sex-specific forms of the DSX protein, DSX^M and DSX^F. Male specific DSX^M represses female specific genes and activates male specific genes. DSX^F represses male specific genes and activates female specific genes. These two gene products differ in the C terminal ends. This difference provides the sex specific response, otherwise the proteins are identical. The conserved amino terminal domain is responsible for DNA binding (Schutt and Nothiger 2000). It has been demonstrated that this system is well conserved across the *Drosophila* genus (Erickson and Cline 1998). Figure 1.1 gives a graphic summary of the *Drosophila* sex determination pathway.

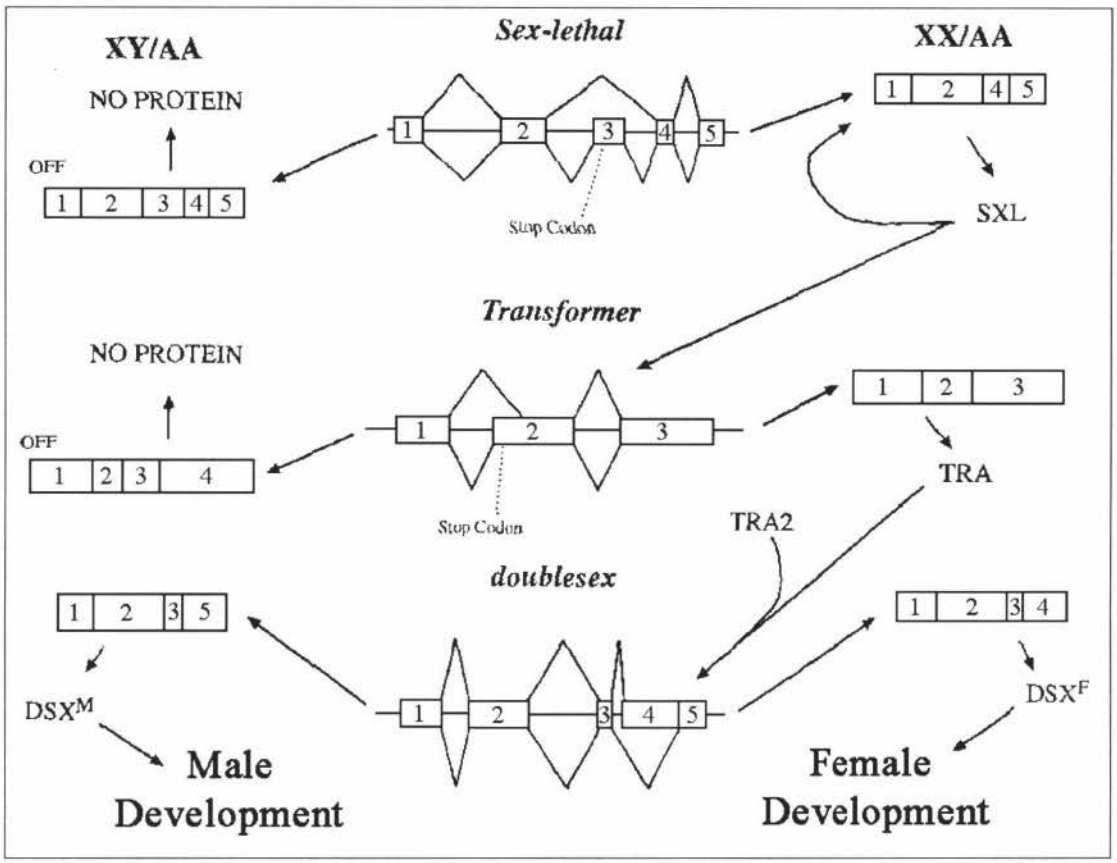

Figure 1.1: The pathway of sex determination in *D. melanogaster*.

In females the X:A ratio signals for female specific splicing of the mRNA of the *Sxl* gene. The SXL protein then promotes female specific splicing of *tra*. TRA then promotes female specific splicing *dsx*, which controls global female gene expression. In males *sxl* is not spliced. An in-frame stop codon leads to translation non-functional protein, which is degraded. Without SXL protein, *tra* is not spliced. In the same way as *Sxl*, an in-frame stop in *tra* codon leads to non-functional protein which is degraded. In the absence of TRA, *dsx* defaults to a male specific form. DSX^M then controls the global expression of male specific genes.

(Figure adapted from Saccone et al 2002)

1.4.2 Other Dipteran flies

Across the greater group of Diptera, comparisons have found that the *Drosophila* system is not widely conserved (reviewed in Schutt and Nothiger 2000).

As *sex-lethal* is the master switch gene it has been a focal point in the studies of other insect sex determination systems. In the housefly *Musca domestica* the *sex-lethal* gene has been found to be highly conserved. However the SXL protein is expressed in the same form in both sexes in the housefly (Meise *et al.* 1998). In addition to the differences in *sxl*, the primary sex determination signal is different in *M. domestica*. The determination of sex in housefly is dependent on the M and F genes. M determines maleness, and is the primary switch. F is required for female development, and is thought to represent a key gene, which is analogous in function to *sxl* (Hilfiker-Kleiner *et al.* 1993). The activity of the M element has been demonstrated to consist of two parts. One is located on the short arm of the Y chromosome, and the other on the long arm. The separate activities of these elements appear to be additive, where the presence of only one copy will result in an intersex phenotype (Hediger *et al.* 1998). Neither M nor F have been characterised at this time.

The system seen in *M. domestica* appears to be similar to *L. cuprina* and could be similar to *C. capitata*. It is probable that the *L. cuprina* sex determination system is more like that of *C. capitata* then the *M. domestica* system. This is because the M factor of *M. domestica* has been found in different linkage groups in different strains, meaning that the gene can be translocated and remain active. *L. cuprina* and *C. capitata* both appear to have M factors that are immobile on the Y chromosome (Shearman 2002).

In contrast to the interspecific sequence similarities and functional differences observed in *sxl*, there have been studies which have shown that there is a high degree of difference in the coding sequence of *tra*. Even within the *Drosophila* genus *tra* exhibits a high degree of divergence, and is considered to be one of the fastest evolving *Drosophila* genes (O'Neil and Belote 1992). However evidence suggests that *tra* has functional relatives in other species (Pane *et al.* 2002), and that *tra-2* has functional conservation in a species as distantly related as humans (Dauwalder *et al.* 1996).

Studies have indicated that *dsx* may be highly conserved across a broad range of species (Marin and Baker 1998, Graham 2003). A *dsx* homologue has been identified in *Bactrocera tryoni* (Queensland fruit fly) (Shearman and Frommer 1998), and evidence suggests that the distantly related *Megaselia scalaris* also exhibits sex-specific *dsx* RNAs (Kuhn *et al.* 2000). Evidence exists for the conservation of *dsx* homologues across greater evolutionary distances, given similarities seen in the *dsx* of *D. melanogaster* and the *Mab-3* gene of *C. elegans* (Marin and Baker 1998)

This evidence indicates that sex determination cascades evolve from the bottom up. The most ancestral and well conserved genes (e.g. *dsx*) occupy the bottom of the pathway, and the more recently evolved or co-opted genes occupy the top. This provides evidence that the master control genes and primary signals at the top of sex determination pathways can be variable between different species (Marin and Baker 1998, Graham *et al.* 2003)

1.4.3 Medfly

Male development is determined by the presence of the Y chromosome in *Ceratitis capitata*. Specifically there is a dominant male determining region located on the long arm of the *C. capitata* Y chromosome, near the centromere (Willhoeft and Franz 1996). This appears to be similar to the sex determination system found in both *M. domestica* and *L. cuprina*. It is currently unknown whether this region contains one or several male determining factors, although theoretically one would be sufficient.

Studies with *C. capitata* have demonstrated that the *sex lethal* protein sequence is highly conserved, but uniformly expressed in both sexes (Saccone *et al.* 1998). Further, no evidence has been found for sex-specific splicing. This indicates that *sxl* has no role in sex determination in medfly.

Recent studies with *C. capitata tra* have demonstrated that while the sequence is not conserved, its function is similar to *D. melanogaster*. At the sequence level *C. capitata tra* only has 32-40% identity to *D. melanogaster tra* (Graham 2003). Pane *et al.* (2002) have demonstrated that *C. capitata tra* is required for female specific splicing of *dsx*. In addition to this the presence of TRA/TRA2 binding elements indicate that a *C. capitata tra-2* homologue may exist (Pane *et al.* 2002). Pane *et al.* (2002) also demonstrated that *tra* exhibits a self-regulation loop much the same as *D. melanogaster sxl*. As a result of this *tra* work a model for *Ceratitis capitata* sex determination has been suggested (Figure 1.2).

Compared to *D. melanogaster*, the *C. capitata dsx* control function is quite well conserved. TRA/TRA-2 binding sites are observed in similar positions in the *dsx* sequences of *D. melanogaster and C. capitata* (Saccone t al 2002). In addition to this the structure of *dsx* mRNA is conserved between the two species (Saccone et al 1998).

1.5 Male Determination and SIT

The medfly male determining gene would allow females to be transformed into males by induction during early development. This could decrease the cost of SIT by eliminating the waste of resources, which results from the removal females from the release population. In addition it will alleviate the problem of breakdown of sexing strains as a result of Y linkage collapsing. The system may be adapted to work in related pest species such as *Ceratitis rosa*, which is a significant pest in South Africa. Utilisation of this in SIT would involve the creation of a construct containing the male determining gene under the control of a heat-shock promoter. Medfly successfully transformed with this construct would normally not express this copy of the male-determining gene. However after incubation at a temperature that activates the promoter, expression of the transformed male-determining gene would then be activated. This heat shock would have to be applied during early development for male development to be properly induced. Thus heat shock of a population of a strain containing such a construct would result in an entirely male population.

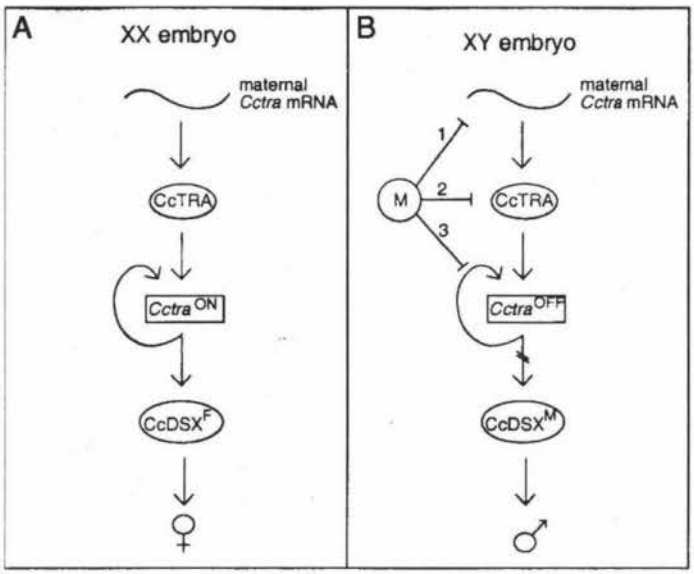

Figure 1.2: A model for medfly sex determination.

In the female medfly *tra* mRNA is spliced into an active from that is capable of autoregulating its own expression, there by maintaining female expression (A). In the male a male determining factor prevents splicing of *tra*, so an in-frame stop codon leads to an inactive protein (B).

The Male determining factor of medfly could act in one of three ways; prevention of translation of *tra* mRNA (1), direct inhibition of the TRA protein (2), or interference with TRA autoregulation to cause its function to be switched off (3). (Figure from Pane et al. 2002)

1.6 cDNA Subtraction

Subtractive cloning is an effective method for isolating differentially expressed genes in populations of mRNA or DNA. The technique is regularly used to identify novel genes expressed in cancer cells by comparing with gene expression of non-cancer cells. It has also been used for the identification of developmental genes by comparison of cells at different developmental stages. In insects the technique has been used successfully to identify a novel cold shock recovery gene (Goto 2001), and a novel early developmental patterning gene (Taylor 2000) in *D. melanogaster.*

The subtraction method requires two populations of mRNA or DNA, a 'tester' sample and a 'driver' sample. The tester sample contains the target fragments while the driver sample contains everything in the tester except the target sequences. The method works by denaturing the tester and driver populations, allowing them to hybridise together with an excess of driver, then all driver/driver and tester/driver hybrids are removed. This process is then repeated a number of times to ensure efficient subtraction. In general there are two approaches to subtraction, which differ in the method for removing driver hybrids. Traditional methods make use of biotinylated driver and remove hybrids with streptavidin and phenol extraction (Ausubel *et al.* 1997, Sive and St. John 1988). More recently PCR based subtraction methods have become available. PCR based subtraction techniques have several advantages over traditional methods. Firstly the PCR based method is quicker and simpler to use. Secondly the PCR based method is more sensitive, and can isolate rare fragments. Thirdly, commercial cDNA subtraction kits are available, which make the process significantly more efficient.

PCR based cDNA subtraction makes use of two techniques to produce a subtracted and enriched library, subtractive hybridisation and suppression PCR. Subtractive hybridisation allows removal of sequences that are common to two populations of cDNAs. Suppression PCR allows only specific target sequences to be amplified. The Clontech PCR-Select™ cDNA subtraction kit utilises amplification primers embedded in adapters, which are ligated onto samples of the tester and driver cDNAs. Through a combination of hybridisations and PCR subtraction is achieved. Figure 1.3 illustrates the events of subtraction using the Clontech kit.

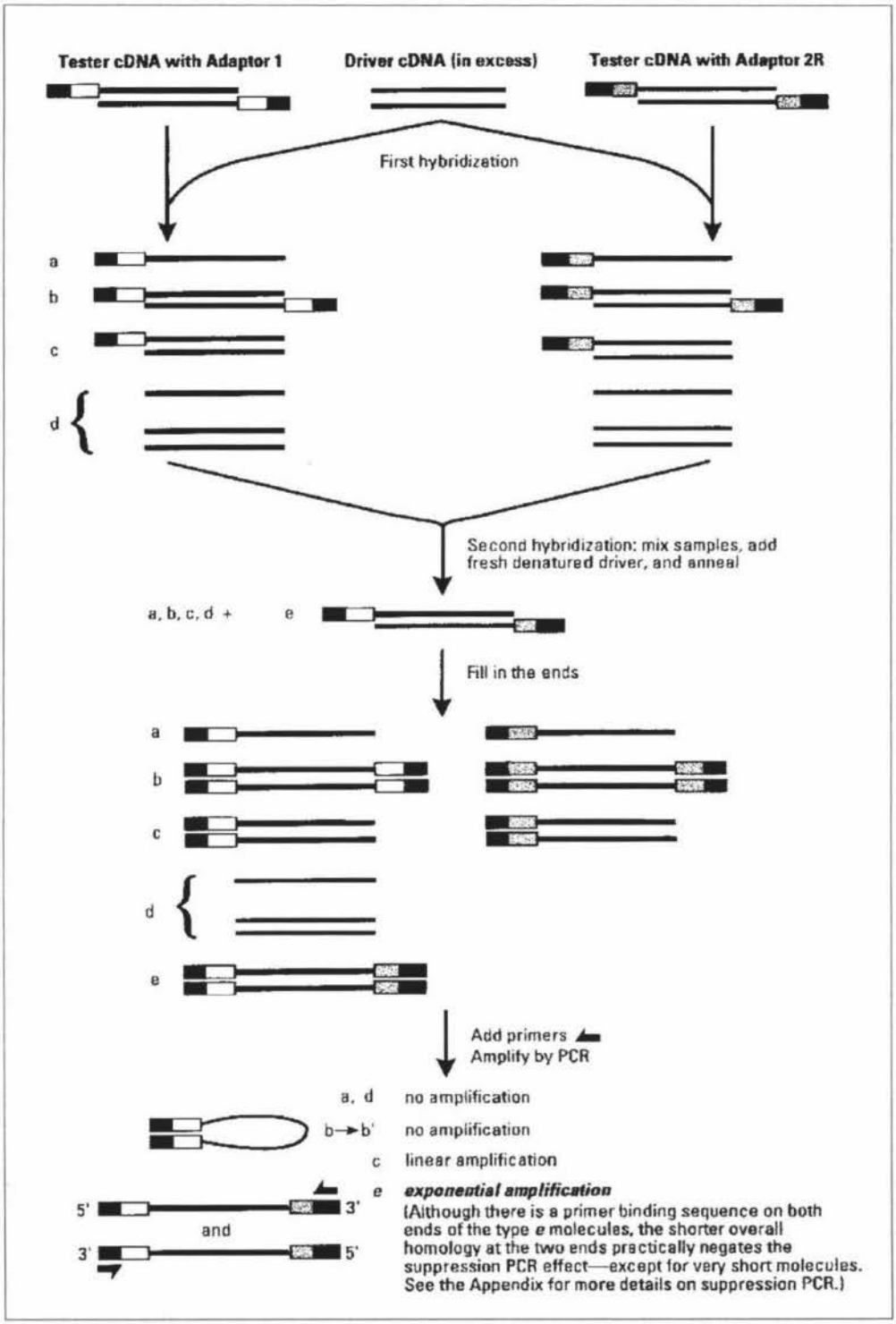

Figure 1.3: A schematic diagram of the events of PCR-Select cDNA subtraction. Bold lines represent *Rsa* I digested cDNA. Solid boxes represent the parts of two adapters that are the same, and contain a common PCR priming site. The clear and shaded boxes represent the parts of the two adapters that are different these contain specific nested PCR primers, that are unique to each adapter. (Figure taken from CLONTECH PCR-Select^{TM} cDNA Subtraction Kit Users Manual)

1.7 Research Objectives

The aim of the research detailed herein was to attempt to isolate the male determining gene of *Ceratitis capitata* by screening a subtracted cDNA library for likely candidates.

This aim was to be achieved by through the following objectives:

- Screening an existing subtracted cDNA library for male-specific candidates
- confirming candidates by Southern hybridisation with total genomic DNA targets
- sequence candidates, and identify known homologues

When the first subtracted library was found to be of limited quality the aim of this research was expanded to include the production of a subtracted cDNA library. The objectives of the project at this time were to:

- produce a subtracted, male-specifically enriched cDNA library
- screen subtracted library by colony hybridisation
- confirm candidates by Southern hybridisation
- sequence candidates, and identify known homologues

After the second subtracted library was found to lack any male-specific sequences a contingency experiment was conducted. The aim of this experiment was to identify male determining gene candidates through the identification of male-specific genes encoding RNA binding proteins. This would be achieved screening genomic DNA for Y-linked genes encoding RNA binding proteins by low stringency Southern hybridisation.

2 Methods

2.1 mRNA Extraction

Samples of medfly total male RNA and total female RNA were obtained from Australia for the purpose of mRNA extraction. mRNA was purified from total medfly RNA samples using an oligo-(dT) spin column based mRNA Purification Kit from Amersham. The protocol supplied with the kit was followed.

2.2 cDNA Subtraction

Subtracted cDNA libraries were made using the Clonetech PCR-Select™ cDNA Subtraction Kit. The initial library was made prior to the work described here, and therefore the details of its making are omitted. The repeat library was made as set out in the user manual for the kit. In general the protocol set out in the user manual was followed. The following is a brief dissection of the protocol, out lining deviations and specific points.

2.2.1 cDNA synthesis

First-strand synthesis was performed on the male and female mRNA obtained by mRNA purification. *AMV reverse transcriptase* (20 units/µl), as supplied with the subtraction kit, was used. [α-^{32}P] dCTP was not used to monitor the reactions. Control mRNA, supplied with the subtraction kit, also underwent first-strand synthesis. The cDNAs produced from this RNA were used for control reactions throughout the subtraction.

All samples from the first-strand synthesis were then put trough second-strand synthesis to produce double stranded cDNA. Second strand synthesis was carried out using the Second-Strand enzyme cocktail (DNA polymerase I, 6 units/µl; RNase H, 0.25 units/μL; *E. coli* DNA ligase, 1.2 units/μL) supplied with the subtraction kit.

6 µl of second-strand synthesis product was kept from each sample for subsequent quality control.

2.2.2 *Rsa* I digestion

A standard restriction digest using *Rsa* I was conducted to produce shorter, blunt ended fragments for subsequent ligation with adapters. After *Rsa* I digestion, 5 µl samples of the digested cDNA were checked against undigested cDNA by gel electrophoresis (Section 2.9.1). This test was to confirm that the *Rsa* I digestion was successful, and to check the size distribution of cDNAs after digestion.

Following digestion the DNA was purified by phenol:choloroform:isoamyl alcohol extraction. This was done using reagents, and following the protocol set out in the user manual for the subtraction kit.

2.2.3 Adapter ligation

At this point in the protocol tester and driver cDNAs were separated. Adapters were only ligated to the tester cDNAs. For each tester cDNA population two adapter ligation reactions were run, one reaction with adapter 1, the other with adapter 2R. Both these adapters are supplied with the cDNA subtraction kit, and are the key to the PCR based method of subtraction.

Before proceeding with the subtraction a ligation efficiency analysis, as described in Section V C of the CLONTECH PCR-Select cDNA Subtraction Kit user manual, was performed. This PCR based test utilises primers specific for the adapter sequences, and requires a set gene specific primers. In this test primers for medfly *pgd* gene were chosen for the gene specific primers (Table 2.1).

2.2.4 Hybridisations

In the first hybridisation each of the tester cDNA samples was hybridised with an excess of driver cDNA. cDNAs common to both populations hybridise, leaving cDNAs specific for the tester population in a single stranded form. The first hybridisation was incubated for 8 hours at 68°C, as recommended by the user manual as the optimal incubation time.

For the second hybridisation the two tester populations (with different adapters) were combined with out allowing tester/driver hybrids from the first hybridisation to denature, in the presence of fresh driver cDNA. The cDNAs specific to, and common to the tester populations anneal producing double stranded cDNAs that have both adapters.

2.2.5 PCR amplification

Immediately prior to PCR cycling, samples were incubated at 75°C for 5 minutes. This was done so that the adapter sequences, which are single stranded until this point, can be filled in. This creates priming sites found in the antisense strands of the adapters. Differentially expressed cDNAs were selectively amplified by PCR. Selective amplification is achieved by using primers designed for the adapters. Only double stranded cDNAs with both adapters (i.e. one on each end) should be exponentially amplified.

Due to cost restraints the 50X Advantage cDNA Polymerase Mix, recommended in the cDNA Subtraction kit user manual, was not used. Standard *Taq* DNA polymerase was used in its place, as the manual advises this can be done. PCR reactions with standard *Taq* required significantly more PCR cycles. In general between 5 and 10 extra cycles were required to produce PCR products. In several cases no product could be seen after twice the recommended number of cycles, in these situations PCR was repeated with fresh solutions.

The PCR amplification involved two rounds of PCR with different primers that were supplied with the cDNA subtraction kit. The primary PCR used PCR Primer 1, which anneals to both adapters allowing amplification of any sequence with adapters on each end. The second round of PCR makes use of two nested primers. Each of these nested primers is specific to one adapter each. This leads to the amplification of only sequences that have one of each adapter. The nested primers are supplied with the subtraction kit.

2.2.6 Analysis of Subtraction

Upon completion of the cDNA Subtraction two PCR based tests were carried out, as outlined in the subtraction manual. These tests asses the effectiveness of the subtraction using serial PCR, samples were taken from the PCR reaction after increasing numbers of cycles. The subtraction efficiency test gave an indication of how effectively common sequences had been removed. The enrichment test indicated whether target sequences have been enriched for, in this case whether or not there had been an enrichment of male-specific sequences.

Medfly specific primers were needed for both of these tests, one set for a universally expressed gene and one set for a male-specific gene. GeneBank was searched using these parameters, and primers were designed from sequences found (see Table 2.1)

2.3 Cloning

2.3.1 Subcloning

pGEM®-T easy

The pGEM®-T easy vector (Promega) (appendix 1) was used for subcloning PCR products. The vector was chosen for its improved ligation efficiency with PCR products. This is due to a 3`-T overhang at the insertion site, which is designed to make use of the 3`-A overhang found in *Taq* amplification products. Ligations were carried out as set out in the technical manual supplied with the pGEM vector system. Ligations were incubated at 4°C overnight, for maximum ligation efficiency.

pDrive

The pDrive vector (Qiagen) (appendix 1) is similar to pGEM®-T easy in that it is designed to make use of the A overhang of *Taq* PCR products. pDrive however uses a U overhang on each end of the insertion site. This U overhang has a higher ligation efficiency with the A overhang of the PCR products. Ligations were carried out as described for the use of this vector in the Qiagen® PCR Cloning Handbook supplied with the vector. Ligations were incubated overnight at 4°C, for maximum efficiency.

pBluescript KS

The vector pBluescript KS (Stratagene) (appendix 1) was also used for some subcloning. Prior to ligation both vector and inserts were digested with *Not* I. Digests were then checked by gel electrophoresis, and concentrations estimated. 10µl ligations, containing: 1 µL of vector (200 ng), 7µl of insert, 1µl of ligation buffer, and 1µl of T4 DNA ligase; were incubated overnight at 16°C.

2.3.2 Transformation

Chemical Transformation

Chemical transformations were carried out using frozen DH5α competent cells (obtained from Mr Atupattu). 1 μL of ligation solution was added to 200 μL of cells and placed on ice for 30 minutes. The cell/vector solution was then heat shocked at 42°C for 30 seconds. Following heat shock 800 μL of SOC (tryptone 20g/L, yeast extract 5g/L, NaCl 0.6g/L, KCl 0.2 g/L, with 9.52g/L of 1M $MgCl_2$ and 10mL/L of 2M glucose solution) was added to the cells and they were allowed to recover for 90 minutes at 37°C while shaking at 200 rpm. After recovery 100 μL of transformed cells were plated out. The remainder of the transformation reaction was spun for 30 seconds at 13000 rpm. All of the supernatant except about 100 μL was discarded, and the pellet was resuspended in the remainder and plated on a new plate. Transformations were plated on LB Amp/IPTG/X-gal plates for overnight incubation, to make use of the blue-white selection capabilities of both vectors. Plates were incubated at 37°C overnight.

Electro-Transformation

For the purpose of high yield transformation, ElectroMAX™ DH10B™ cells (Life Technologies) were used. The transformation procedure supplied with the cells was followed. Electroporation was carried out at 2.5 volts with 200 Ω of resistance and 25 μF of capacitance, in a BioRad genepulser with a BioRad pulse controller. Electroporation cuvettes from EquiBio were used. Following electroporation 1 mL of SOC was added to the eletroporation cuvette, the SOC/cell mixture was then transferred to a microcentrifuge tube. Form this point the cells were treated in the same way as previously described for the chemical transformation of DH5α cells.

2.3.3 Plasmid DNA Preparation

Alkaline lysis minipreps, as described in Ausubel *et al.* (1997) (Unit 1.6), were performed to isolate small amounts of plasmid DNA (less than 20μg). Diagnostic restriction digestions were carried out on the resulting plasmid preparations, and checked by gel electrophoresis. Where RNA contamination was seen to be a problem, 1μl of 10mg/ml RNase was included in digestion reactions.

2.4 Plating of Subtracted library

Transformed colonies resulting from subtraction were individually picked from initial selection plates and plated on LB/IPTG/X-Gal/Amp plates with 1 cm spaced grid lines, allowing 52 colonies per plate. Due to a large number of colonies, it was decided to screen the library and identify potentially important clones before making glycerol stocks of clones of interest. During the screening process the colonies were maintained on LB/IPTG/X-Gal/Amp plates. On a weekly basis colonies were individually picked and transferred to new plates, under sterile conditions. These plates were incubated overnight at 37°C then stored at 4°C.

2.5 PCR Amplification of DNA

The oligonucleotide primers that were used in PCR reactions described herein are tabulated in Table 2.1.

2.5.1 Standard PCR

PCR with *Taq* polymerase (Roche) was carried as per manufacture's instructions, unless otherwise stated. All PCR reactions were carried out with 50µl total volume. Unless otherwise stated PCR reactions were at 1.5 mM $MgCl_2$ concentration.

Cycling conditions:

Taq polymerase with *M13* forward and reverse primers (see Table 2.1)-

	Temperature	Time	Number of cycles
Initial denaturation	94°C	2 minutes	1
Denaturation	94°C	30 seconds	30
Annealing	45°C	30seconds	
Extension	72°C	120 seconds	
Final extension	72°C	7minutes	1

Table 2.1: Oligonucleotide primers used in this study.

Primer	Sequence 5'-3'	Tm (°C)	Use
PCR primer 1 [1]	CTAATACGACTCACTATAGGGC		Primary PCR of cDNA subtraction protocol
Nested PCR primer 1 [1]	TCGAGCGGCCGCCCGGGCAGGT		Secondary PCR of cDNA subtraction protocol
Nested PCR primer 2R [1]	AGCGTGGTCGCGGCCGAGGT		Secondary PCR of cDNA subtraction protocol
G3PDH 5' [1]	ACCACAGTCCATGCCATCAC		Subtraction control PCR
G3PDH 3' [1]	TCCACCACCCTGTTGCTGTA		Subtraction control PCR
M13 forward [2]	CCCAGTCACGACGTTGTAAAACG	52	Standard PCR of clones
M13 reverse [2]	AGCGGATAACAATTTCACACAGG	48	Standard PCR of clones
Sxl forward [3]	CAGGATATGACAGATCGCCA	60	Amplification of *sxl*
Sxl reverse [3]	CCGAGCTGAGCATATAGTG	60	Amplification of *sxl*
MedflyPgd + [3]	CCGTTATGGGTCAGAATCTC	60	Amplification of *Pgd*
MedflyPgd - [3]	CTCACTTTAGTTCCCGTACC	60	Amplification of *Pgd*
CcXDHfor [3]	GTTTCTTCCGTTGTGCCGTG	62	Subtraction efficiency test
CcXDHrev [3]	CCACCAACGAACGGCGATAC	64	Subtraction efficiency test
Mssp-a2F1195 [3]	CAGGAAATGGGGCATAATAG	58	Male-specific enrichment test
Mssp-a2rev1347 [3]	TACACATCAATAGGGCTACC	58	Male-specific enrichment test
ELAVF1 [3,4]	TAYGGTTTYGTIAATTAYGT [6]	51	Degenerate PCR for *elav*
ELAVF2 [3,5]	GTIGGTTTYATHMGWTTYGA [6]	55	Degenerate PCR for *elav*
ELAVR1 [3,5]	AAIGGACCRAAYAAYTGCCA [6]	57	Degenerate PCR for *elav*
ELAVR2 [3,4]	GCTTCATCRTAATTIGTCAT [6]	53	Degenerate PCR for *elav*

[1] primers supplied with cDNA subtraction kit

[2] primers ordered from sigma primers

[3] primers ordered from Genset oligos

[4] external *elav* primer set

[5] internal *elav* primer set

[6] degenerate primer key: H = A+C+T, I = inosine, M = A+C, R = A+G, W = A+T, Y = C+T

2.5.2 Hot Start PCR

Where PCR with standard *Taq* polymerase failed, or where a protocol called for a hot start PCR, HotStarTaq polymerase (Qiagen) was used. The protocols for using HotStarTaq were downloaded from the Qiagen website, and followed exactly. In situations were hot start PCR failed, the PCR was repeated following the protocol for including Q-solution. The Q-solution is designed to alter the melting behaviour of DNA for use with templates that do not work well with standard conditions.

Cycling conditions:

HotStarTaq with M13 primers-

	Temperature	Time	Number of cycles
Initial activation	95°C	15 minutes	1
Denaturation	94°C	30 seconds	30
Annealing	45°C	30seconds	
Extension	72°C	60 seconds	
Final extension	72°C	10 minutes	1

2.5.3 Gradient PCR

In situations were PCR had previously failed, or when optimisation of PCR conditions was required, gradient PCR was used to determine the optimum annealing temperature. For most templates, two rounds of gradient PCR were done. The first round of PCR covered as wide temperature range as possible the second covered a much smaller range.

In all cases standard *Taq* DNA polymerase was used.

2.6 Purification of PCR Products

2.6.1 PCR Purification

PCR reactions that required purification were passed through Concert™ Rapid PCR Purification columns (Life Technologies). The protocol supplied by the manufacturer was followed in all cases.

2.6.2 Gel extraction

Gel extraction was used to purify single PCR products from smears and to separate restriction fragments. The Concert™ Gel Extraction system (Life Technologies) was used, and the manufacturer's protocol was followed.

2.7 DNA and RNA Quantitation

2.7.1 Comparison with Lambda Ladder

In many cases accurate measurement of DNA concentration was not required. Therefore to determine an estimate of the concentration samples were gel electrophoresed alongside *Hind* III/*Sac* II Lambda ladders. The DNA quantity was estimated by comparison to the intensities of the ladder bands, which were of a known mass.

2.7.2 Spectrophotometric Analysis

Where DNA concentrations needed to be accurately determined, samples were measured by absorption of ultraviolet light at 260 nm. RNA was also quantified in this manner. The dilution required to obtain an accurate measurement of the DNA was based on the estimate of concentration by comparison with Lambda standards.

2.7.3 Fluorometric Analysis

Concentrations of DNA samples in the ng/mL range were determined using a Hoefer DyNA Quant 200 fluorometer. The manufacturer's procedure for fluorometric analysis was followed. Fresh dye solution (1.0 μg/mL, Hoechst 33258 in 1X TNE) was prepared before use. The fluorometer was blanked on 2 mL of dye solution, and calibrated using 100 μg/mL calf thymus DNA stock. Calibration was repeated until stable consecutive readings could be taken. Three separate readings were taken for each DNA sample of unknown concentration.

2.8 Restriction Endonuclease Digestions

Restriction endonuclease digestions were carried out as recommended by the manufacturer for each enzyme used. Between 2 and 5 units of endonuclease were used per 1 μg of DNA. Reaction volumes were adjusted so that the final volume was 10 times the volume of enzyme added, to prevent high glycerol concentrations. In order to remove RNA from genomic DNA digestions, 1 μL of a 1 μg/ml solution of DNase free RNase (Roche) was included in the digestion reactions.

Digestions were usually carried out for 3 hours at an appropriate incubation temperature. However some genomic digestions required 5 hours for complete digestion. To monitor this requirement, test samples of genomic DNA digestions were taken after 2 hours and analysed by gel electrophoresis.

2.9 Gel Electrophoresis

2.9.1 Standard DNA gels

DNA was size fractionated on either TBE (89 mM Tris-HCL, 89 mM Boric acid, 2.5 mM Na_2EDTA) or TAE (40 mM Tris acetate, 1.4% (v/v) glacial acetic acid, 1 mM EDTA (pH 8.0)) gels, at 1% – 2% agarose. Standard electrophoresis sample loading dye was used (Ficoll 400 20% (w/v), EDTA (pH 8.0) 0.1 M, and bromophenol blue 0.24% (w/v)). For molecular size reference *Hind*III / *Sac*I ladder was run on these gels. In general gels were run at 50-100 volts until the marker dye was 1-2 cm from the end of the gel.

Gels were stained in 2 μg/ml ethidium bromide for 15 minutes, then destained in water. Visualisation was carried out under UV light, and photographs taken using the Gel Documentation System (Alpha Innotech).

2.9.2 Nusieve Agarose gels

Where separation of small DNA fragments was required, gels made with NuSieve® GTG® agarose were used. NuSieve® GTG® agarose is capable of resolving DNA fragments from 10 to 1000bp, here it was used to separate fragments below 200bp.

Agarose solutions where made up as per instructions supplied with the agarose. As very small DNA fragments were being resolved on these a 25bp ladder (Life Tech.) was used as a reference marker. NuSieve® GTG® agarose gels were processed in the same way as standard gels.

2.9.3 Gel Electrophoresis of RNA

RNA was size fractionated in formaldehyde agarose gels as per the protocol found in Sambrook *et al.* (1989) (pg 7.43)

Where possible, two separate sets of identical samples were run together. One set containing ethidium bromide (1µl of 1mg/ml solution) for visualisation under UV light. The other, not including ethidium bromide, was for subsequent Northern transfer.

All solutions to be used for RNA work were treated with 0.2ml DEPC per 100ml of solution. All glassware was baked overnight at 180°C, and a dedicated RNA gel box was used.

0.24-9.5 kb RNA ladder (Life Technologies) was run on these gels and photographed alongside a ruler so that the sizes of bands seen on subsequent RNA blots could be estimated. This ladder was handled as per instructions found in the product manual supplied with it.

2.10 Transfer of Nucleic Acids to Membranes

2.10.1 Southern Transfer of DNA

Southern transfer was carried out using Hybond-XL (Amersham) nylon membrane. 20x SSC was used for the transfer buffer (3 M NaCl and 300 mM sodium citrate (pH 7.0)). The protocol for standard capillary blotting, supplied with the membrane, was followed exactly.

Before Southern transfer, gels were processed as outlined by the neutral transfer protocol for Southern blotting gel treatments found in the user manual for the Hybond-XL membrane.

Following transfer, DNA was immobilised on the membranes by UV crosslinking. Crosslinking was carried out using an Ultra Lum ultraviolet trans-illuminator machine

using optimised settings. Membranes were then dried and stored between two pieces of 3MM paper until required for hybridisation.

2.10.2 Northern Transfer of RNA

In general Northern transfers were carried out in the same way as the Southern transfers, using the same method, types of buffers, and membrane. However precautions such as using DEPC treated solutions, and baking glassware at 180°C overnight, were taken to prevent degradation by RNase. Following electrophoresis RNA gels were washed in DEPC treated water prior to transfer.

2.10.3 Colony Lifts

Colony lifts were carried out so that large numbers of clones could be screened quickly, without having to perform large numbers of PCR reactions. Colony lifts were carried out as outlined in the manual supplied with the Hybond-XL membrane (Amersham). Membranes were oriented on the plates by making a series of holes around the edges, corresponding to matching marks on the plate. The membranes were also labelled with the number of the corresponding plate.

2.11 Hybridisation of Membranes

2.11.1 Probe labelling

Probes made from DNA templates were labelled using the random primer based Ready-To-Go™ DNA labelling beads (-dCTP) (Amersham). In most cases approximately 50 ng of DNA were labelled for probe. In these circumstances the standard protocol was followed. Some hybridisations required more probe (up to 100ng), so the protocol for labelling between 10 ng and 1μg was used.

cDNA probes were made from RNA by first-strand cDNA synthesis incorporating ^{32}P-dCTP. SuperScript™ II RNase H$^-$ Reverse Transcriptase (Invitrogen) was used. The protocol supplied with the enzyme was modified to suit the use of α-^{32}P dCTP.
The following components were added in an RNase free microcentrifuge tube:

1 μL random primers (50-250 ng)

1 μL total RNA (1 ng-5 μg)

1 μL dGTP, dATP, dTTP mix (20 mM each)

1 µl dCTP (120 µM)

volume made up to 12 µL with DEPC treated water.

The mixture was heated at 65°C for 5 minutes, and chilled on ice. After a short centrifugation to collect contents of tube, the following was added:

4 µL 5X First-Strand Buffer

2 µL 0.1 M DTT

1 µL RNaseOUT™ ribonuclease inhibitor (40 units/µL)

5 µL α-^{32}P dCTP

Contents were gently mixed, then incubated at 42°C for 2 minutes, following which 1µl of SuperScript™ II (200 units) was added. The reaction was then incubated for 1 hour at 42°C. After incubation the reaction was inactivated by heating to 70°C for 15 minutes.

All probes were purified using ProbeQuant G-50 Micro Columns (Pharmacia Biotech), which remove unincorporated nucleotides from the labelling reaction. Following purification probes were denatured in a boiling water bath for 2 minutes, and placed on ice for 2 minutes before being added to hybridisation buffer.

2.11.2 Prehybridisation

Membranes were prehybridised in 25-30 mLs of appropriate buffer, either Denhardt's buffer (5x SSC, 5xDenhardt's solution, 0.5% (w/v) SDS), or Modified Church and Gilbert Buffer (0.5M phosphate buffer (pH 7.2), 7% (w/v) SDS, 10mM EDTA(pH8.0)) were used. Prehybridisation was carried out in hybridisation tubes for 1-2 hours in a 65°C rotating incubator. Blocking agents did not have to be added to prehybridisation solutions as it was not required when using nylon membranes. Prehybridisation solution was then removed before adding hybridisation solution.

2.11.3 Overnight hybridisation

Hybond-XL (Amersham) nylon membrane was used for all hybridisations.

The protocol for hybridisation in tubes, as set out in the manual supplied with the membrane, was followed in all cases. Both Denhardt's buffer, and Modified Church and Gilbert Buffer type hybridisations were performed. Overnight hybridisations were carried out in 20–25 mLs of the appropriate buffer, in a rotating incubator, at 65°C.

2.11.4 Stringency washing

Stringency washes were carried out as follows:

-Low stringency wash (2x SSC, 0.1% (w/v) SDS), twice at 5-10 minutes each.

-Medium stringency wash (1x SSC, 0.1% (w/v) SDS), twice at 15-20 minutes each.

-High stringency wash (0.1x SSC, 0.1% (w/v) SDS), twice at 20-30 minutes each.

High stringency wash buffer was heated to 65°C before use. All washes were carried out with agitation. Washes were carried out in boxes due to the greater effectiveness of washing in large volumes. Washes were continued until little radioactivity was detectable on the membrane.

2.11.5 Probe stripping

Probe striping was performed following the hot SDS procedure found in the Hybond-XL (Amersham) manual. For difficult probes SDS concentrations were increased up to 0.5%, until no probe could be detected after overnight exposure. Membranes were left in stripping solution (0.1% (w/v) SDS) for 6 to 12 hours, with agitation. Following probe stripping membranes were exposed to X-ray film overnight, if any probe was still detected probe striping was repeated.

2.11.6 Low stringency hybridisation

For the purpose of screening a library with a probe with low sequence similarity, low stringency hybridisation was used. In low stringency hybridisation formamide is included in the hybridisation buffer so that hybridisation can occur at lower incubation temperatures. This allows probes that are of lower sequence similarity to the target DNA to bind.

Membranes were prehybridised in approximately 30ml of 10x Denhart's solution at 68°C for 1- 2 hours.

Probes were made as stated previously, and added to hybridisation buffer immediately before overnight hybridisation.

Buffer for overnight hybridisations consisted of: 43% deionised formamide, 5x Denhart's solution, 5x SSC, 0.1% SDS, and 50mM sodium phosphate (pH 7.0)

Membranes were hybridised overnight at 37°C, in a rotating incubator.

Fewer Stringency washes were carried out subsequent to hybridisation.

First, two washes in 2x SSC, 0.1% SDS at room temperature with agitation for 5 minutes each. Second, two washes 2x SSC, 0.1% SDS at 50°C, with agitation for 30 minutes each.

2.12 Autoradiographs

Membranes, after having been adequately washed, were wrapped in plastic and exposed to X-ray film for between 12 and 24 hours, at -80°C. In most cases BioMax MS film (Kodak), and a BioMax MS intensifying screen (Kodak) were used for exposure of membranes. Where BioMax film was not used, X-OMAT™ AR (Kodak) film was used with a standard intensifying screen. An automatic X-ray film processor was used to develop the autoradiographs.

2.13 Sequencing of Selected Clones

PCR products were diluted to a concentration of 4ng per 100bps in 20µl, and plasmids were diluted to a concentration of 600ng in 20µl, and run by the Massey University DNA sequencing unit. Initially only BigDye Terminator sequencing was carried out, using M13 forward and reverse primers. Failed sequences that seemed to have arisen as a result of regions of high GC content were resequenced by dRhodamine sequencing.

Sequence electropherograms were analysed using the ABI Prism EditView 1.0.1 programme. Sequences were manipulated using both the DNAsis, and Oligo 4.0 programmes. Sequence searches were carried out using the BLAST search engine.

3 Results

3.1 Subtracted Library 1

A subtracted cDNA library was produced by Dr Max Scott, using the CLONTECH PCR-Select cDNA Subtraction kit (unpublished data). For this subtraction tester cDNA was produced from 1st instar male RNA and driver cDNA was produced from adult female RNA. One hundred transformed cDNA clones resulted from this subtraction. These cDNA fragments were cloned into pGEM-T.

3.1.1 PCR amplification of 100 clones

The 100 subtracted cDNA fragments where PCR amplified out of the pGEM-T plasmid using M13 forward and reverse primers (Table 2.1). Standard PCR conditions (Section 2.5.1) were found to be unfavourable for the amplification of a large number of these fragments. Following optimisation, it was found that all the fragments amplified at a magnesium concentration of 1.25 mM at an annealing temperature of 45°C (otherwise conditions were standard for the use *Taq* polymerase (see section 2.5.1)). Figure 3.1 shows examples of the PCR optimisation results.

Many of the PCR reactions produced non-specific amplification products. The principal product was considered to be the most intense band for observed for each clone after gel electrophoresis of the PCR products. If additional bands were observed these were considered to be the result of non-specific amplification (see lane 10 of Figure 3.1). Gel extraction (Section 2.6.2) was utilised to purify PCR reaction samples containing non-specific amplification products. Where an absence of non-specific amplification products was observed PCR reaction samples were purified using PCR purification columns (Section 2.6.1).

3.1.2 Southern Hybridisation of 100 clones

Two sets of the 100 clones were size separated by electrophoresis (Section 2.9.1) on identical 1.5 % agarose gels. Following electrophoresis the gels were Southern transferred (Section 2.10.1). The two membranes were hybridised overnight under identical conditions (Section 2.11). One was hybridised with ^{32}P-labelled adult male total cDNA probe, and the second was hybridised with ^{32}P-labelled adult female total

cDNA probe. Autoradiographs were taken of each of the membranes (Section 2.12). These hybridisation screens were performed with both randomly primed probes and oligo-dT primed probes, with identical results. Figures 3.2 and 3.3 show the results of the 100 clone hybridisations.

The majority of the 100 clones hybridised equally well with both male and female probes. However clone #4 showed significantly stronger hybridisation with the male cDNA probe. Also 20 clones exhibited slightly stronger hybridisation to male DNA. In addition there were 14 clones which did not hybridise well with either DNA probe.

3.1.3 Sequence analysis of selected clones

A selection of clones were chosen for sequence analysis, these included; clone #4, a random selection of clones that exhibited a slight difference in hybridisation or failed to hybridise to either probe, and four clones that hybridised well to both probes. The inclusion of the latter four was an attempt to identify a highly expressed autosomal gene common to both sexes (for example tubulin) to be used as a control for subsequent hybridisation analyses.

A large number of these clones did not produce quality sequence when using the dGTP BigDye terminator sequencing reaction. It was hypothesised that this was due to GC rich stretches, located primarily in the adapter sequence regions of these clones. To overcome this, these clones were sequenced using dRhodamine sequencing reactions, however a number of clones still did not produce sequence of a suitable quality.

The majority of clones showed no sequence similarity to any published sequences when BLAST nucleotide searches were conducted. This was probably a result of most of the fragments being 3' UTR. Also, the very small size of several fragments following deletion of vector and adaptor sequences could be a contributing factor to the lack of BLAST hits. However several clones exhibited very high identity matches to mitochondrial genes. In addition, the sequencing demonstrated that several clones were of sub-optimal size (less than 200bp). The results obtained from the selected clones studied are summarised in Table 3.1.

(See Appendix 2 – Sequence data, located on CD in the rear cover, for sequence information)

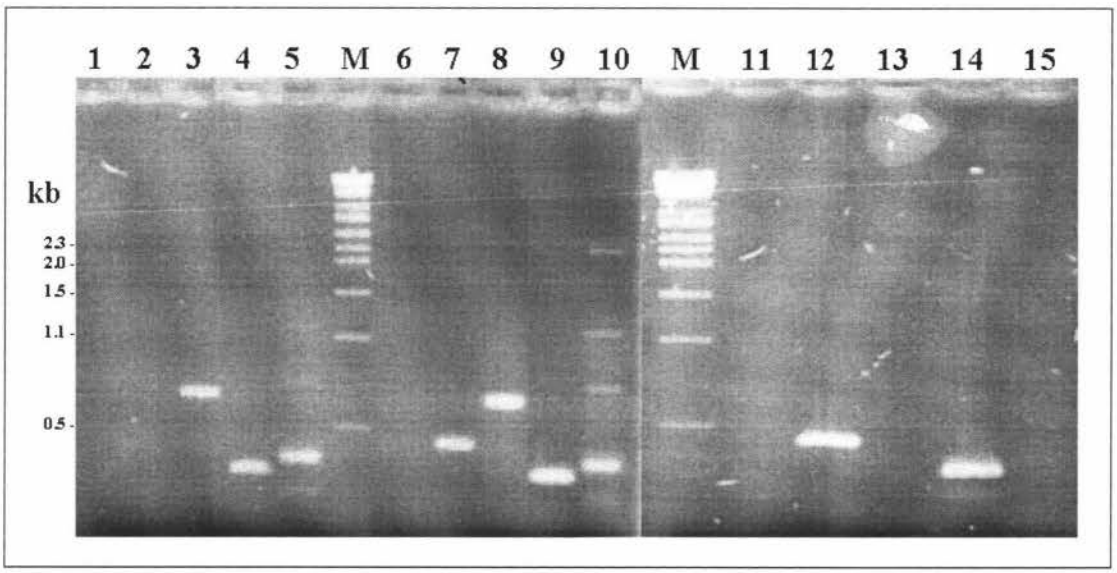

Figure 3.1: **PCR amplification of initial library clones was found to be most efficient at a magnesium chloride concentration of 1.25 mM.**

PCR reactions were performed with *Taq* polymerase using different magnesium concentrations at an annealing temperature of 45°C. The PCR products were size separated on 1.5% TBE agarose gels for 1 hour at 90 V. The magnesium concentrations and templates used were: 1.75 mM $MgCl_2$ with, Negative no DNA control (1), Positive control (2), clone #4 template (3), clone #3 template (4), clone #1 template (5).

1.25 mM $MgCl_2$ with, Negative no DNA control (6), Positive control (7), clone #4 template (8), clone #3 template (9), clone #1 template (10).

1.50 mM $MgCl_2$ with, Negative no DNA control (11), Positive control (12), clone #4 template (13), clone #3 template (14), clone #1 template (15).

(M) denotes *Hind* III / *Sac* II molecular weight marker.

These results indicate that to reliably obtain PCR product from these clones a magnesium chloride concentration of 1.25 mM should be used. However use of this concentration can lead to a less specific PCR reaction as seen in (10).

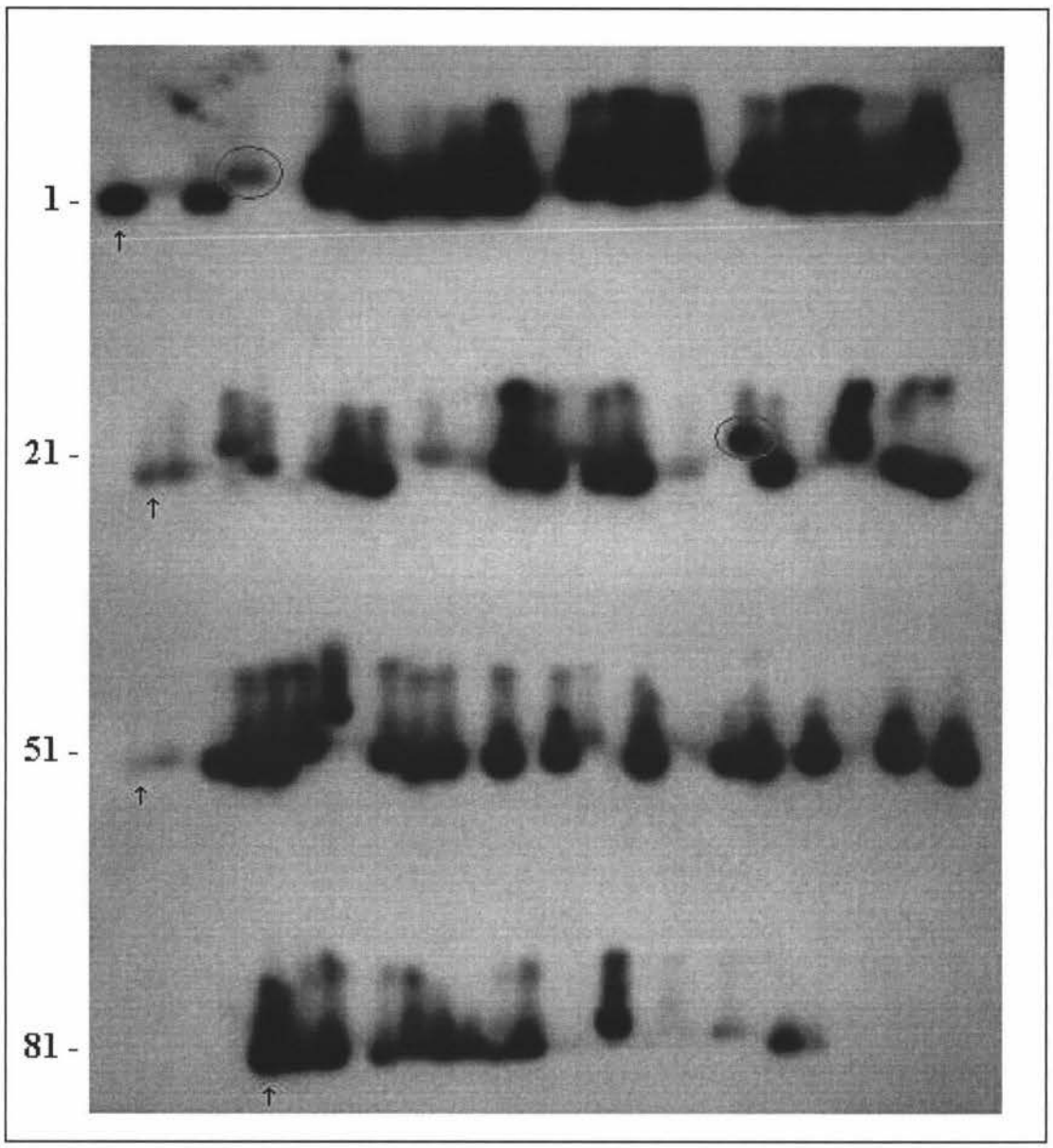

Figure 3.2: Southern analysis of 100 clones probed with ^{32}P labelled male cDNA.

Approximately 2.5 μg of each cDNA clone PCR product was gel electrophoresed and Southern transferred to a nylon membrane. The membrane was probed with 5 μg of ^{32}P-labelled total adult male cDNA. Hybridisation was carried out overnight at 65°C and the autoradiograph was exposed for 15 hours.

The number of the left most lane of each row is indicated to the left of the blot, and the position of each of these identifier lanes is marked with an arrow.

Clone #4 (top row), and clone #42 (second row) are highlighted as these two clones were found to be of most interest.

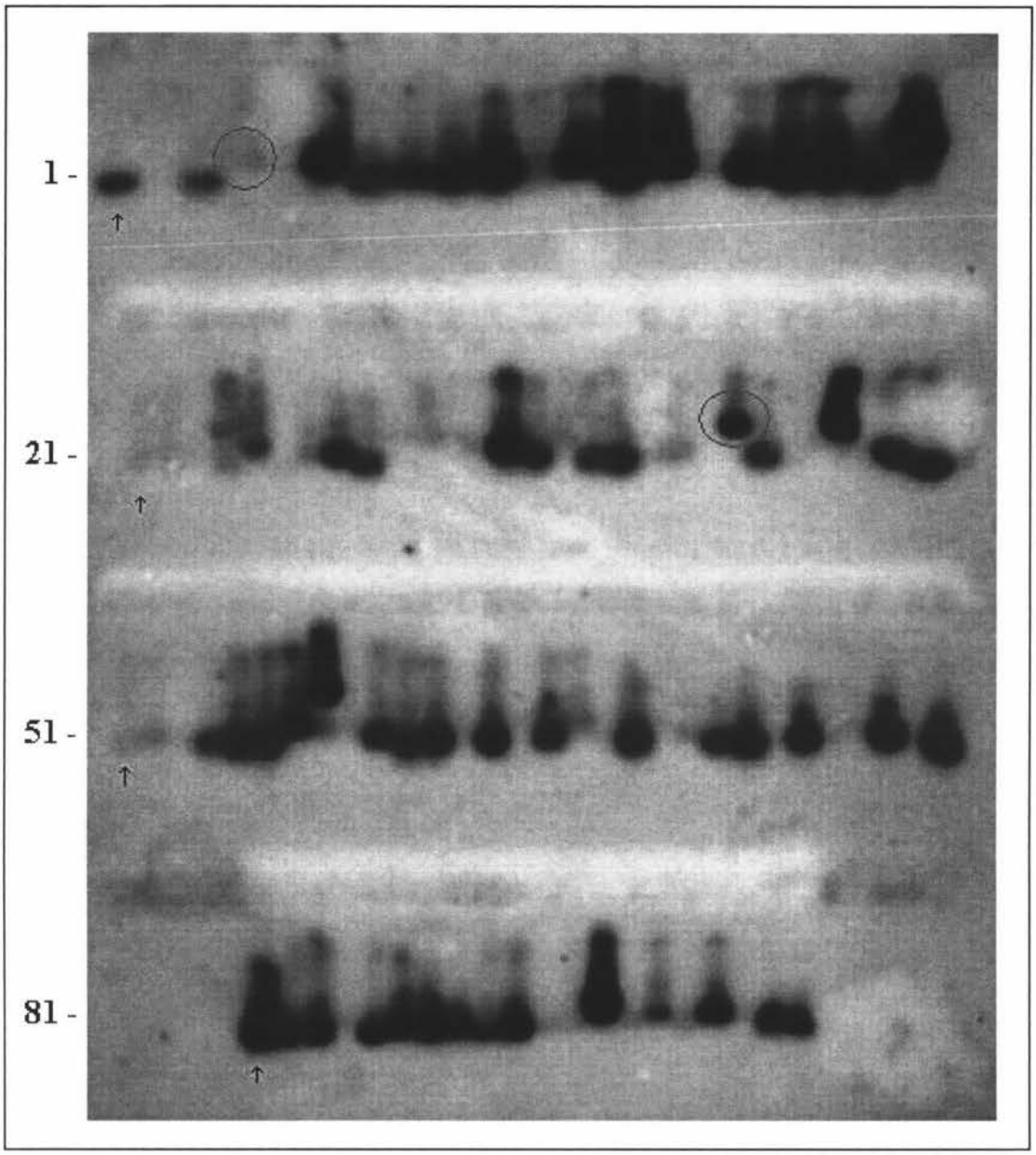

Figure 3.3: Southern analysis of 100 clones probed with ^{32}P labelled female cDNA.

Approximately 2.5 µg of each cDNA clone PCR product was gel electrophoresed and Southern transferred to a nylon membrane. The membrane was probed with 5 µg of ^{32}P-labelled total adult female cDNA. Hybridisation was carried out overnight at 65°C and the autoradiograph was exposed for 15 hours.

The number of the left most lane of each row is indicated to the left of the blot, and the position of each of these identifier lanes is marked with an arrow.

Clone #4 (top row), and clone #42 (second row) are highlighted as these two clones were found to be of most interest.

3.1.4 Southern hybridisation analysis of selected clones

More sensitive hybridisation analyses were conducted to confirm positive results, and to clarify the ambiguous results observed for several clones that exhibited slight hybridisation differences, or failed to hybridise with either male or female probe. For this experiment the clone of interest was used to probe membranes of genomic DNA targets. Northern hybridisation analysis was not used due to a lack of a sufficient amount of quality RNA.

Male and female genomic DNA samples were separately digested with the restriction endonucleases *Eco*RI and *Pst*I (Section 2.8), separated by gel electrophoresis (Section 2.9.1), and transferred to nylon membranes (Section 2.10.1). These membranes were individually probed with a selection of ^{32}P labelled clones of interest, as identified from 100 clone screens. With one exception (see Section 3.1.5.2), none of the clones tested in this way exhibited male specific hybridisation.

3.1.5 Results from two specific clones

Clone #4

From the results of the 100 clone screen, clone #4 represented the only clear candidate for a potential male-specific gene. However results of a hybridisation with genomic DNA targets clearly show that clone #4 did not favour binding to male DNA (see Figure 3.4). As a result clone #4 can be considered to be non male-specific.

Clone #42

Clone #42 was selected for further study as it hybridised well to both male and female ^{32}P labelled cDNA. This was done in an attempt to identify highly expressed autosomal genes to use as controls for later experiments. A clone #42 probe produced a clear male-specific band when hybridised with digested genomic DNA targets, result shown in Figure 3.5A. However the same probe was used for Northern hybridisation analysis and male specificity was not seen (Figure 3.5B)

Sequence analysis was difficult, as neither the Bigdye terminator nor dRhodamine reactions could sequence through a short GC rich region midway through the approximately 500 bp fragment. By compiling several forward and reverse sequencing runs the composition of this clone was modelled. The 5` half of the

fragment was found to be 93 % identical to the ADP/ATP translocase of *Lucilia cuprina* (Figure 3.6 shows the alignment of these two sequences). Further comparison of these sequences demonstrated that the differences were primarily located in the third position of each codon, and that the resultant amino acid sequence was identical. BLAST searches using the sequence of the 3` half of the fragment failed to produce any matches.

Sequencing results demonstrated that clone #42 was comprised of two unrelated fragments. These fragments were separated to identify which component was causing the male-specific band. Each sequence was removed from the pGEM-T vector by enzyme digestion. Both *Rsa* I, and *Not* I sites were identified that separated each sequence from the other and the vector. The fragments were gel extracted (Section 2.6.2), ligated into the pBluescript plasmid and cloned into DH5α (Section 2.3). This transformation produced 12 ampicillin positive colonies, two of which were chosen to produce hybridisation probes. These two clones were given the identifiers BS1, and BS8. BS1 contained the ADP/ATP translocase fragment, flanked by *Sac* II and *Sma* I restriction sites. BS8 contained the fragment from the unknown gene flanked by *Not* I sites. Each was digested using the respective enzymes, and the resultant 275 bp fragments were gel extracted and used to make probes for Southern hybridisation against membrane bound genomic DNA (Section 2.11). These hybridisations were unclear due to extremely weak hybridisation, however faint male-specific bands were seen in the hybridisation of the unknown fragment.

At this time fresh medfly genomic DNA from male, female, and male F_{18} flies was obtained from Dr Gerald Franz, IAEA labs, Seiberodorf, Austria. The male F_{18} genomic DNA sample was from males of the Family 18 strain of medfly. This strain has a Y chromosome that has been extensively deleted, but maintains the male determining signal. The new DNA samples were endonuclease digested with *Eco*RI and *Pst* I (Section 2.8), and transferred to a nylon membrane (Section 2.10.1). The membrane was hybridised with a clone #42 probe (Section 2.11). This hybridisation did not produce any male-specific banding (see Figure 3.7). The restriction fragment length polymorphism (RFLP) seen in the *Eco*RI digested F_{18} male DNA (lane 4 of Figure 3.7) is not unusual as such RFLPs are commonly seen in different strains of medfly.

Control blots were performed using the pBluescript vector to probe both old and new genomic Southerns. As both the pBluescript and pGem-T are pUC based plasmids this probe would be suitable to act as a control for both. These control hybridisations showed that the male-specificity seen was due to hybridisation of plasmid DNA to the genomic digests, probably as a result of contamination of the genomic DNA. Figure 3.8 shows this result. Additionally, the hybridisation with fresh genomic DNA revealed that the plasmid DNA hybridised with uncontaminated medfly genomic DNA. Other laboratories have also noticed non-specific hybridisation between pUC DNA probes and medfly genomic DNA (M. Scott, personal communication).

Table 3.1: Summary of results obtained from clones chosen for individual study.

Clone No.	Male screen result	Female screen result	Sequence similarity	Other notes
4	Medium	Weak	No significant similarity	No sex-specificity seen when used to probe genomic targets
2	Weak	Very weak	No significant similarity	No sex-specificity seen when used to probe genomic targets
5	Very weak	Very weak	No significant similarity	No sex-specificity seen when used to probe genomic targets
11	Weak	Weak	Mitochondria, cyt B	No sex-specificity seen when used to probe genomic targets
15	Weak	Very weak	No significant similarity	Short fragment ~180bp
20	Strong	Strong	Mitochondria, 1-rRNA region	
26	Very weak	Very weak	No significant similarity	Very short fragment ~90bp
36	Weak	Weak	No significant similarity	Poly-T region lead to poor sequence
39	Weak	Very weak	No significant similarity	Short fragment ~150bp
41	Very weak	Very weak	No significant similarity	Short fragment ~110bp
42	Strong	Strong	ADP/ATP translocase	Fusion of fragments
46	Strong	Strong	Mitochondria, 1-rRNA region	
53	Very weak	Very weak	No significant similarity	Poly-T region lead to poor sequence
68	Very weak	Very weak	No significant similarity	Poly-T region lead to poor sequence
84	Very weak	Very weak	No significant similarity	Fragment ~ 300bp
93	Strong	Strong	Mitochondria, 1-rRNA region	

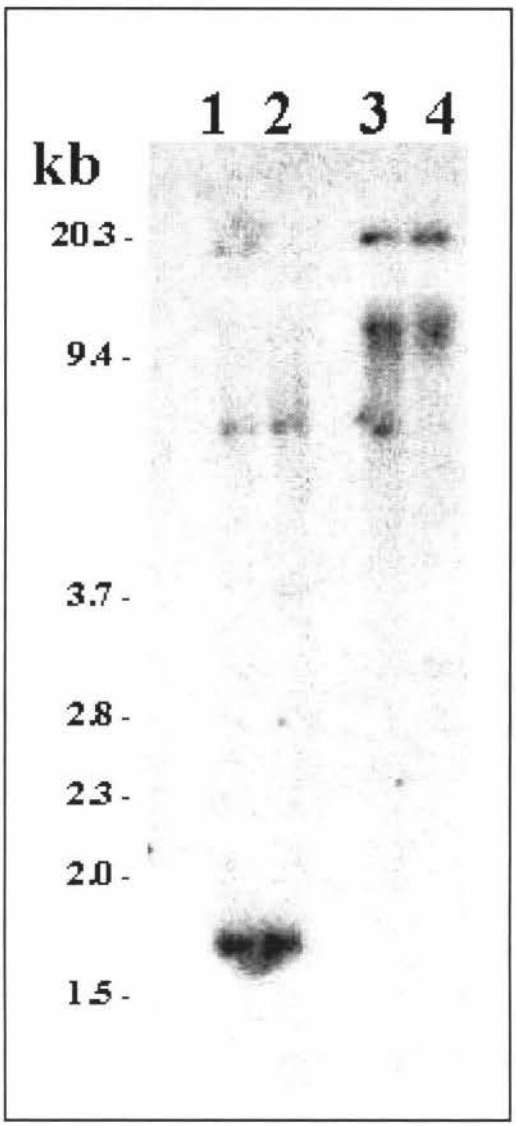

Figure 3.4: Southern hybridisation analysis demonstrates that clone #4 exhibited no male-specificity.

The membrane of genomic DNA contained 10 µg of: *Eco*RI digested adult female genomic DNA (1), *Eco*RI digested adult male genomic DNA (2), *Pst*I digested adult female genomic DNA (3), and *Pst*I digested adult male genomic DNA (4). 50 ng of ^{32}P labelled PCR product of clone #4 was used to probe the membrane overnight, before 12 hour exposure

No male-specific bands are seen on this autoradiograph, ruling out the possibility of any male-specific expression of the gene represented be clone #4.

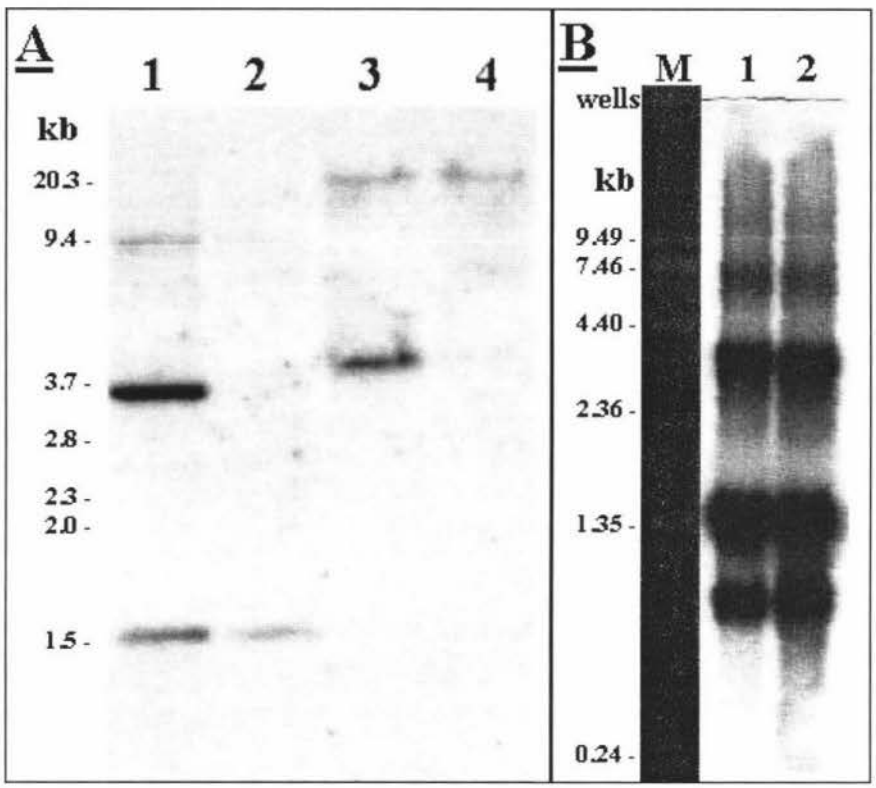

Figure 3.5: Hybridisation analysis with clone #42 illustrating conflicting results as to the potential male-specific nature of the clone.

(A) Southern hybridisation of genomic DNA digestions; 10μg of male DNA digested with *Eco*RI (1), 10 μg of female DNA digested with *Eco*RI (2), 10 μg of male DNA digested with *Pst*I (3), 10 μg of female DNA digested with *Pst*I (4), probed with 50 ng of ^{32}P labelled PCR product of clone #42. Male-specific bands are seen at approximately 3.7 kb. This is very odd due to the fact that clone #42 hybridised equally well to both male and female probes in the 100 clone screens.

(B) Northern hybridisation of total RNA, female (1), male (2), probed with 50 ng of ^{32}P labelled PCR product of clone #42. RNA molecular weight marker (M) was separately gel photographed and aligned with the autoradiograph. These results show no male-specificity, contradicting the results of the Southern analysis.

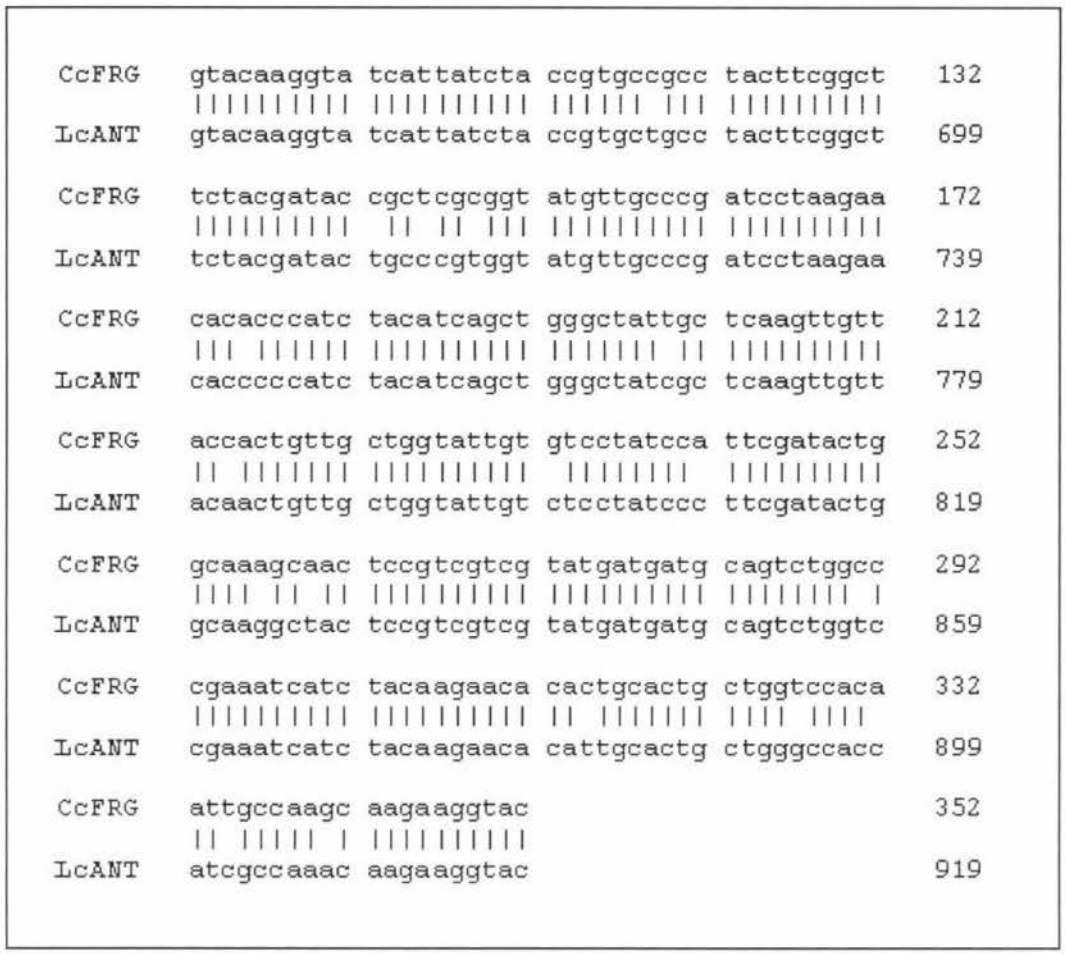

Figure 3.6: 260 base pairs of the clone #42 show a 93% sequence similarity to the ADP/ATP translocase of *Lucilia cuprina*.

This result was obtained by performing a nucleotide search on the BLAST search engine with the clone #42 sequence. Following this initial search the unmatched sequence was edited out and the Blast search was repeated using this edited sequence (CcFRG). The most similar match was the ADP/ATP translocase of *Lucilia cuprina* (LcANT), although the ADP/ATP translocases of several *Drosophila* species also showed significant sequence similarity (*D. pseudoobscura* 86%, *D. melanogaster* 86%, *and D. subobscura* 85%).

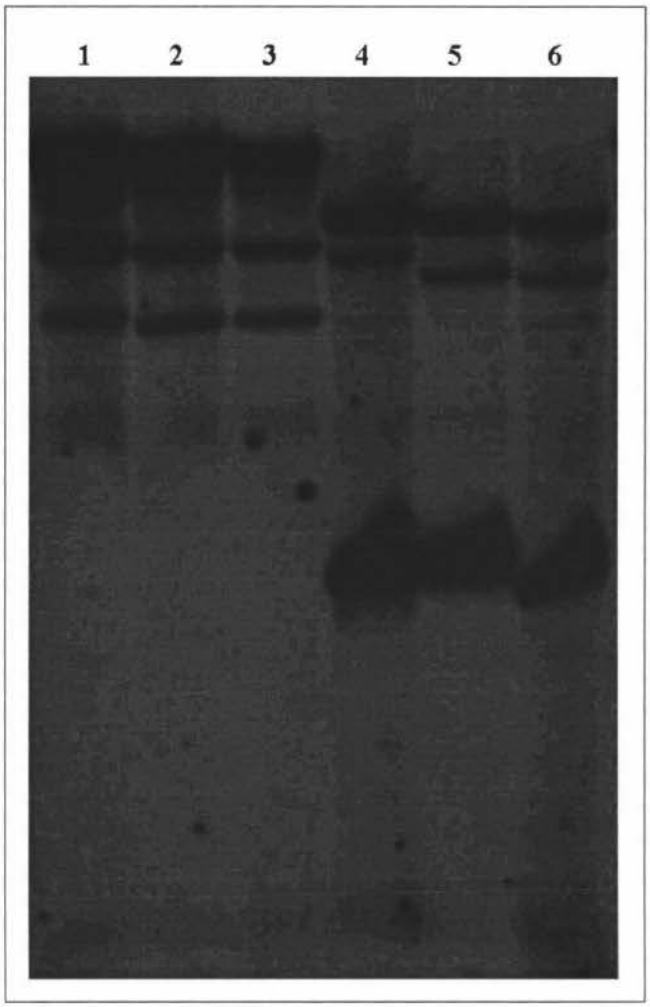

Figure 3.7: Clone #42 hybridisation with medfly genomic DNA indicates that the original genomic samples had been contaminated.

10 μg each of; *Pst*I digested F_{18} male DNA (1), *Pst*I digested female DNA (2), *Pst*I digested male DNA (3), *Eco*RI digested F_{18} male DNA (4), *Eco*RI digested female DNA (5), *Eco*RI digested male DNA (6), were Southern transferred to a nylon membrane. 50 ng of ^{32}P labelled clone #42 PCR product was used to probe the membrane, and an autoradiograph was taken. The autoradiograph shows that no male-specific hybridisation had occurred.

The genomic DNA used for the Southern blot was isolated at the IAEA laboratory in Austria.

(The F_{18} male DNA was taken from a strain that has a mostly deleted Y chromosome that still contains the male determining region.)

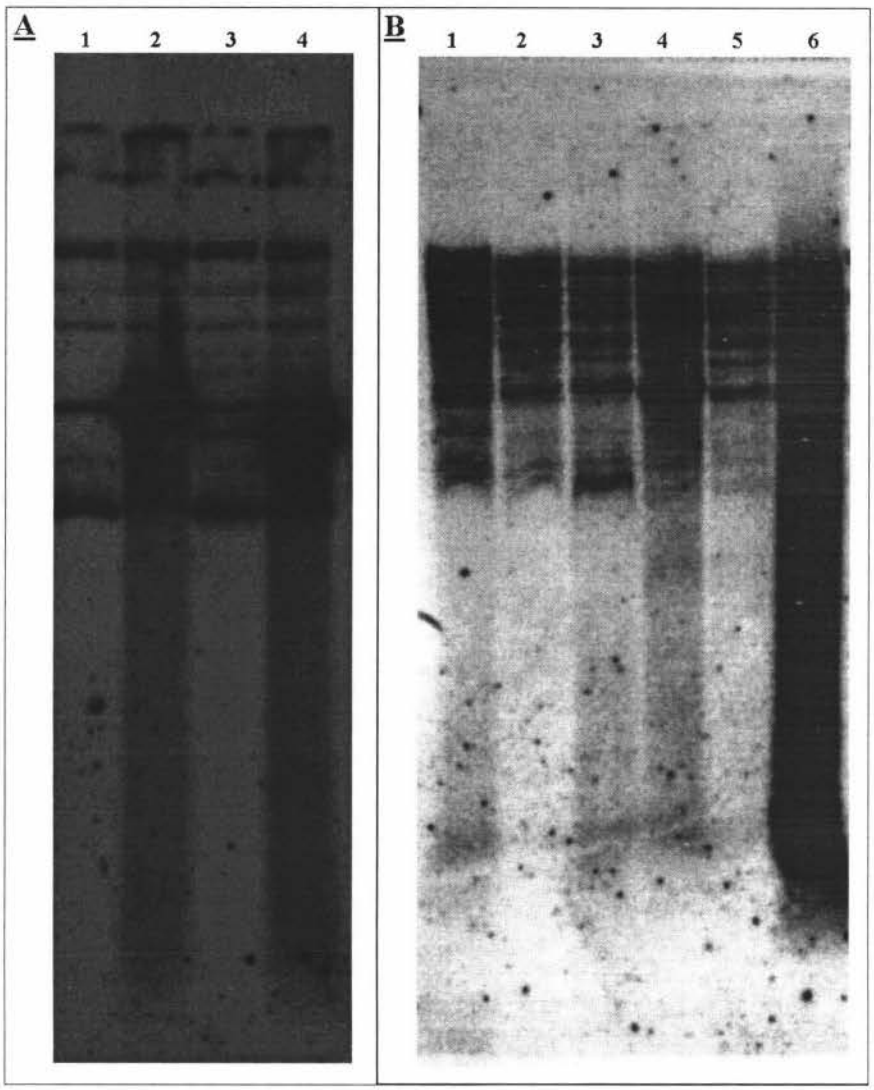

Figure 3.8: Control hybridisation analysis using pBluescript probes illustrates contamination of original genomic DNA samples.

(A) 50 ng of ^{32}P labelled, linearised pBluescript plasmid was used to probe the original membrane of medfly genomic digestions; *Pst* I digested female DNA (1), *Pst* I digested male DNA (2), *Eco* RI digested female DNA (3), *Eco* RI digested male DNA (4). The autoradiograph shows a male hybridisation bias of the pBluescript probe.

(B) 50 ng of ^{32}P labelled, linerised pBluescript plasmid was used to probe a membrane of digestions of genomic DNA obtained from Austria; *Pst* I digested F_{18} (deleted Y strain) male DNA (1), *Pst* I digested female DNAb(2), *Pst* I digested male DNAb(3), *Eco* RI digested F_{18} male DNAb(4), *Eco* RI digested female DNAb(5), *Eco* RI digested male DNA (6). This autoradiograph indicates that medfly genomic DNA contains sequences homologous to pUC based vectors, as a significant amount of hybridisation of plasmid to genomic DNA is seen.

3.2 Subtracted Library 2

As no male-specific cDNA sequences were obtained from the first subtraction it was decided to repeat the procedure. Medfly is a prohibited organism in New Zealand, and existing RNA samples where degraded, new RNA samples were sourced from outside the country. Also a new ClonTech PCR-select^{TM} cDNA subtraction kit was obtained.

3.2.1 mRNA purification

Total medfly male and female RNA was purified by Dr Max Scott from a wild type strain raised at the West Australia Department of Agriculture laboratories. These RNA samples were stored as pellets in ethanol at -20°C. Before mRNA purification these samples were pelleted, the ethanol removed, and the pellets resuspended in DEPC treated milli-Q water.

Before beginning mRNA purification the quality of the new RNA was assessed, and compared to the initial RNA sample, by denaturing formaldehyde gel electrophoresis (Section 2.9.3). It was clear that the fresh RNA was of significantly better quality than the initial sample (Figure 3.9).

mRNA purification was conducted (Section 2.1) with the starting amounts of RNA being 720 µg for male and 772 µg for female. As poly-A RNA represents approximately 1% of the total RNA complement the mRNA purification could be expected to yield around 7µg of mRNA for each sample. The actual yields obtained were 4.0 µg of male mRNA and 5.1 µg of female mRNA. The discrepancy was due to an amount of RNA being lost from each sample due to rotor buckets jamming in a horizontal position during the first spin of the RNA purification system.

3.2.2 cDNA subtraction

3.2.2.1 *Rsa* I digestion check

Following *Rsa* I digestion of cDNA fragments after second strand synthesis, a PCR based digestion check was carried out to ascertain the effectiveness of the digestion and the size distribution of the resulting fragments (Section 2.2.2). The results of the digestion check are shown in Figure 3.10. The results show an overall decrease in the size of fragments after *Rsa* I digestion, indicating that the digestion was successful. The fragment sizes of the digested samples ranged from approximately 2 kb to less

then 0.1 kb. This is significant in that comparing these results with the results obtained for the previous subtraction (M. Scott, personal communication) it can be seen that the size distributions of the new cDNAs include larger sequences. The digestion check from the original subtraction showed a size distribution from about 1 kb. The presence of very small sequences found in the first subtracted library was probably a result of the small sizes of fragments after *Rsa* I digestion. Since there was a better size distribution (i.e. more larger fragments) seen in the new subtraction after *Rsa* I digestions the subtraction was continued.

3.2.2.2 Ligation analysis

A ligation analysis test (Section 2.2.3) was conducted to ensure that at least 25% of cDNAs had adapters ligated to both ends. If the percentage is lower, then the cDNA subtraction efficiency will be greatly reduced. This analysis was based on a test for the adapter/cDNA junction. PCR products obtained by using a gene primer and a primer for the adapters were compared to the product from the same template using forward and reverse gene primers. The presence of ligated adapter allows the first PCR reaction to succeed. In addition the product of that reaction will be larger than that of the gene primer reaction. This system allows for an easy visual confirmation of the success of the adapter ligation.

For this test there was a need to design primers for a uniformly expressed medfly gene. Primers were supplied with the subtraction kit, but these are designed for mammalian systems, and will not work with medfly cDNA template. As a result of the earlier *Rsa* I treatment (Section 2.2.2) the fragment between the primers could not contain *Rsa* I restriction sites. This constraint in addition with avoiding the standard pitfalls of primer design such as hairpin loops, lead to difficulties finding a suitable primer. A set of primers that could amplify a fragment of the medfly *pgd* gene were used. This gene encodes the housekeeping enzyme 6-phosphogluconate dehydrogenase (Scott *et al.* 1993). Unfortunately these primers consistently produced anomalous results, and it was found that the primers were not suitable for use in this test as their placement meant that no product would be produced between them.

The ligation analysis test was successful with the skeletal control samples, using the primers supplied with the subtraction kit. This result is shown in Figure 3.11. From this result it was estimated that the ligation efficiency was close to the lower limit.

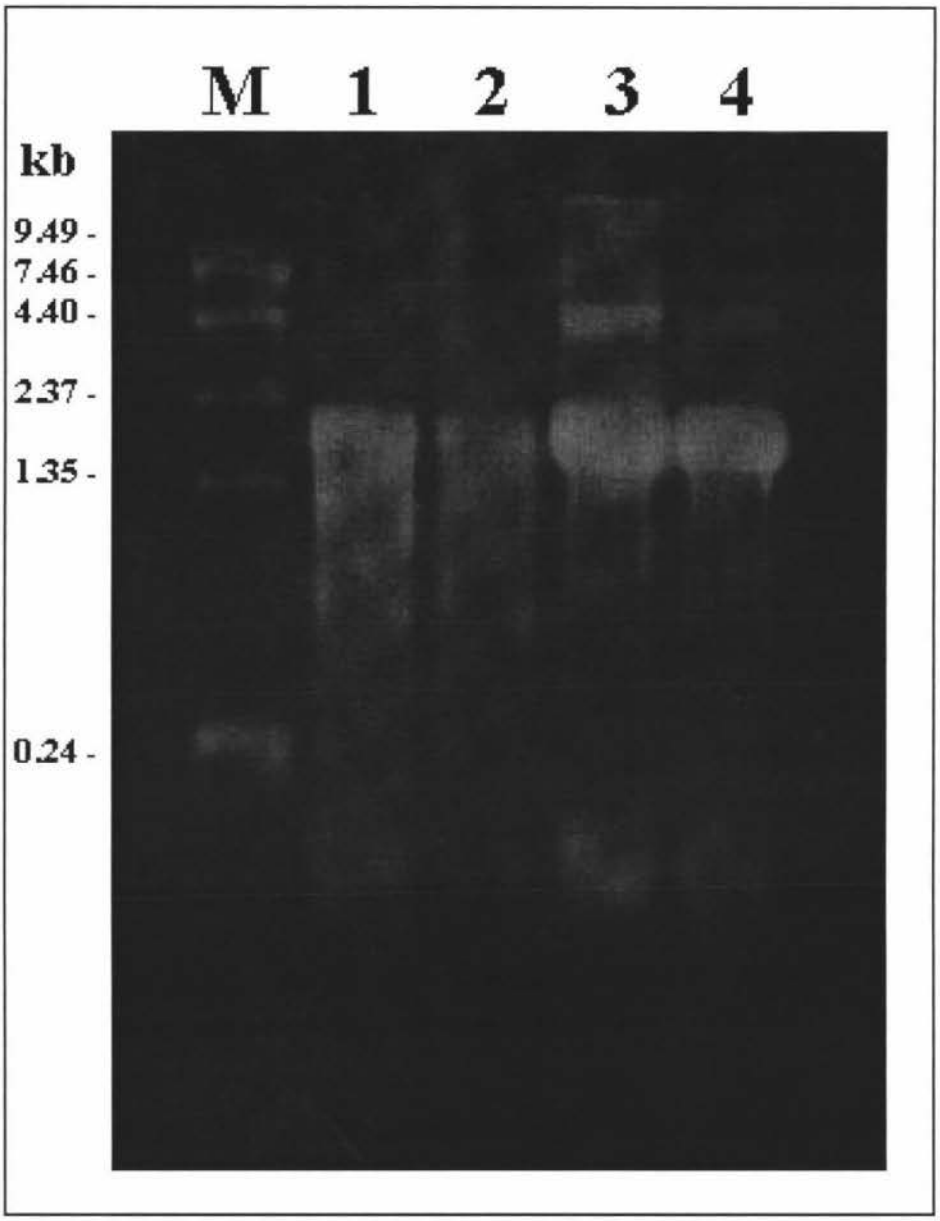

Figure 3.9: Assessment of RNA quality by agarose gel electrophoresis demonstrates that newly obtained RNA samples were of higher quality then existing samples.

Gel photograph of total RNA samples, 10 µg of each, electrophoresed under denaturing conditions on a 1 % formaldehyde agarose gel for 3 hours at 75 V; (1) old male total RNA, (2) old female total RNA, (3) new male total RNA, (4) new female total RNA, (M) RNA size markers.

The gel indicates that the new RNA samples were of suitable quality for mRNA purification, while old samples had degraded a significant amount.

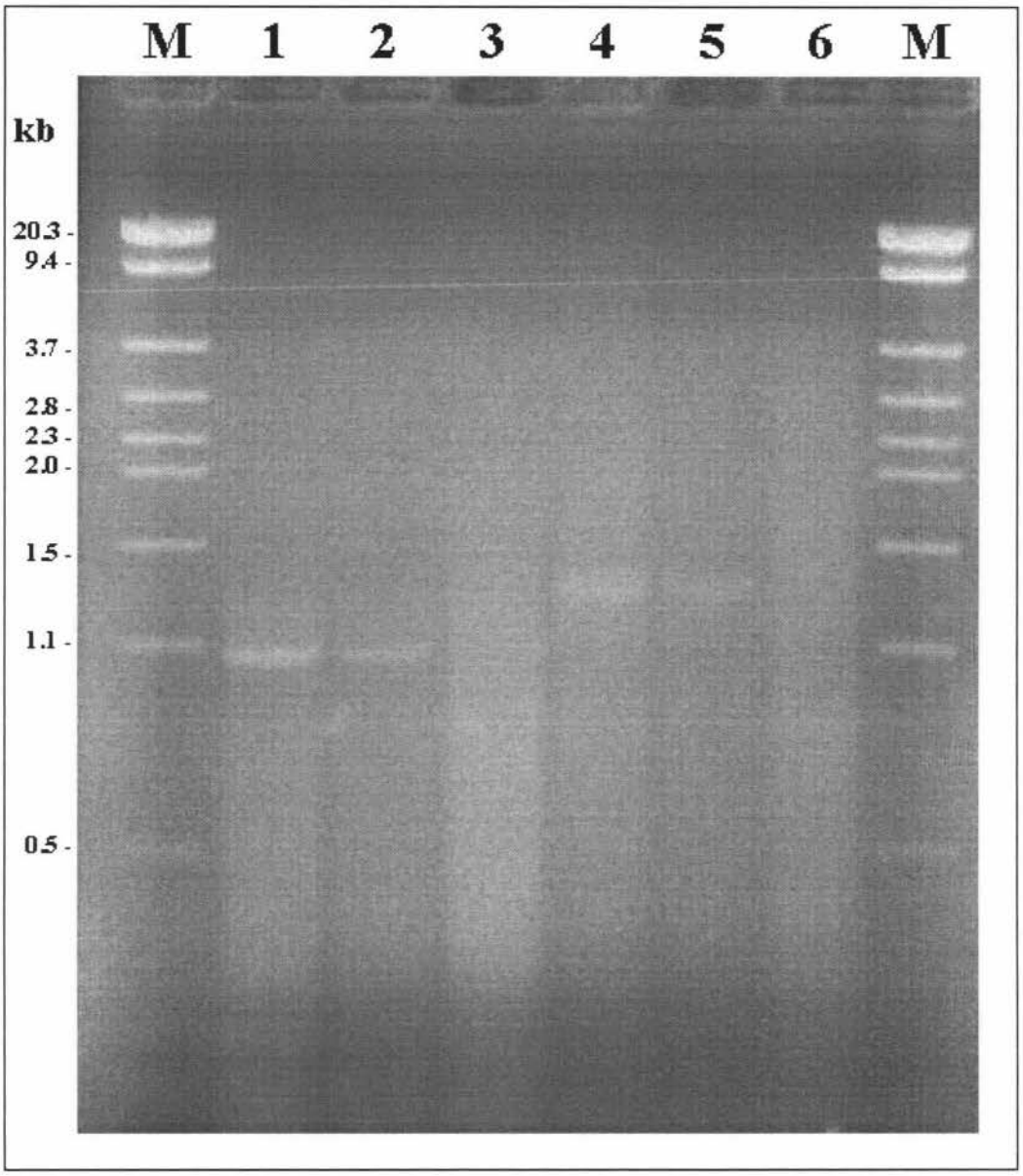

Figure 3.10: Gel electrophoresis analysis of cDNA samples digested with *Rsa*I.

Each sample was electrophoresed on a 1% TAE agarose gel for 2.5 hours at 50 V. 5 μL was run for each digested sample; *Rsa*I digested male medfly cDNA (1), *Rsa*I digested female medfly cDNA (2), *Rsa*I digested control skeletal muscle cDNA (3). 2.5 μL was run for each undigested sample; undigested male medfly cDNA (4), undigested female medfly cDNA (5), undigested control skeletal muscle cDNA (6). *Hind* III / *Sac* II molecular size marker (M). A decrease in the size distribution of the smear of DNA fragments seen in the undigested samples indicates a successful digestion.

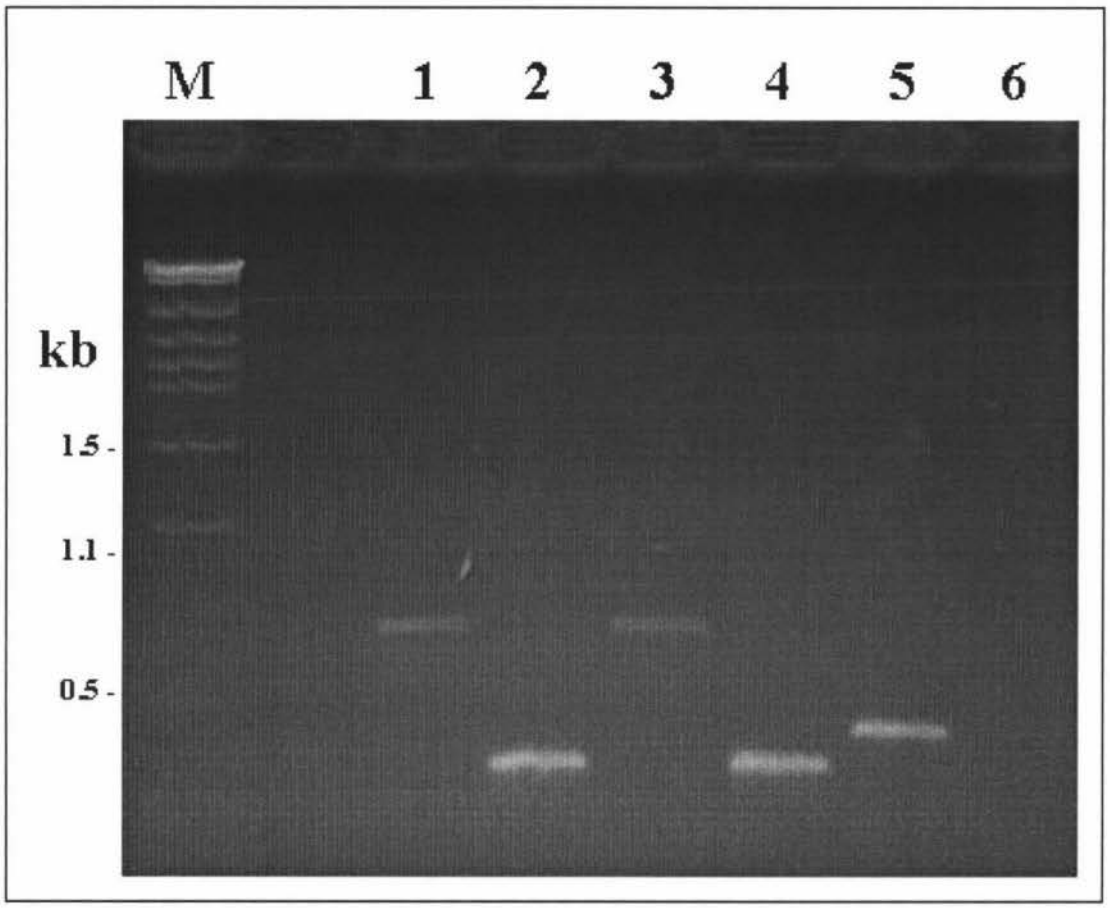

Figure 3.11: PCR based ligation analysis of skeletal muscle control samples.

Products of the PCR based adapter ligation test, performed on control subtraction samples, were electrophoresed on a 2% agarose gel at 90V for 1.5 hours. The templates and primer sets used were as follows; skeletal muscle control cDNA with ligated adapter 1 template PCR amplified using G3PDH 3' primer and PCR primer 1 (1), skeletal muscle control cDNA with ligated adapter 1 template PCR amplified using G3PDH 3' and 5' primers (2), skeletal muscle control cDNA with ligated adapter 2R template PCR amplified G3PDH 3' primer and PCR primer 1 (3), skeletal muscle control cDNA with ligated adapter 1 template PCR amplified using G3PDH 3' and 5' primers (4), positive PCR control (5), negative 'no DNA' PCR control (6), *Hind* III / *Sac* II molecular weight marker (M).

3.2.2.3 Primary PCR

Initial attempts at primary PCR failed to produce products. Several variations of the PCR protocol were trialed, but all failed to produce product. Gradient PCR was performed to identify optimum conditions (Section 2.5.3). The results of this are shown in Figure 3.12. The samples for the reverse subtraction did not produce any PCR product. The forward subtraction, however, did produce product. Subsequent attempts to reproduce this result using identical PCR conditions found to be optimal in the gradient PCR failed, as did further PCR experiments with the reverse subtracted template. Due to time constraints it was decided to continue the cDNA subtraction using the primary PCR product from the gradient PCR (lanes 4 and 5, Fig. 3.12). Therefore at this point the reverse subtraction was abandoned, eliminating the ability to perform a differential screen on the resulting forward subtracted library.

3.2.2.4 Secondary PCR

Secondary PCR was successful on the first attempt. The results are shown in Figure 3.13. These results were similar to the expected result illustrated by the cDNA subtraction kit manufacturer. It is stated that a successful subtraction will show a different banding pattern when experimental and unsubtracted control samples are compared. In this case a difference can be observed in the banding pattern between 1.1 and 0.5 kb.

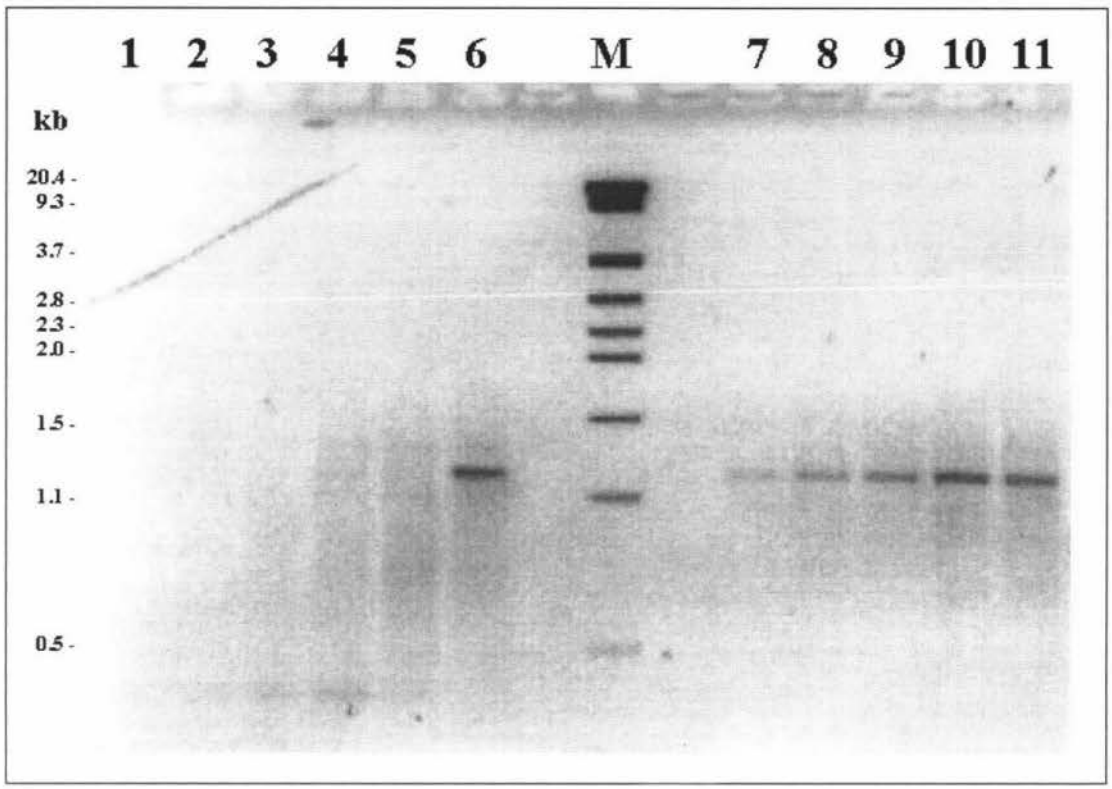

Figure 3.12: Gel electrophoresis analysis of gradient PCR products.

Gradient PCR was performed in order to find working conditions for the primary PCR step of cDNA subtraction. 10 µl of each PCR product was run on a 2% agarose gel at 75 V for 1.5 hours. The template for the PCR was cDNA obtained from the second hybridisation step of the cDNA subtractions. The primer used was the PCR primer 1 from the subtraction kit. The PCR annealing temperature gradient was; 40.4°C (1), 41.4°C (2), 44.4°C (3), 48.3°C (4), 52.6°C (5), 54.7°C (6), using forward subtracted cDNA as template, and; 40.4°C (7), 41.4°C (8), 44.4°C (9), 48.3°C (10), 52.6°C (11), using unsubtracted tester control cDNA as template. (M) Indicates *Hind* III / *Sac* II molecular size marker.

This gel indicates that the optimum annealing temperature for primary PCR is between 48 and 53°C.

The expected result is a smear from approximately 0.2 – 2.0 kb, with no major bands seen. This result is seen in lanes 4 and 5. The band seen at around 1.3 kb in lanes 6 to 11 is probably highly abundant ribosomal sequence. As the products containing this band were not needed for the secondary PCR step they caused no problem.

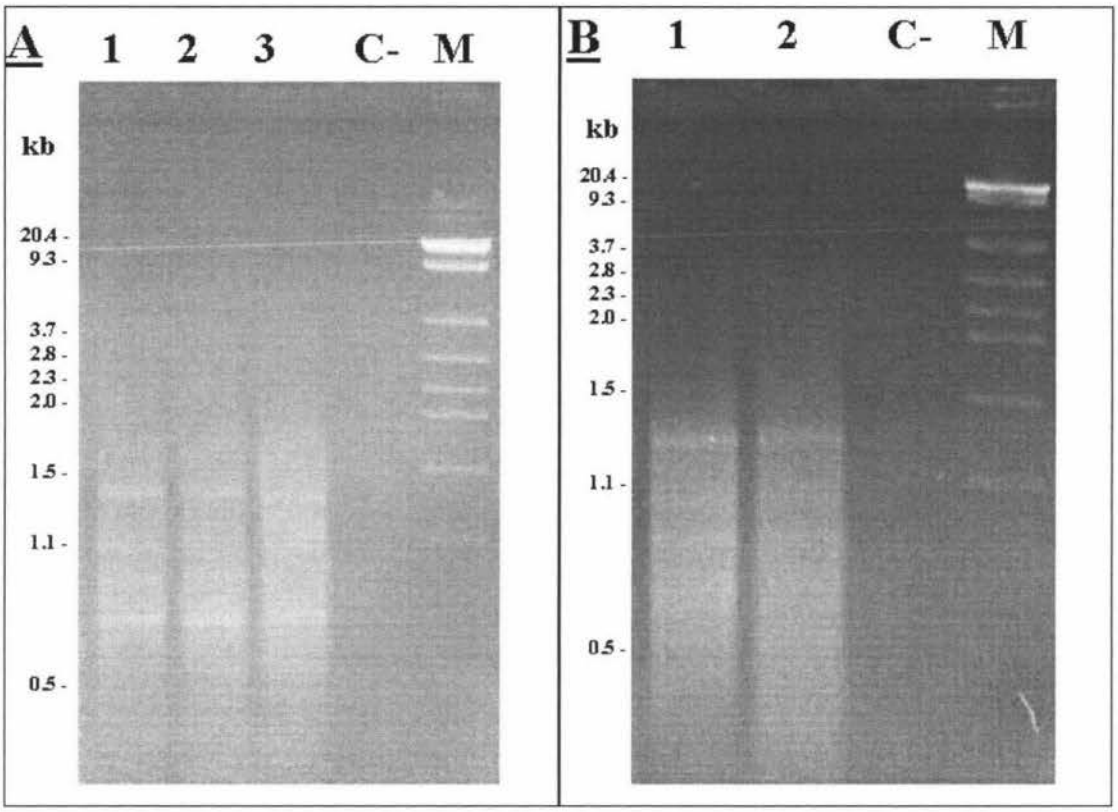

Figure 3.13: Analysis of secondary PCR by gel electrophoresis indicates that PCR was successful.

PCR products of the secondary PCR step of the cDNA protocol were electrophoresed at 75 V for 2 hours on 2% agarose gels. These products were produced using the nested PCR primers of the cDNA subtraction kit.

(A) Products of secondary PCR with experimental samples using; 48.3°C annealing temperature primary PCR product as template (1), 52.6°C annealing temperature primary PCR product as template (2), 54.7°C annealing temperature primary PCR product as template (3).

(B) Products of secondary PCR with control samples using; 48.3°C annealing temperature primary PCR product as template (1), 52.6°C annealing temperature primary PCR product as template (2). In both cases, (C-) denotes no-DNA negative PCR control, and (M) denotes *Hind* III / *Sac* II molecular size marker.

Both control and experimental samples show an expected smear, with an increase in observable banding over the primary PCR products.

3.2.2.5 Subtraction efficiency test

The subtraction efficiency test (Section 2.2.6) detects if unwanted sequences have been subtracted. In this case it illustrates whether non male-specific genes had been removed. This PCR based test was conducted using primers for medfly *xanthine dehydrogenase* gene (see Table 2.1), which is not male-specifically expressed (Pitts and Zwiebel 2001). This test was carried out twice. Once with samples taken after 25, 30, 35, 40, and 45 cycles of PCR, to identify the exponential phase of the PCR reaction. And a second time over a shorter range (30, 32, 34, 36, and 38 cycles) to get clearer results. Results of these tests are shown in Figure 3.14. The fragment seen at approximately 500 bp represents the expected size of the PCR product. In both cases it is clear that fragments in the lanes of subtracted samples are not present, or significantly weaker than in corresponding unsubtracted samples. This is a good indication that subtraction of non-sex specific sequences have occurred.

3.2.2.6 Male specific enrichment test

This test required PCR primers for a medfly male specific gene. A Genebank database search was conducted to find a medfly male-specific gene sequence. This search located a small number of male specific microsatellite fragments along with a family of genes known as Male Specific Serum Polypeptides (MSSPs) (Christophides *et al.* 2000). *mssp-α2* (Genbank accession number Y19145) was chosen as it is expressed solely in male fatbodies, unlike other members of the family which have a small amount of female expression. The primers created for the mssp - α2 gene are shown in Table 2.1.

The results of the male specific enrichment test are shown in Figure 3.15. These results indicate that there has been enrichment of male-specific sequences. This is evident as bands in the 10, 15, and 20 cycle lanes are more intense in the subtracted samples then in the unsubtracted. By the 25 cycle samples there appears to have been equalisation of PCR products.

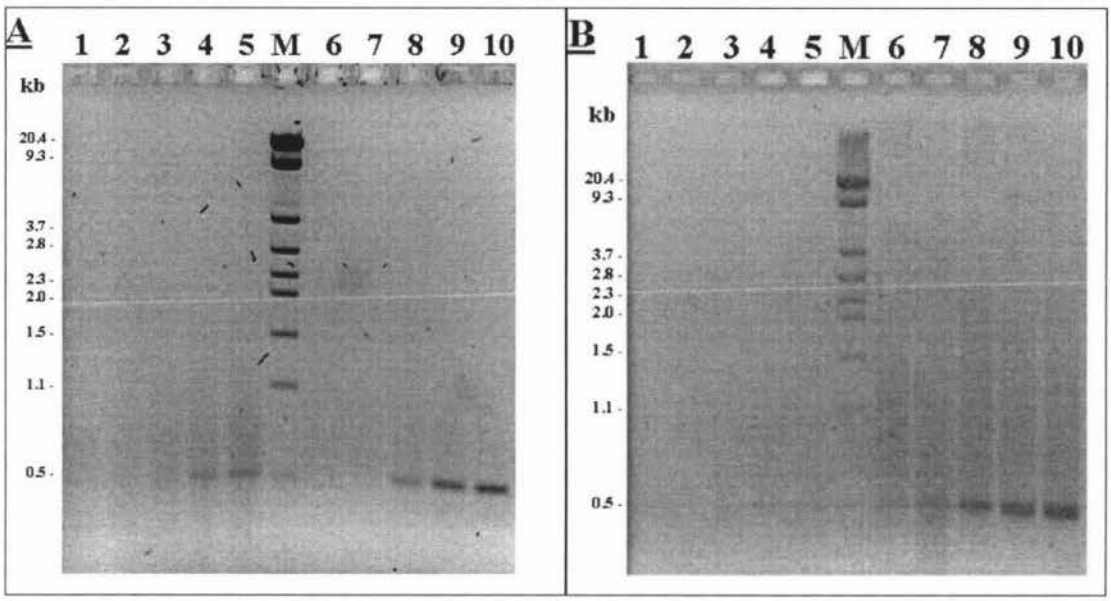

Figure 3.14: PCR based subtraction efficiency test indicates effective removal of sex-specific cDNAs.

PCR products obtained from the subtraction efficiency test using PCR primers for the medfly xanthine dehydrogenase gene were electrophoresed on 2 % TAE agarose gels at 75 V for 2 hours.

On both gel photographs lanes 1 to 5 are products of PCR using subtracted cDNA as template, lanes 6 to 10 are products of PCR using unsubtracted control cDNA as template, and lane M denotes *Hind* III / *Sac* II molecular size marker.

(A) Samples of PCR product were taken after; 25 cycles (1), 30 cycles (2), 35 cycles (3), 40 cycles (4), 45 cycles (5), 25 cycles (6), 30 cycles (7), 35 cycles (8), 40 cycles (9), 45 cycles (10).

(B) Samples of PCR product were taken after; 30 cycles (l), 32 cycles (2), 34 cycles (3), 36 cycles (4), 38 cycles (5), 30 cycles (6), 32 cycles (7), 34 cycles (8), 36 cycles (9), 38 cycles (10).

The CcXDH primers used produced the expected band at approximately 500 bp. If the subtraction was successful, when a subtracted sample is compared to an unsubtracted sample taken after the same number of cycles a loss of this band should be seen. However after sufficient cycles of PCR product will be seen in both samples.

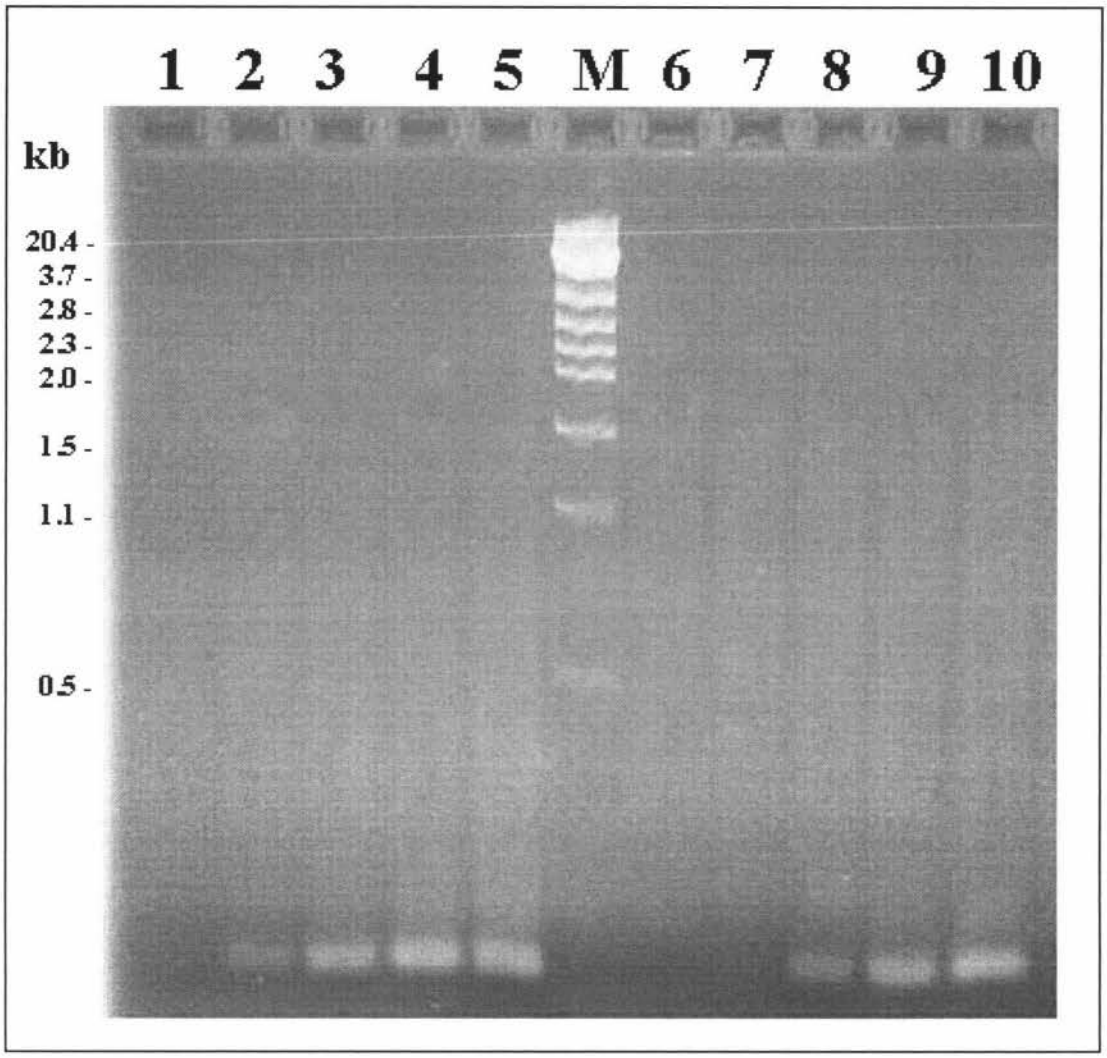

Figure 3.15: PCR based male-specific enrichment test indicates some enrichment of male-specific cDNAs.

PCR products obtained from the male-specific enrichment test using primers for the medfly male-specific serum polypeptide α2, electrophoresed on a 2 % TAE agarose gel for 2 hours at 75 V.

Lanes 1 to 5 are products from subtracted cDNA template, lanes 6 to 10 are products from unsubtracted cDNA template, and M denotes *Hind* III / *Sac* II molecular size marker. Samples of PCR product were taken after; 5 cycles (1), 10 cycles (2), 15 cycles (3), 20 cycles (4), 25 cycles (5), 5 cycles (6), 10 cycles (7), 15 cycles (8), 20 cycles (9), 25 cycles (10).

3.2.3 Cloning the subtracted library

The subtracted library was ligated into the pGem-T easy vector, and transformed into both DH5α chemically competent cells, and DH10b electro-competent cells (Section 2.3). Blue/white selection was used to identify transformants containing the vector. 45 transformants were obtained from the DH5α transformation, and 489 colonies from the DH10b transformation.

All of these colonies were plated on ampicillin selection plates (Section 2.4), and colony lifts were taken from these plates (Section 2.10.3)

3.2.4 Colony screens

Membranes obtained by colony lifts were screened using [^{32}P] labelled probes made from total adult male and female total cDNA. A representative example of these results is shown in Figure 3.16. The results indicate that no colonies hybridised significantly more strongly with male probes then with female probes. There were however several colonies that showed a difference in hybridisation intensity, which is hypothesised to be the result of experimental error such as incorrect loading. In addition to this a large number of colonies failed to hybridise with either probe.

3.2.5 Screen of selected clones

40 colonies were selected for more sensitive Southern DNA hybridisation analysis. These colonies were chosen on the basis that there was either a slight difference in the hybridisation in the previous experiment, or that they failed to hybridise to either male or female DNA. The cDNA inserts were amplified by PCR, size separated by agarose gel electrophoresis, and transferred to a nylon membrane. The membrane was hybridised first with ^{32}P labelled male cDNA then stripped and reprobed with ^{32}P-labelled female cDNA. The PCR fragments hybridised equally well with both probes, demonstrating that Southern hybridisation is a more sensitive test then the colony hybridisations. The results clearly illustrate a lack of any sex specific hybridisation (Figure 3.17).

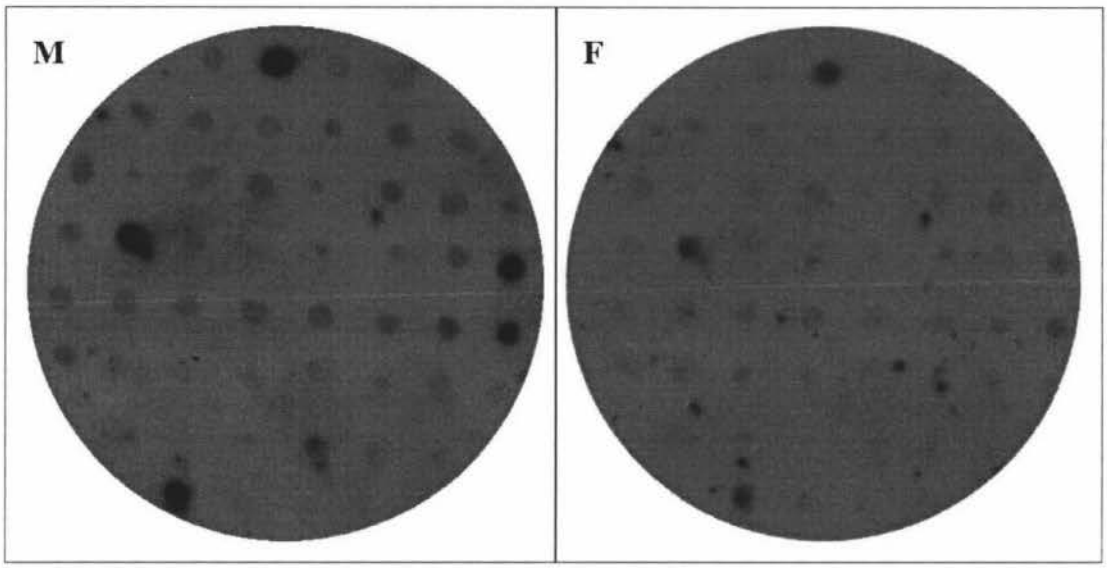

Figure 3.16: Colony screens demonstrate a lack of male-specific hybridisation of new subtracted cDNAs.

These autoradiographs show an example of colony lift membranes hybridised with ^{32}P-labelled total male (M), and total female (F) cDNA probes overnight, and exposed for 15 hours. These two blots are representative of all 24 individual membranes.

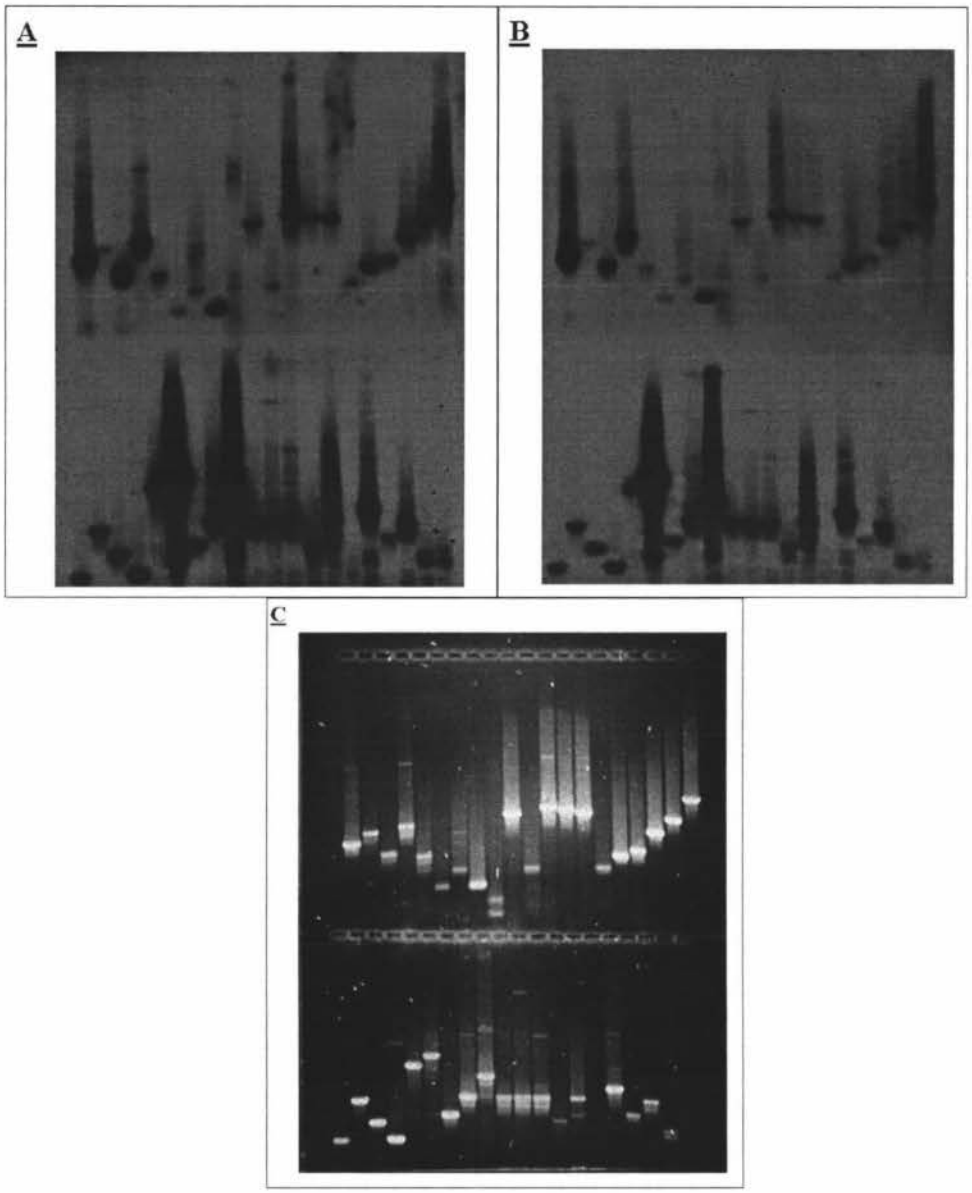

Figure 3.17: Hybridisation analysis of 40 selected clones confirm evidence that cDNAs of the second subtracted library hybridise equally well to both male and female cDNA probes.

Results of gel electrophoresis and Southern hybridisation of PCR amplification products of 40 selected clones are shown. (A) Autoradiograph of the blot of the 40 selected clones hybridised with a total male cDNA probe. (B) Autoradiograph of the blot of the 40 selected clones hybridised with a total female cDNA probe. (C) Gel photograph of results of gel electrophoresis of PCR products of the 40 selected clones before Southern transfer (shown for comparison).

No difference was seen in the hybridisation of the male or female probes to these clones. This indicates that no male-specific sequences were represented here.

3.3 Alternative approach to male determining gene identification

Medfly *transformer* (*tra*) expression is controlled by sex-specific RNA splicing. It is hypothesised that the male determining gene could encode an RNA binding protein. This alternative approach involved identifying Y-linked RNA binding genes from medfly, by low stringency hybridisation using fragments of known RNA binding genes as probes. PCR probes for medfly *sex lethal* (*Sxl*) were available (see Table 2.1), however no other known RNA binding genes similar to *Sxl* had been identified in medfly (*C. capitata sxl* is not Y-linked).

The *embryonic lethal, abnormal vision* (*elav*) gene of *D. melanogaster* encodes an RNA binding protein related to *Sxl*. Using the sequence for *D. melanogaster elav*, PCR probes were designed to isolate medfly *elav* (see Table 2.1)

It was proposed to use *Sxl* and *elav* probes in low stringency hybridisation experiments to identify if a related male-specific gene exists in medfly.

3.3.1 Isolation and cloning of medfly elav gene

Total medfly cDNA was produced by reverse transcription of total RNA, using both randomly primed and oligo-dT primed reactions. *Elav* sequences were then amplified from these cDNA populations by PCR using a set of degenerate primers (see Table 2.1). Initially no product of the expected size was obtained. However positive control PCR reactions with medfly *Sxl* primers were successful. Thus the cDNA synthesis appeared to have been successful. The external *elav* PCR was repeated with a fresh aliquot of *Taq* polymerase, and a lower annealing temperature of 45°C. The internal *elav* primer set (see Table 2.1) PCR reaction was also performed. Both of these reactions produced product, as shown in Figure 3.18A. PCR product from the external primer reaction was diluted 1 in 500 and used as template for the internal *elav* primer PCR reaction. This reaction produced a 375 bp band as seen in Figure 3.18B. The 375 bp fragment was gel extracted, ligated into pDrive, and used to transform DH5α. 5 transformants were randomly picked for plasmid DNA preparations (Section 2.3.3). The transformant exhibiting the strongest band following *Eco* RI digestion was sequenced to confirm its identity. This sequence demonstrated that the clone was not a fragment *elav*. Due to time constraints the

pursuit of *elav* was abandoned, and the low stringency hybridistions were performed with *Sxl* only.

3.3.2 Low stringency screens

α-^{32}P labelled probe was made using an *Sxl* PCR product, and used to probe medfly genomic digests under low stringency hybridisation conditions. No male-specific bands were seen. This indicates an absence of any Y-linked RNA binding proteins closely related to *Sxl*, which is not sex-linked in medfly. These results are shown in Figure 3.19

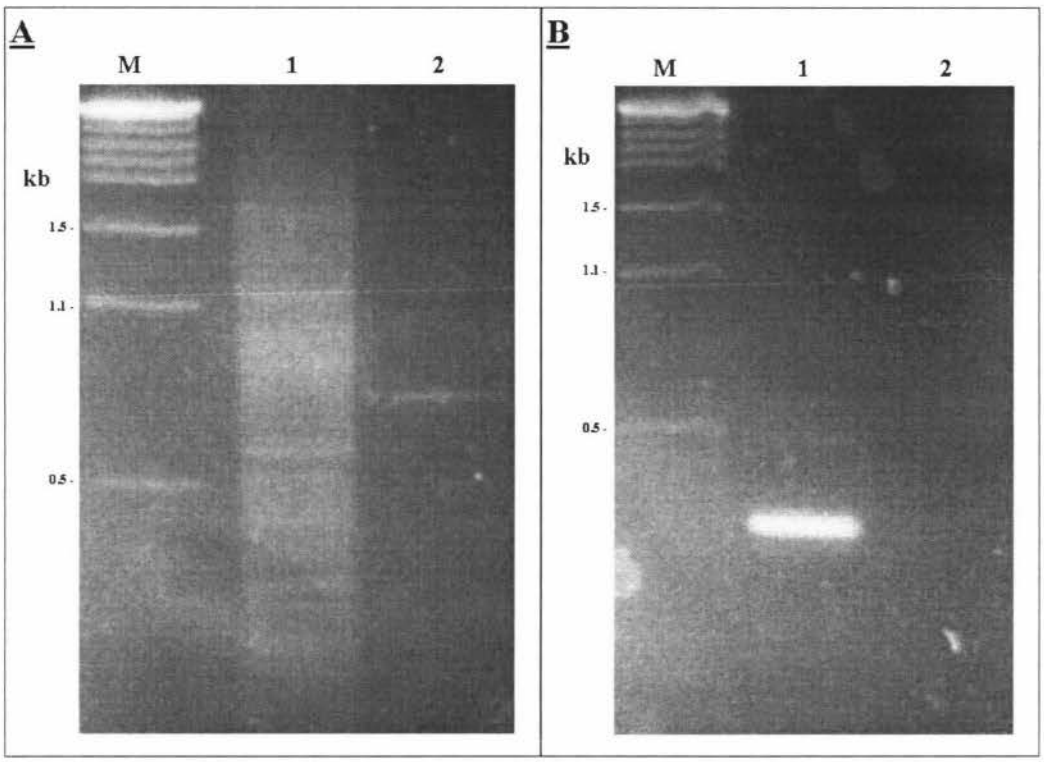

Figure 3.18: Medfly *elav* fragment obtained using degenerate primer PCR.

Products of PCR using primers for the *elav* gene electrophoresed on 2% agarose gels for 1.5 hours.

(A) Products from PCR using medfly cDNA as template and; *elav* external primer set (1), *elav* internal primer set (2). (M) denotes *Hind* III / *Sac* II molecular weight marker.

(B) Products from PCR using *elav* external primer product as template and; *elav* internal primer set (1). (2) denotes the no-DNA negative control, and (M) denotes *Hind* III / *Sac* II molecular weight marker.

The *elav* primers were expected to produce the band seen at approximately 375 bps.

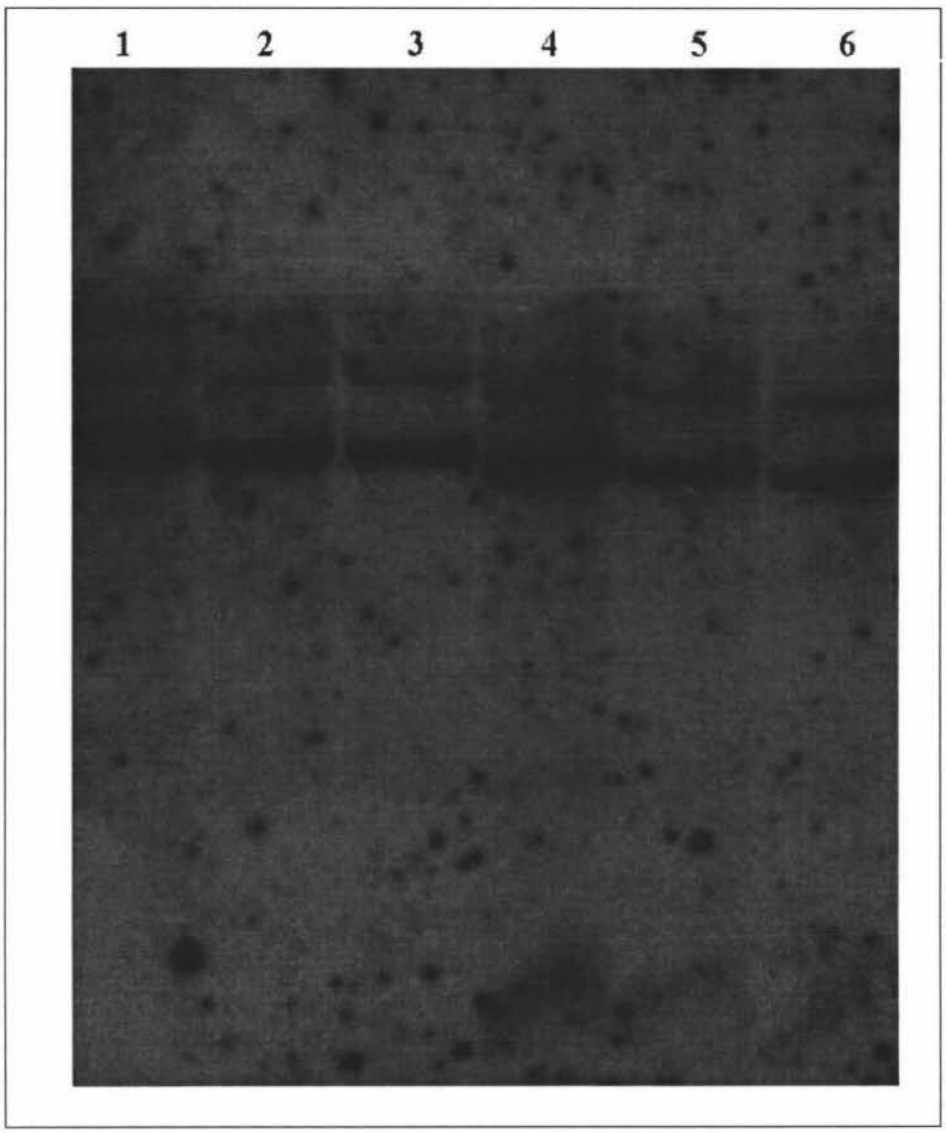

Figure 3.19: Low stringency hybridisations fail to identify a male-specific gene encoding an RNA binding protein of the *Sxl* family of RNA binding proteins.

Autoradiographs of overnight exposures of membranes with target medfly genomic DNA hybridised with an *Sxl* probe under low stringency hybridisation conditions at 37°C.

(1) male F18 strain genomic DNA digested with *Pst* I, (2) female genomic DNA digested with *Pst* I, (3) male genomic DNA digested with *Pst* I, (4) male F18 strain genomic DNA digested with *Eco* RI, (5) female genomic DNA digested with *Eco* RI, (6) male genomic DNA digested with *Eco* RI.

The lack of any male bias in the hybridisation of this probe to the genomic targets at these hybridisation conditions indicates the absence of a gene encoding a male-specific RNA binding protein of the *Sxl* family of proteins.

4 Discussion

4.1 Quality of subtracted Male-specific libraries

The results obtained from the cDNA libraries indicate that there had been a significant lack of subtraction. This is especially true for the first subtraction where a number of mitochondrial sequences and housekeeping genes were found. It is possible that these sequences were differentially expressed between males and females, however this is unlikely. The presence of these sequences in the subtracted library was more likely due to these genes being highly expressed and the subtraction procedure being unable to completely remove them.

In addition to the lack of subtraction it appears that no male-specific enrichment had occurred. No male-specific sequences were found after screening either of the subtracted libraries. This contradicts the male-specific enrichment test results obtained from the second cDNA subtraction (section 3.2.2.6).

It was therefore concluded that both attempts at creating subtracted cDNA libraries enriched for male-specific sequences were unsuccessful.

4.1.1 Subtraction efficiency tests

In retrospect the subtraction efficiency tests could have been engineered to be more informative. The subtraction efficiency test itself should have been carried out using PCR primers for the medfly ADP/ATP translocase identified from the first subtracted library. The presence of the ADP/ATP translocase fragment in the first library indicates that this gene is expressed very highly and because of this was not fully subtracted out. The second subtraction was carried out in order to produce a more efficiently subtracted library. Hence the use of the ADP/ATP translocase in the efficiency test would have been a good indication of both the efficiency of the second subtraction compared to the first.

It would also have been informative to screen the colonies obtained from the second subtraction with probes for the genes used in the subtraction efficiency tests. Showing, with hybridisation screens of the subtracted library, that fragments of the gene used in the actual subtraction efficiency test (CcXDH) would have provided evidence to support the results of that test. Also, given that it was indicated that there

had been enrichment for male-specific fragments, based on results of the male-specific enrichment test using MSSP-α2 gene primers, and that subsequent screens contradicted this result. It would have been advantageous to have evidence of the presence of the MSSP-α2 in the final subtracted library to support the results of the male-specific enrichment test. Both these screens could be considered controls for the subtraction efficiency tests, and if they had been done soon after the subtracted library had been produced would have given a better indication of the quality of the second library.

4.1.2 Tester and driver cDNAs

It is believed that male determination might only require a pulse of high activity of the male determining gene during early development. This pulse of activity would initiate male determination, which would then autoregulate itself. Thus high levels of expression of the male-determining gene would no longer be necessary in somatic cells. However it is still possible that there would be low levels of expression of the male determining gene throughout the life cycle, particularly in germline cells.

If this is the case it is important that the mRNA used to prepare cDNA for the subtraction was taken at a stage in development when the male-determining gene was active. For this reason, in the original subtracted library, total adult female cDNA was used to drive subtraction of total cDNA from first instar male larvae. Female killing systems allow a male only population of larvae to be produced, however no such method allows for the selection of female larvae. This means that the clones produced from this subtraction should represent both male-specific genes and early developmental genes expressed in first instar larvae. Ideally the subtraction should be performed using cDNA from male and female embryos, but no plausible method of separating large populations of embryos exists.

The second subtraction was performed using only adult medfly RNA as time did not allow for the production of a first instar male larvae RNA sample. Therefore if the medfly male determining gene is only expressed at very low levels during the later part of the life cycle there is a good chance that it could have been lost during the second subtraction.

4.1.3 Sample complexity

Another factor that could have lead to the failure of these subtraction attempts is the complexity of the samples that were compared. The complexity relates to the total number of different cDNA sequences that constitute a cDNA population (Davidson 1986). For example, where two cell types from the same organism are being compared, the complexity will be low due to the lower number of genes that are expressed in the specific tissues being studied. However when total male and female cDNAs are compared the complexity will be high due to the large number of mRNAs representing all the genes expressed in that organism. It is important to make clear that it is not the number of differentially expressed sequences, but the total number of sequences in the two sample populations for comparison that contribute to the complexity of the sample. The problem of complexity represents one of the critical parameters for subtraction. An increase in the complexity of the samples being compared leads to a drop in the efficiency of subtraction (Ausubel et al 1997, Section 5.9). In general as the complexity of cDNA populations increase the number of rounds of subtraction needed to adequately remove common sequences also increases. The cDNA subtraction kit from Clontech is designed mainly for use with mammalian systems and the hybridisations are optimised so that only two rounds are required. It is probable that there were not enough rounds of subtraction to remove all of the sequences common to both medfly sexes. This would explain the presence of sequences found in the initial subtracted library.

4.2 Screening subtracted libraries

In general the screening of PCR amplified cDNA subtracted clones with ^{32}P labelled male and female cDNA was successful and quite sensitive. However very few clones showed any difference in their sex specificity and no clone hybridised only with labelled male cDNA. With no obvious candidate for the male determining gene the clones that show a difference between male and female hybridisation were studied more closely. On closer inspection the majority of clones showed no sex specificity. However 2 clones, #4 and #42 from the first subtraction gave anomalous results.

4.2.1 Clone 4

Clone 4 showed a significant male hybridisation bias after the initial screens. Conversely no difference was seen in Southern and Northern hybridisations using clone 4 labelled with ^{32}P to probe male and female total RNA. Further Southern hybridisations showed that clone #4 was not Y-linked. The difference seen in the 100 clone screen was probably due to bad transfer of cDNA the membrane filter. Most likely due to an air bubble over the clone during transfer

4.2.2 Clone 42

Clone #42 displayed significant hybridisation to both male and female hybridisation probes after screening the 100 clones. However when this clone was used to probe a membrane of genomic DNA a male-specific band was seen. Further study showed that this seemingly male-specific band was that result of a contamination of the male genomic DNA digestion with a pUC based plasmid. The flanking pGem-T regions of the #4 clone hybridised with this contaminant to produce the band seen only in the male lane. Control hybridisations using pBluescript to probe membranes of genomic DNA showed that medfly DNA contains some repeat sequences that have sequence similarity with pUC vectors.

Further anomalous results were obtained from sequencing reactions with clone #42. Comparing forward and reverse sequencing reactions showed quite clearly that this clone was a fusion of two unrelated fragments. One showing high sequence similarity to *L. cuprina* ATP/ADP translocase, the other having no significant sequence matches on Genbank. Due to unreliable sequencing, the connecting stretch between the two could not be properly identified. However in each case the readable sequence terminates a short way into sequence resembling PCR primer 1 in the sequence of adapter 1 from the cDNA subtraction system. It appears that during the subtraction an anomalous event occurred were two separate, short fragments joined at some point in the adapter 1 sequence.

The presence of the ADP/ATP translocase in the subtracted library is most likely due inadequate removal as a result of it being highly expressed. The subtraction method can fail to properly subtract out genes which are very highly expressed, and ADP/ATP translocase has been described as the most abundant integral protein of the mitochondrial inner membrane (Louvi and Tsitilou 1992).

The difference in the sequence similarities observed for the *L. cuprina* and *D. melanogaster* ADP/ATP translocases to the *C. capitata* fragment appear to be due to codon bias. *C. capitata* and L. cuprina share an AT rich genome, and a similar codon bias. Therefore sequences from these two that are well conserved across different species will be more similar to each other then either will be to *D. melanogaster*. At the protein level there is almost no difference between the three species mentioned. When the predicted protein sequence of the *C. capitata* fragment was compared to that of the other two it was found that it was identical to the *L. cuprina* sequence and differed by only one amino acid from *D. melanogaster*. Studies with the ADP/ATP translocases of several species show that the protein sequence is well conserved (Louvi and Tsitilou 1992, Zhang et al 1999).

4.2.3 Colony screens

The colony screens were largely uninformative, as many colonies did not hybridise well with either male or female ^{32}P labeled probes. Screens of the PCR products of a selected group of clones showed that the cDNA fragments of the subtracted clones did hybridise well to both male and female cDNA, but no male-specific bias was seen. None of the selected clones showed any hybridisation bias. The lack of hybridisation of ^{32}P labeled cDNA seen in the colony screens was probably due to excess cellular debris, from bacterial colonies that were too large, interfering with either the transfer of DNA to the membranes or the UV crosslinking of the DNA to the membranes. An attempt at solving this problem was made by repeating the colony lift procedure with newly grown colonies of a smaller size. This was unsuccessful, as the colonies would no longer grow at an even rate, leading to plates with both large and small colonies. With the results from the screen of 40 selected clones indicating that there were no male-specific sequences in the second subtracted library, and due to time constraints it was decided to stop individual screens at 40.

4.3 Sequencing of clones obtained by cDNA subtraction

Many of the clones that were sequenced did not produce sequence of very high quality. This appeared to be largely due to GC rich regions causing the sequencing protein machinery slip along, or detach from the template DNA. It appeared that the relatively GC rich adapter sequences from the cDNA subtraction where often the cause of this. This could be seen in a dramatic decrease in the height of peaks seen on electropherograms at the region of the adapter sequences. A large number of the clones that were difficult to sequence also contained poly-T stretches within short distances of the adapter regions. These poly-T stretches represent the poly-A tails of the mRNAs used to make the cDNA for subtraction. Where this occurred it was impossible to get reliable sequence of any region after the poly-T stretch using the BigDye terminator sequencing reaction. Using the dRhodamine sequencing reaction it was possible to get reasonable sequence for most of the clones that showed GC region difficulties. However where poly-T stretches occurred it was impossible to sequence through them. It is likely that this problem could be overcome with new enzymes used in v3.1 kits.

4.4 An attempt to identify the medfly male determining gene by identification of genes encoding male-specific RNA binding proteins

The theory that the medfly male determining gene was likely to encode an RNA binding protein was based on the observation that many of the genes known to operate in sex determination cascades encode this type of protein. *Sxl*, which functions as the master switch gene of the sex determination pathway of *D. melanogastor*, operates by sex-specifically splicing RNA. While it is known that *Sxl* does not function in this way in *C capitata* it was thought that the male determining gene could operate in a similar way, by binding RNA. Primers were available to PCR amplify a fragment of the *Sxl* gene for use in a low stringency hybridisation experiment. This experiment was based on the hypothesis that the male determining gene would be similar to *Sxl*. If so, hybridisation to genomic DNA under low stringency an *Sxl* probe should identify a Y-linked gene. However no male-specific hybridisation was observed. The hybridisation experiments, designed to attempt to identify any male-specific genes

encoding RNA binding proteins distantly related to SXL, failed to identify any candidates. It was therefore concluded that the male determining gene could be from a different family of RNA binding proteins to *Sxl*. However shortly after this work was carried out Pane et al (2002) published work done with the *C. capitata tra* gene. This work showed that *tra* operates as the master switch gene of the medfly system, in much the same way as *Sxl* works in *D. melanogastor*. From their work with the *tra* gene Pane et al developed a model for sex determination in medfly (see Figure 1.3). In this model they suggest three ways in which the medfly male determining gene could function. These are: prevention of translation of *tra* mRNA, direct inhibition of the TRA protein, or interference with TRA autoregulation to cause its function to be switched off. The latter two of these options involve protein/protein interactions. It follows then that if the function of the medfly male determining gene is one of these, the gene will not nesecceriaIy encode an RNA binding protein. Therefore experiments designed to identify Y-linked male-specific RNA binding genes will fail to isolate the male determining gene of medfly.

Evaluation of the normal function of *Sxl* puts further doubt on the possibility of the success of the low stringency screens for male-specific RNA binding proteins. This gene has normal function in neuronal cells (Erickson and Cline 1991), and in most species appears to have no role in sex determination. Combined with theory that *Sxl* has been co-opted into the sex determining pathway of drosophilids reasonably recently in evolutionary history (Shearman 2002, Schutt and Nothinger 2000)), this leads to the conclusion that RNA binding proteins of this family unlikely to have a role in the sex determination pathways of other species.

4.5 Future directions

At the time of writing there are no plans to further pursue any work in relation to this project. However if at a future time this work were to be revisited the most likely avenue of study to pursue would be to identify the male determining through yeast two-hybrid analysis (Ausubel 1997, Unit 13.14).

As the work presented in this thesis did not identify an RNA binding male determining gene it follows that the gene may have a different mode of activity. The model for medfly male determination suggested by Pane et al. (2002), also implicates protein/protein interactions as a mode of activity for the male determining gene. It is therefore logical to test this. With the recent characterization of the medfly *tra* gene (Pane et al. 2002) it would be practical to perform a two-hybrid library screen, using the medfly TRA protein as the 'bait'. This type of screen should identify any proteins that interact with TRA. This approach would have the added benefit of identifying the medfly TRA-2 protein if it exists. However if the male determination gene product does interact with RNA, specifically that of *tra,* then this interaction could be detected using yeast three-hybrid analysis (Bernstein *et al.* 2002). Using this system the proteins that interact with *tra* RNA can be identified in a similar way to yeast two-hybrid systems.

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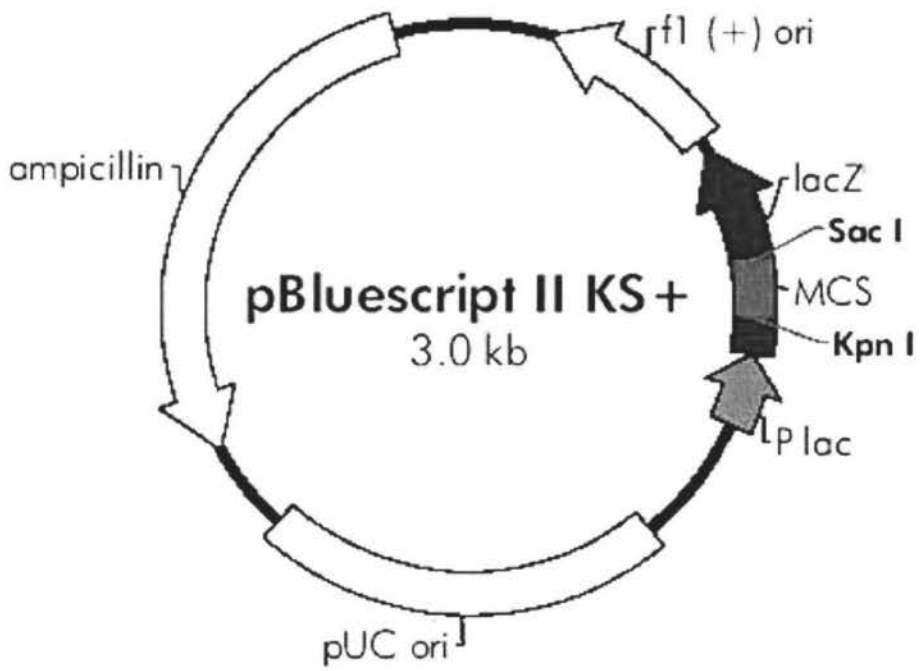
Appendix

1 Plasmid maps

pBluescript

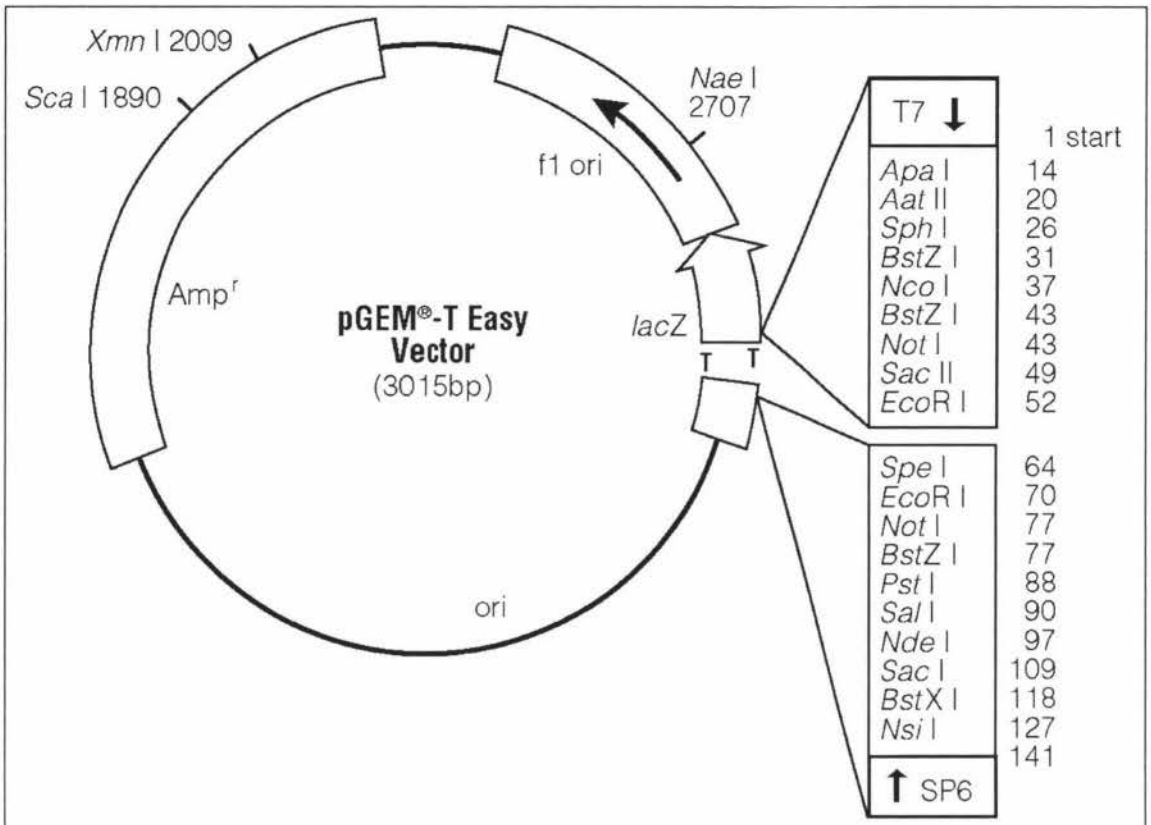
(Stratagene)



f1 (+) origin	135–441
β-galactosidase α-fragment	460–816
multiple cloning site	653–760
lac promoter	817–938
pUC origin	1158–1825
ampicillin resistance (bla) ORF	1976–2833

pGem-T easy

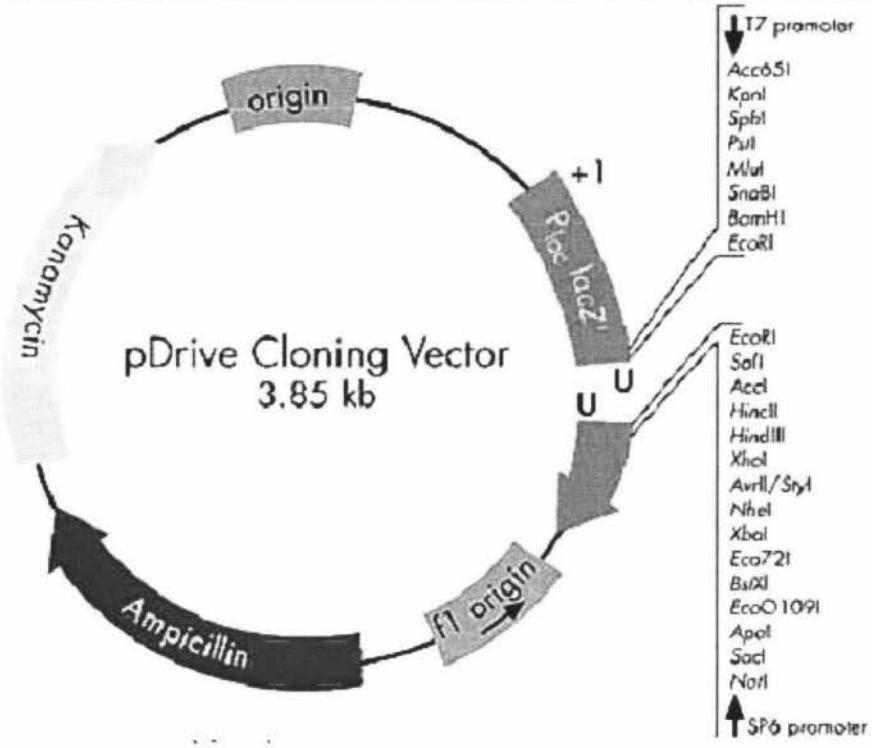
(Promega)



T7 RNA Polymerase transcription initiation site	1
SP6 RNA Polymerase transcription initiation site	141
T7 RNA Polymerase promoter (–17 to +3)	2999–3
SP6 RNA Polymerase promoter (–17 to +3)	139–158
multiple cloning region	10–128
lacZ start codon	180
lacoperon sequences	2836–2996, 166–395
lacoperator	200–216
b-lactamase coding region	1337–2197
phage f1 region	2380–2835
binding site of pUC/M13 Forward Sequencing Primer	2956–2972
binding site of pUC/M13 Reverse Sequencing Primer	176–192

pDrive

(Qaigen)



Multiple cloning site	266–393
LacZ α-peptide	216–593
T7 RNA polymerase promoter	239–258
T7 transcription start	256
SP6 RNA polymerase promoter	398–417
SP6 transcription start	400
Ampicillin resistance gene	1175–2032
Kanamycin resistance gene	2181–2993
pUC origin	3668
Phage f1 origin	588–1043
Primer binding sites:	
M13 forward (–20)	431–447
M13 forward (–40)	451–467
M13 reverse	209–224
T7 promoter primer	239–258
SP6 promoter primer	400–418