GENETIC TRANSFORMATION OF THE MOSQUITO AEDES AEGYPTI USING A TRANSPOSABLE GENETIC ELEMENT

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by

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ABSTRACT

Tropical diseases represent some of the most serious health care problems in the world today. These diseases are commonly transmitted by insect vectors such as the mosquito *Aedes aegypti*, the vector of the arboviral diseases yellow fever, dengue and dengue haemorrhagic fever (DHF). The application of synthetic insecticides has been very successful in the control of insect vectors. However, the development of multiple resistance to these compounds has led to the study of alternative vector control strategies to complement the existing methods. Results of previous attempts at genetic control involving the release of sterile males, have often fallen short of the predicted success of these programmes. In an attempt to avoid the problems associated with previous methods of genetic control the potential of germline manipulation is being studied, whereby any genetic modification of individuals introduced into a population could be transmitted through subsequent generations.

The aim of the research reported in this thesis was to investigate the possibilty of introducing the P element, a transposable genetic element isolated from *Drosophila melanogaster*, into the germline of *Ae.aegypti*, for use as a DNA transformation vector in germline manipulation. As in *Drosophila*, the P element was to be introduced into the germline cells of the mosquito by means of a technique of micro-injection into early embryos. The P element used was engineered to carry a resistance gene for the antibiotic G418, to permit selection of transformed individuals, and a multiple cloning site to facilitate the introduction of a specific DNA sequence. A micro-injection system has been developed as part of this research. This system has been designed specifically for introduction of DNA into the embryos of *Ae.aegypti* which differ considerably from those of *Drosophila*. Post-injection survival rates, comparable to those obtained with *Drosophila*, have now been achieved and the sensitivity of wild type *Ae.aegypti* larvae to the antibiotic G418 determined. Individuals successfully reared from injected embryos have been mated to wild type mosquitos to give rise to subsequent generations showing a degree of resistance to the antibiotic.

Genomic DNA has been isolated from adults arising from injected embryos and their subsequent progeny, and probed with the foreign vector DNA plasmid. Foreign DNA sequences have been detected in G_0, G_1, G_2 and G_3 individuals. Reprobing 3 of these genomic integration events, with isolated regions of the vector plasmid suggests that each event represents an integration of sequences carried on a single fragment of this plasmid. The integration events do, however, appear to be very unstable, disappearing or varying in size from one generation to the next. An assay system for P element function has only produced negative results to date. The results of this assay, coupled with the data obtained from probing restriction enzyme digests of genomic DNA from transformants suggest that the integration events are not P mediated. Possible mechanisms of integration and rearrangement of sequences are discussed.

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CHAPTER 1

INTRODUCTION

1.1 THE MOSQUITO AEDES AEGYPTI

Aedes aegypti is a mosquito of great medical importance in terms of its disease bearing capacity, being the major urban vector of the arboviral diseases yellow fever, dengue and dengue haemorrhagic fever (DHF). Its high vectorial capacity, coupled with its urban environment, make it a primary target in many insect control programmes. The peri-domestic habitat of this mosquito has led to its distribution around the world from its native Ethiopian region, chiefly by means of shipping and commerce. Its distribution is limited by latitude, very rarely occurring beyond the latitudes of 45°N and 35°S, so that it is widespread throughout humid, frost-free areas of south and central America, south east US and the Caribbean, southern Europe, Africa and the islands of the Indian Ocean, southern Asia, Australia and the Pacific Islands.

The habitat of the aquatic *Ae.aegypti* larva is virtually limited to man-made containers. These include discarded tyres and cans, roof gutters, pots, basins and particularly latrine pits and water storage jars and tanks so that the species is prevalent in areas with this type of sanitary facility and where water storage is commonplace. Larvae may also be found in rot holes of trees and in the holes of recently felled trees in the vicinity of dwellings. Both male and female adults of the species remain close to the breeding site from which they originated, frequenting dwellings in the immediate vicinity. *Ae.aegypti* is rarely found in desert regions or in forests unless it is introduced by man, though some strains have been found breeding in forest areas of its native

Africa, independently of man.

Eggs are deposited on moist surfaces usually along the water line of the aforementioned containers. Following completion of development of the embryo, approximately 4 days after oviposition, the eggs hatch on submergance in water, brought about by increased rainfall or disturbance of the oviposition receptacle. Development and physiology of the embryo will be considered in more detail in Chapter 3, section 3:2. The larvae develop through 4 instars, feeding on organic matter, and require a water surface free of scum or oil to permit their oxygen uptake. The larval stage may be completed in 6-8 days depending on seasonal temperature and the adult mosquitos emerge after a pupal stage of 1-5 days duration. The adult female will then take a blood meal after approximately 4 days, feeding during daylight hours.

1.2 VIRAL PATHOGENS TRANSMITTED BY AEDES AEGYPTI

Yellow fever

Aedes aegypti is commonly known as the Yellow Fever Mosquito since it was recognized as the major vector of the urban form of yellow fever. This arboviral disease is acute and often fatal, characterized by severe headaches, aches in the bones and fever, followed by deep jaundice, internal haemorrhage and vomiting. Development of an effective vaccine led to a decrease in the incidence of the disease from the 1940's which ultimately resulted in progressive neglect of surveillance and immunization in the early 1960's and relaxation of control and eradication measures. The number of reported cases has thus fluctuated annually due to repeated epidemics, with 3,361 cases reported in Africa in 1986 (WHO, 1988). At the outset of an epidemic, vector control strategies must be employed making this a good example of the need for such control strategies even though effective vaccines against the pathogen are available.

Dengue and Dengue Haemorrhagic Fever (DHF)

Ae.aegypti is the worlds most important arthropod reservoir for the dengue virus. Dengue virus serotypes 1 and 2 are the causative agents of dengue, a non-fatal febrile disease. Viral serotypes 3 and 4 were isolated from patients suffering from the more dangerous DHF, in which the febrile phase of dengue is followed by haemorrhagic manifestations and shock, with a mortality rate of 10-20%. The syndrome is endemic in Southeast Asia and outbreaks of epidemic proportions have occurred in Latin America and the West Indies. In Malaysia between 1973 and 1987, a relatively low number of annual cases (500-800) rose to 2-3000 in incidences of epidemics (Cardosa, 1988). Annual increases in reported cases in Thailand has led to speculation that the number of cases in the period 1988-1997 should average 124 per 100,000 people annually (Ungchusak and Kunasol, 1988).

Neurotropic Viruses

Other *Ae.aegypti* borne viruses include the neurotropic viruses such as the St. Louis virus, which attack the central nervous system causing encephalitis i.e. inflammation of the brain and spinal cord resulting in high fever, stupor, coma and spastic paralysis.

While molecular biology is being employed to tackle the development of vaccines to some of these viruses (dengue-WHO, 1988), vector control programmes are currently the primary weapon available in combating the spread of these diseases. It is apparent, in the light of experience gained from the extensive yellow fever vaccination programme, that control of the insect vector will still be an essential component of such public health programmes, even after the development and administration of an effective vaccine.

1.3 CURRENT METHODS OF INSECT VECTOR CONTROL

The application of highly effective synthetic insecticides in the late 1940's represented a major breakthrough in insect control for public health programmes. The application of the insecticide may be targeted at either the adult mosquito by means of airborne administration or residual spraying of surfaces to provide a lethal contact dose, or at the larval stage of the life cycle by means of application into standing water sites or into irrigation or drainage systems.

The first types of synthetic chemical insecticides used were organochlorine compounds such as DDT and dieldrin. However, in 1947, DDT resistance was reported in the housefly and in the mosquito species *Culex molestus*. In 1955, dieldrin resistance was reported in *Anopheles gambiae*. More cases of resistance to the organochlorine compounds were reported annually (Brown and Pal, 1971) leading to the use of organophosphorous and carbamate compounds in a bid to overcome this problem. However, in the last 35 years, following the continuous use of any of the 3 groups of synthetic insecticides, resistance has developed in many species so that in 1983, resistance to one or another was found in 93 species of mosquitos (Brown, 1983). This resistance was found to be heritable and due to an allele of a single gene (Georghiou, 1969). Other undesirable factors in the use of insecticides such as adverse environmental consequences, short term effectiveness and expense, coupled with the development of resistance, necessitate the application of other control methods. An integrated control strategy would also optimize the use of synthetic chemical insecticides, ensuring the minimium number of applications, and thereby maintaining its effectiveness for as long as possible.

Environmental control has proved effective in many control programmes and may take the form of modification or manipulation. Modification entails permanent physical transformation of the environment such as drainage and land levelling. Manipulation is based on planned recurrent activity, aimed at producing temporary conditions unfavourable to breeding e.g. water salinity changes, regulation of water levels in reservoirs and removal of vegetation. Both methods are aimed at eliminating breeding habitats of the mosquito. Relocation of human settlements and revision of hygiene practices are used in conjunction with these methods. The advantages of

environmental control include its long term effectiveness and so relatively low cost, and its low adverse environmental impact. However, high capital cost, requiring resources beyond many control programmes, and the need for local co-operation may limit the success of such proposed schemes.

Biological control exploits the destruction or prevention of development of mosquito larvae by natural forces. These forces may include natural predators of the larvae such as fish e.g. *Gambusia affinis* and *Poecilia*, or arthropods e.g. mosquitos of the genus Toxorhynchites. Some spore forming bacteria and in particular certain strains of *Bacillus thuringiensis* and *Bacillus sphaericus* produce toxins which are lethal to mosquito larvae but inocuous to most other organisms, and which can be produced on an industrial scale. Pathogenic agents being studied include parasitic nematodes and protozoa, fungi and viruses. The successful use of these agents in control programmes requires an in depth knowledge of the ecology of a particular mosquito population though the disadvantages in terms of adverse environmental impact should usually be less than those resulting from chemical control.

Genetic Control

The success of the control programme involved in the virtual elimination of the screw worm fly, *Cochliomyia hominivorax* in some areas of the USA and the island of Curacao (Baumhover et.al., 1955) has drawn wide attention to genetic control methods. The technique used in the screw worm project involved mass rearing and release of adult males treated with ionizing radiation such that all gametes produced carry one or more dominant lethal conditions. Since the discovery of chemicals capable of inducing sterility in insects (LaBreque et.al., 1960), chemosterilized males can also be used in control programmes (reviewed by Grover, 1985). Essential factors to be considered in the design of such control programmes must include competitiveness of sterile males released, both in terms of mating and life span, permanence of sterility and hazards pertaining to chemosterilant residues in insects sterilized by chemical means. Of the projects carried out which attempted population suppression by the release of sterile males, those involving the Mexican fruit-fly, *Anastrepha ludens* in California, *Anopheles*

albimanus in El Salvador and the medfly, *Ceratitis capitata* in California (reviewed by La Chance, 1979), all fell short of the predictions of success for these programmes. This shortfall was found to be due to the inability of the sterilized individuals to compete with the wild type population, re-establishment of a population following heavy mortalities and migration of untreated neighbouring populations. Several trials of mosquito control using the irradiation method of sterilization have been carried out but with little success (Davidson, 1974). A field release of irradiated *Ae.aegypti* male pupae over a period of 43 weeks produced little effect on a wild population in Florida (Morlan et.al., 1962). This was attributed to reduced male fitness due to the irradiation dosage administered and to the inadequate dispersal of release points. While short flight range of a species suggests an advantage in terms of migration of untreated neighbouring populations, this should also be considered in the choice of release points when the species has such a short range as *Ae.aegypti*. In the absence of a rapid method to distinguish male and female pupae, both must be released following sterilization. This may be problematic if the female is a disease vector or is responsible for crop damage or infestation of livestock. Male and female pupae of *Ae.aegypti* can easily be separated mechanically on the basis of their size differences.

The nature of genetic control programmes is such that non-scientific factors may be responsible for their failure. Political intervention and disruption of genetic research may be the cause since the choice of species and target regions are often the outcome of complex political and international considerations. An example of such a situation occurred in 1975. Despite extensive preparation by the Research Unit on Genetic Control of Mosquitos, a control programme involving the release of chemosterilized *Ae.aegypti* males at Sonepat, India, was abandoned due to a government decision following adverse propaganda (Grover, 1985).

Other mechanisms of genetic control exploit the naturally occurring sterility mechanisms of cytoplasmic incompatibility, hybrid sterility and translocation chromosomes (Davidson, 1974). However, all of the methods of genetic control considered so far rely on the repeated mass release of individuals and so require extensive rearing facilities. A primary factor for consideration of any control programme must be its long term effectiveness so that the number

of repeated applications will be kept to a minimum thus limiting its cost. In the case of genetic control, genetic manipulation targeted at germline cells should ensure the perpetuation of any modifications throughout subsequent generations. For example, germline manipulation of genes controlling functions related to disease transmission could result in an inheritable inability of the insect vector to support a particular pathogen. Given a sufficient selective advantage, such manipulated individuals may ultimately replace the original vector population subsequent to their release, hence interrupting the transmission of the pathogen. In the design of a control programme involving germline manipulation, factors to be considered include the isolation of potentially useful target genes, their introduction into the genome and their subsequent establishment in a target population.

Germline manipulation requires an effective eukaryotic DNA vector to facilitate integration of DNA sequences into the mosquito genome. In *Drosophila melanogaster*, the transposable genetic element 'P' has been isolated from this species and used successfully to transform flies of the same species with a phenotypic marker gene at the level of the germline (Rubin and Spradling, 1982), thus serving as a eukaryotic DNA vector. Isolation of transposable elements endogenous to the mosquito genome could identify similar DNA vectors for use in the germline transformation of the mosquito in insect control programmes. While a putative transposable element has been isolated from the Ae.aegypti genome (Warren, 1989), further characterization of this element is required to determine its potential as a DNA vector. In the absence of an endogenous mosquito transformation vector and in the light of the successful transformation of distantly related species of *Drosophila* (Scavarda and Hartl, 1984, Brennan et.al., 1984 and Daniels et.al., 1985) with the P element, this element was considered for use in the germline transformation of the mosquito. To appreciate the barriers which may arise in the use of the P element in species outside of the family Drosophilidae, the nature of the P element, and its transposition within the genome must be considered.

1.4 THE P ELEMENT

The P element first came to light when the syndrome of hybrid dysgenesis was studied in *D,melanogaster*. Hybrid dysgenesis was defined by Kidwell and Kidwell (1976) as a " syndrome of correlated genetic traits that is spontaneously induced in hybrids between certain mutually interacting strains, usually in one direction only." The genetic traits involved include sterility and reduced fertility, hypermutability, chromosomal structural changes, transmission ratio distortion, increased female recombination and male recombination which is normally absent in *D. melanogaster* (Kidwell et. al., 1977). A striking feature of these dysgenic traits is the difference in their frequencies in the progeny of reciprocal strain crosses. Strains can be designated as either P or M according to whether dysgenesis results from their use as either the paternal (P) parent or as the maternal (M) parent i.e. dysgenic traits occur in the progeny of a cross between P strain males and M strain females but not in the reciprocal cross (Kidwell et. al., 1977). Hybrid dysgenesis was found to result from interaction between chromosomally linked factors (P factors) and a particular type of cytoplasm (M type). This cytoplasmic determination is referred to as cytotype by Engels (1979).

DNA insertions of 0.5 - 1.4kb were isolated from the mutant *white* eye locus of dysgenic hybrids (Rubin et.al., 1982). These DNA sequences were proposed to belong to a family of transposable elements dispersed over all the major chromosome arms (Engels, 1979) and were labelled 'P elements'. Transposition of these elements was proposed as the cause of hybrid dysgenesis, with P elements present but their transposition repressed in P strains, and P elements absent from the M strains. Derepression of P element transposition occurs when the P elements are introduced into the M cytotype which would account for the non-reciprocality of dysgenic crosses. The stability of mutations in the P cytotype and their reversion in the M cytotype supports this model. Mechanisms of repression of P element transposition will be considered in the light of a more detailed discussion of P element structure and function.

P element structure

Rubin et.al. (1982) proposed that the transposition of P elements was mediated by a transposase protein whose production is induced in hybrid dysgenesis and which is encoded by sequences elsewhere in the genome. In 1983, O'Hare and Rubin isolated a 2.9kb DNA element, present as multiple highly conserved copies, from the genome of a *D.melanogaster* P strain. The complete sequence of this 'P element' was determined, along with the sequence of the insertions from dysgenic mutations of the *white* locus (isolated by Rubin et.al., 1982). The sequence of each of these smaller insertions appeared to have arisen from a single internal deletion of the large 2.9kb element. The intact autonomous 2.9kb 'P element' was shown to encode the trans-acting transposase function required for mobilization of the smaller deleted P elements. Spradling and Rubin (1982) introduced DNA containing 2.9kb elements into the developing embryos of an 'M' cytotype strain and demonstrated integration of these elements into the genome. In the same series of experiments, they also demonstrated the ability of the 2.9kb elements to mobilize the smaller deleted P elements present at the singed locus of the M strain used, thus confirming the role of the 2.9kb elements in transposition.

The intact 2.9kb P element has precise inverted repeats of 31bp at its termini with adjacent 8bp direct repeats of genomic DNA sequences, but no long direct repeats like many other eukaryotic transposable elements (e.g. *copia*-like elements, Finnegan, 1985). The protein coding region of the element is made up of 4 open reading frames, ORF 0, 1, 2, and 3, (O'Hare and Rubin, 1983) all of which contribute information in the production of the transposase protein (Karess and Rubin, 1984).

Germline specificity of P transposition

In addition to cytotype dependent repression of transposition, the transposition of P elements is also tissue specific, being limited to germline cells (Engels, 1983). This may be due to a germline specific promoter or post-transcriptional event, or the requirement of germline specific co-factors in the production of a functional transposase protein. The possibility of a germline

specific promoter was ruled out by transforming *white* locus mutant flies (w^-) with a fusion of the wild type *white* gene (w^+) and the P element coding sequences, under the control of the promoter, hsp-70, which is known to be active in both somatic and germline tissue. w^+ transformants were obtained which gave rise to spontaneous w^- revertants at high rates indicating that the fusion element was capable of catalyzing its own excision. Somatic excision events, detectable as clonal patches of w^- tissue in a wild type eye were not detected, confirming that the P element function of excision was not active in somatic cells (Laski et.al., 1986).

Germline specificity was, in fact, determined to be controlled at the level of mRNA splicing (Laski et.al., 1986). Analysis of polyA$^+$ RNA transcripts from dysgenic and non-dysgenic embryos and natural P strains, revealed a number of bands ranging in size from 0.5kb to greater than 4kb (Karess and Rubin, 1984). The smaller RNA species are thought to arise from transcription of deleted P elements. Transcripts of 2.5kb and 3kb were detected in polyA$^+$ RNA isolated from wild type strains transformed with an autonomous P element (Karess and Rubin, 1984 and Laski et.al., 1986) and in the polyA$^+$ RNA isolated from dysgenic and nondysgenic embryos and natural P strains. The 2.5kb transcript was found to be spliced twice resulting in the production of a continuous open reading frame including ORF 0, ORF 1 and ORF 2. A third splice to include all 4 open reading frames in this continuous ORF was not apparent. The 3kb transcript is identical to the 2.5kb transcript except that it is extended through the normal P element polyadenylation signal and is terminated at a site adjacent to the P element sequences. The third splice required to join all of the 4 ORF's is germline specific. Artificial splicing of this third intron results in transposase production in somatic cells (Laski et.al., 1986).

Two proteins expressed by the P elements were identified in *Drosophila* tissue culture cells transformed with P elements with and without artificial splicing of the third intron (Rio et.al., 1986). A protein of 87kd is encoded by mRNA in which all 3 introns are removed to permit the continuous translation of the 4 ORF's. A 66kd protein, encoded by the first 3 ORF's was isolated from cells transformed with P elements without artificial removal of the third intron. Transposition and excision of P element sequences in cells in which the 87kd protein is

produced, argues strongly that this protein represents the P element encoded transposase protein required for the mobilization of P sequences involved in the syndrome of hybrid dysgenesis.

P element transposition

The mechanism of P element transposition is not yet clearly understood though several lines of evidence must be considered in the proposal of possible models. Models for transposable element transposition include:- (i) conservative transposition i.e. the transposition of a P element to a new site which requires its precise excision from the original site; (ii) replicative transposition i.e. a second copy of the P element appears at a new site by the formation and resolution of a co-integrate structure and (iii) transposition occurs by means of an RNA intermediate and reverse transcription. Conservative transposition should involve an equal number of transposition and excision events though this is apparently not the case, with P elements producing new insertions approximately 30 - 500 times more frequently than it undergoes precise excision (Engels, 1988). Replicative transposition involves replication of the P element at the site of a P mediated double stranded break in the target DNA. The resulting co-integrate structure is then resolved to form a new copy of the P element. Although the 8bp direct repeats found at the ends of the P elements (O'Hare and Rubin, 1983) are consistent with this mechanism, high frequencies of chromosome rearrangements as a result of occasional failure of co-integrate structures to be resolved, are not observed (Engels and Preston, 1984). Finally production of a new P element copy by means of reverse transcription of an RNA intermediate also seems unlikely since full length P element transcripts are not produced and long terminal repeats are not present in the P element sequence.

Control of cytotype

O'Hare and Rubin (1983) proposed a mechanism for control of cytotype whereby the P elements code for both the transposase protein and also for a regulator molecule which leads to the suppression of transposition. However, most of the P element coding sequence is required for

the expression of a functional transposase so the coding sequence for the regulator molecule must be extremely short or must overlap that of the transposase. Rio et.al. (1986) proposed a model in which the 66kd protein, produced as a result of splicing only the first and second introns from the transposase transcript and therefore lacking information from the third intron, may serve as a regulator molecule, negatively regulating transposition by competitive binding to the P element termini or by direct binding to the 87kd transposase molecule. The isolation of identical P element transcripts from dysgenic and non-dysgenic embryos (Karess and Rubin, 1984) is consistent with this model.

Nitasaka et.al. (1987) studied a 'Q' strain which carries only deleted P elements, and hence does not produce transposase, in its genome but is still able to repress the transposition of complete P elements. Their data showed that the ORF 3 was unnecessary for suppression of P transposition but that defective P elements with only ORF's 0, 1 and 2 could suppress transposition. This is consistent with the putative DNA binding domains, on polypeptide sequences encoded by ORF's 1 and 2, determined by Rio et.al. (1986).

Additional factors required in transposition

Detection of precise and imprecise excision events in somatic *Drosophila* tissue culture cells mediated by a P sequence with the third intron artificially spliced (Rio et.al., 1986) suggests that no other germline specific proteins are required for transposition. Providing no *Drosophila* specific proteins are required, then it may be possible to express the functional P element transposase in other organisms to mediate P element transformation. To test this hypothesis, Rio et.al. (1988) expressed P elements with either one or all three introns removed, in mammalian cells and yeast respectively. Their results indicated that P element excision did occur in mammalian cells and that double-stranded breaks occurred in yeast chromosomal DNA as a result of transposase expression. Both excision and the production of double-stranded breaks are thought to be functions of transposase. A staggered double-strand break in DNA, initiating forward transposition, could generate the 8bp duplications of genomic DNA found at the ends of the P elements and expected in replicative transposition. However, no forward

transposition was detected in either mammalian cells or yeast cells suggesting that although some P element functions are retained in other organisms, forward transposition resulting in the mobilization of P sequences to new sites, is apparently *Drosophila* specific. Mechanisms which could explain this phenomenon could include the requirement of a *Drosophila* specific protein which must interact with the transposase protein, or *Drosophila* specific post-translational modifications of the transposase protein. A polypeptide of 66kd has been isolated from *Drosophila* tissue culture cells (Rio and Rubin, 1988) which interacts specifically with a 16bp region of the 31bp terminal inverted repeats, which is directly adjacent to the duplication of target site DNA but not dependent on the sequence of this duplication. Since the I.R.'s are essential for transposition, this 66kd protein may be a host factor required for P element transposition. Interaction of the transposase protein and the 66kd protein could occur which may involve binding of the transposase to internal P sequences adjacent to these inverted repeats.

O'Brochta and Handler (1988) have also studied the excision function of the transposase following the introduction of the P element into the embryos of other *Drosophila* species and into the embryos of insects outside of the family Drosophilidae. They found that the frequency of P element excision in drosophilids decreased in species more distantly related to *D.melanogaster*, and was not evident in non-drosophilids, so that excision is apparently phylogenetically restricted. However, RNA transcripts apparently corresponding to the transposase gene were isolated from the transformed non-drosophilid embryos. Analysis of later steps in the production of the functional transposase protein should elucidate the nature of any *Drosophila* specific requirements.

Use of the P element as a eukaryotic DNA vector

Having demonstrated the autonomous transposition of the 2.9kb P element (Spradling and Rubin, 1982), the vectorial capacity of the element was studied, i.e. its ability to introduce exogenous DNA sequences into the *Drosophila* germline (Rubin and Spradling, 1982). A P element construct carrying the wild type *rosy* gene was introduced into the germline of

D.melanogaster by micro-injection of *ry*$^-$ embryos prior to pole cell formation. Transformed flies with the wild type eye colour were found in G1 and subsequent generations. These results demonstrated the integration of exogenous DNA into the *D.melanogaster* genome at a high efficiency and in a controlled manner, without sequence rearrangements. The potential of this system for use in other multicellular organisms was then considered.

Experiments in which P element vectors were used to transform distantly related species of *Drosophila* suggested that the initial invasion event of the P elements was the barrier to the introduction of P elements into a species and that this could be overcome by micro-injection into embryos. Scavarda and Hartl (1984) successfully used a P element vector carrying the *ry*$^+$ marker to transform *D.simulans*, while Daniels et.al. (1985) transformed *D.simulans* with an autonomous P element without a selectable marker and found that the characteristic P element features of increase in copy number (Kidwell et.al., 1981) and transposition to new sites in the genome (Bingham et.al., 1982) were displayed. These features were also displayed by an autonomous P element introduced into the genome of *D.hawaiiensis* (Brennan et.al., 1984). These results suggest that P element mediated transformation may function in many species and possibly outside of the family Drosophilidae. This has led to speculation on their use in the mosquito as a eukaryotic DNA transformation vector for germline manipulation. If P element vectors cannot be introduced into the mosquito genome by means of transposition, investigation of the behaviour of exogenous sequences on introduction into the mosquito cell would nevertheless be valuable. The precise fate of sequences integrated by alternative mechanisms, e.g. random or homologous integration, may be studied and any species-specific responses determined with a view to exploiting such mechanisms for genome manipulation.

Stable integration of a P element based construct, through 5 generations, has been reported in the mosquito *Anopheles gambiae* (Miller et.al., 1987). Recently, integration of the same construct has been reported in the genome of *Aedes triseriatus* (McGrane et.al., 1988) though only through two generations. Neither of these integration events was shown to be P mediated. However, at the time the work described in this thesis was intiated, none of this work on mosquito systems had been undertaken.

1.5 G418 ASSAY OF PUTATIVE TRANSFORMANTS

DNA vectors used in the transformation of *Drosophila* commonly carry a marker gene to allow for selection of lines carrying insertions. The *rosy* gene (*ry*), (Rubin and Spradling, 1983) and the *alcohol dehydrogenase* gene (*Adh*), (Goldberg et al, 1983), both isolated from *D.melanogaster*, are widely used. The *rosy* gene codes for xanthine dehydrogenase, an essential product in the drosopterin eye colour pathway. Introduction of the *rosy* gene into the genome of a *rosy* mutant fly readily restores the wild type eye colour ensuring easily scored transformants. Expression of the *rosy* gene at a level of less than 5 - 10% of the wild type activity is sufficient to complement the *ry*- phenotype suggesting that it is unlikely to be affected by position effects i.e. insertions in the proximity of a cellular promoter, leading to high levels of expression of the *rosy* gene, are not essential for complementation.

The alcohol dehydrogenase gene is used as a marker which allows transformed flies to be selected as adults by virtue of increased ethanol resistance. Unlike the *rosy* gene, relatively high levels of activity are required in adults for survival so that position effects may lead to preferential recovery of specific insertions. An advantage of the *Adh* marker gene is its use in the biochemical screening of large numbers of progeny without scoring flies individually as for a visible phenotype such as the *rosy* gene. However, partial expression of the *Adh* gene may not be detected whereas a partially restored visible phenotype could be.

Little is known of the biochemical eye pigment pathway in the mosquito though it is thought to involve two pathways similar to the ommochrome and pteridine pathways in *Drosophila* and eye colour mutants are available. If the *Drosophila rosy* gene was found to complement a particular eye colour mutant of the mosquito then it could be used as a marker in mosquito transformation experiments. Alternatively any homology of *Drosophila* eye colour loci to mosquito genomic DNA could be used to isolate genes expressed in the eye colour pathways of the mosquito which could also be tested for their ability to complement specific mosquito eye colour mutations. Clearly though, the technology for transformation of the mosquito must be established before unknown markers can be tested in this way. Similarly for the *Adh* gene, the

alcohol dehydrogenase activity of the mosquito must first be studied, mutant phenotypes established and their ability to complement with the *Drosophila Adh* gene determined.

An alternative approach in the search for a suitable marker gene would be to determine a compound which is lethal to wild type *A.aegypti* but for which the gene coding for resistance in other organisms has already been determined. This resistance gene when introduced into the genome of the wild type fly may confer resistance to the otherwise lethal substance. This approach would eliminate the need for the isolation of specific mutant phenotypes although, as for the *Adh* and *rosy* genes, the effectiveness of the marker gene conferring resistance cannot be determined until transformation of the mosquito is readily achieved.

In 1985 Steller and Pirotta demonstrated that the bacterial neomycin resistance gene (*neo*) can be used to render wild type *Drosophila* larvae resistant to the antibiotic G418. G418 is an antibiotic related to gentamycin, neomycin and kanamycin though its toxicity appears to be universal, unlike its aforementioned analogues. G418 is a 2-deoxystreptamine antibiotic which has inhibitory activity against a wide range of prokaryotic and eukaryotic organisms by interfering with the function of 80S ribosomes and blocking protein synthesis. In bacteria, resistance to G418 can be determined by several modifying enzymes, some of which are carried on transposable genetic elements (TGE's). The Tn601 transposon codes for aminoglycoside phosphotransferase-3'(I) (APH(3')-I) which phosphorylates and inactivates a number of aminoglycoside antibiotics, containing the 2-deoxystreptamine moiety, at the 3' hydroxyl position. In 1980, Jimenez and Davies successfully used Tn601 to confer G418 resistance to *Saccharomyces cerevisiae* cells by transformation of spheroplasts. In view of the broad activity of G418 - it is toxic to yeast, fungi, algae, plant and animal cells (Jimenez and Davies, 1980) - recovery of resistant *S.cerevisiae* cells suggested that G418 may be useful as a selective agent for the introduction of marker resistance genes into other eukaryotic organisms. Further support for this theory was provided by Colbere-Garapin et. al. in 1981, when they used the Tn5 transposon coding for the aminoglycoside 3'- phosphotransferase (II), linked to the promoter region of the Herpes Simplex Virus type I thymidine kinase gene, to transform mammalian cell lines (murine, simian, human) to G418 resistance. In 1982, Southern and Berg also transformed

mammalian cell lines with the Tn5 APH(3')-II gene under the control of the SV40 early region promoter.

In their work on the transformation of *Drosophila*, Steller and Pirrotta (1985) used the *Drosophila* heat shock promoter - hsp70 - to drive the *neo* gene from transposon Tn5 (Davies and Smith, 1978), and successfully transformed wild type *Drosophila* to G418 resistance. In view of the wide ranging toxicity of G418 and the successful transformation of a range of eukaryotic cells to G418 resistance the determination of wild type mosquito larvae as sensitive to G418 could enable the hsp70-*neo* construct to be used as a suitable marker in subsequent transformation experiments with mosquitos.

Having identified a potential DNA vector and a marker gene, it remains to introduce these into the developing pole cells of the mosquito. Pole cell formation occurs in early embryonic development so that DNA delivered into the embryo prior to the development of these cells may become incorporated into them and hence into the germline tissue arising from these cells. The primary goal of the research reported in this thesis was to develop a technique for introduction of DNA into the early embryo and to study the fate of this DNA.

1.6 APPLICATION OF GERMLINE TRANSFORMATION

Target genes for use in transformation strategies

Transformation of *Ae.aegypti* with genes which may play a role in the control of insect vectorial capacity would first involve the isolation of potentially useful genes. Transformation of the mosquito genome may in itself be a useful method for the recovery of genes of interest by means of transposon tagging (Searles et.al., 1982). Using a transposable element such as the P element which may transpose at a high rate, insertion mutations could arise. Determination of the phenotypic effect of this insertion mutation would reveal the identity of the mutated gene. The DNA sequences of the mutated locus could then be retrieved from a genomic library

prepared from mutant fly DNA, by virtue of its acquired homology to the P element.

The types of genes which should be considered fall into two categories, with one type resulting in the eradication of the disease bearing population and the second type resulting in vector refractoriness so that the vector population is eventually replaced by a non-vector population. Eradication of a population could be brought about by the introduction of conditionally lethal genes. Once the gene is established throughout the population, control measures could then be applied to which the new population is sensitive. Control measures such as temperature sensitivity may be useful in regions were a natural seasonal temperature increase is expected. Conditional lethals such as insecticide resistance genes or genes controlling stages of the life cycle such as diapause, resulting in hatching in unfavourable conditions, could also be applied. However, the eradication of disease transmitting populations would leave an empty ecological niche which may be readily re-colonized by migration of other vector species. Perhaps a more useful strategy would involve the progressive replacement of a vector population with a non-vector species.

The most obvious target genes for use in the vector replacement strategy are those conferring refractoriness to the specific pathogen. For example, susceptibility of *Ae.aegypti* to the filarial parasite sub-periodic *Brugia malayi* is controlled by a sex-linked recessive gene *fm* (MacDonald, 1965). Isolation and introduction of the dominant gene into a previously susceptible population could render the population refractory to filarial infection. The filarial susceptibility genes, determining the insects ability to support development of different filarial worms all map at approximately the same place on the sex chromosome (Munstermann and Craig, 1979). Cloning and analysis of these genes may prove to be useful in understanding the mechanisms of susceptibility and refractoriness to a range of pathogens. Other genes of interest, which may also be involved in the ability of *Ae.aegypti* to support and therefore transmit pathogens, are those coding for insect immune proteins (Lackie, 1988 review) such as the attacins, cecropins and diptericin, and the cytochrome P-450 genes which are involved in more general detoxification mechanisms. Both putative immune protein genes (Knapp, unpublished data) and P-450 genes (Gerke-Bonet, unpublished data) have been cloned from the *Ae.aegypti* genome.

Similarly, putative *Ae.aegypti* homeobox genes have been cloned (Eggleston, unpublished data) on the basis of their homology with *Drosophila* genes (Gehring, 1987), which have a variety of functions in the control of development. Manipulation of homeotic genes could be used to alter developmental processes. Isolation of promoter sequences controlling these genes may be used to developmentally regulate the expression of unrelated sequences.

The periodicity gene, *per*, has been cloned from *Ae.aegypti* also on the basis of its homology to the *per* genes of *Drosophila* (reviewed by Young *et.al.*, 1985). These periodicity genes may regulate rhythmic processes such as cycles of development or behavioural cycles, manipulation of which could interrupt the cycle of disease transmission.

Control sequences derived from endogenous stage-specific or tissue-specific genes could be used to direct the expression of potentially useful sequences isolated for use in transformation experiments. For example, promoter sequences derived from salivary gland specific genes could be used to direct the expression of a foreign gene exclusively in salivary glands, which could prove to be a useful target for the expression of a foreign gene since it is the final destination of many pathogens in the insect host. To this end, genomic and cDNA clones of a gene expressed specifically in the salivary glands of adult *Ae.aegypti* have been isolated and sequenced by James *et.al.* (1989) so that the ability of its promoter sequences to direct the expression of exogenous genes may be assessed.

Potential drive mechanisms

Introduction of a gene into the mosquito genome is unlikely to be successful as a control method without the use of an effective drive mechanism to force the required gene through the target population. Positive selection of the gene of interest or of a closely linked co-introduced gene is the primary aim in control strategies employing transformed individuals. However, many factors must be considered in determining selection pressures.

For example introduction into the genome of a gene conferring refractoriness to filarial infection, may be expected to result in positive selection for the resulting transformants, driving the gene through the population, since filarial infection may lead to mortality of various genotypes of *Ae. aegypti* (Townson, 1971). However in such an instance, the infection rates must be considered since a low infection rate in the target population would result in similar positive selection of both non-infected mosquitos of the target population and the refractory transformants introduced into the population. Also, a susceptible wild type population suggests that some selection has already occurred against refractory individuals so that their selective advantage in terms of parasite infection must be countered by an additional selected trait.

One possible drive mechanism to be considered is that of meiotic drive. This describes any event occurring during the process of meiosis which leads to preferential recovery of one chromosome over its homologue. This mechanism has been used to drive the marker gene *re* (red eye) into a laboratory cage population of *Ae.aegypti* using meiotic drive at the *Md* locus (Wood et.al., 1977). Meiotic drive is also associated with the syndrome of hybrid dysgenesis (Kidwell et.al., 1977) so that introduction of P element vectors or similar transposable elements into the *Ae.aegypti* genome may result in the occurrence of this trait without additional manipulations of loci unrelated to the vector integration sites.

Genetic traits leading to reduction of heterozygote fitness may serve as an alternative mechanism for driving a gene through a population (Curtis and Graves, 1988). The gene to be driven through the population could be introduced onto a translocation chromosome so that viable and fertile homozygotes would result but heterozygotes with reduced fitness would be selected against. Total sterility of the heterozygote could be achieved by using compound chromosomes instead of translocation chromosomes. The sterile hybrids would then be analogous to those arising from inter-racial crosses. A disadvantage of this mechanism compared to that of meiotic drive is that it would require repeated releases of larger numbers of genetically altered flies into the target population since the frequency of the driven chromosome would not increase as with meiotic drive.

An insecticide resistance gene could be introduced into the genome along with the gene to be driven through the population. Treatment of the sensitive target population with the corresponding insecticide should then lead to the preferential survival of the transformed flies. However, this method would only provide a temporary advantage unless the insecticide resistance gene was tightly linked to the gene of interest. Otherwise, inter-breeding of the released and indigenous populations would ultimately result in linkage equilibrium in which the selective advantage of the resistance gene would favour the wild type and foreign alleles equally. To overcome this problem genes to be introduced into the population could be fused to the gene providing a selective advantage, using molecular biological techniques, so that on introduction into a population, the two genes would effectively be transmitted as a single unit. Introduction of insecticide resistance into a population may also pose problems in terms of eliminating the insecticide of choice from future, conventional chemical control methods.

1.7 SUMMARY

The future of insect vector control strategies appears to lie in an integrated approach, applying a combination of methods ranging from vaccination to total eradication or replacement of disease bearing populations, and involving a variety of techniques. Given the range of problems facing arbiters of control programmes, no single method of control can be considered a panacea. The work presented in this thesis was undertaken in an attempt to investigate the potential of an additional method of vector control to supplement currently available methods. Development of a micro-injection technique for the introduction of exogenous DNA into developing embryos of *Ae.aegypti* and the effective use of DNA vectors and marker genes should ensure that germline transformation is a viable proposition for field research.

PRIMARY AIMS AND STRATEGY EMPLOYED IN THIS RESEARCH

Ae.aegypti was the mosquito of choice in these transformation experiments for the following reasons:-

(a) Rearing of this species is relatively straightforward and healthy stocks were available.

(b) More is known of the genetics and biology of *Ae.aegypti* than for any other insect vector. It has an extensive linkage map and many mutant phenotypes are available for experimental use.

(c) *Ae.aegypti* embryos are laid singly and not in rafts as for *Culex* species, and are laid on moist surfaces rather than directly onto a water surface so that hydrophobicity associated with orientation on the water surface is not a problem in manipulating the embryos.

(d) After a 4 day conditioning period directly after oviposition, *Ae.aegypti* embryos may be stored dry for up to 4 months without any significant loss in their viability, so that field isolates can be obtained with minimal difficulties in their transportation.

The aims of the research were as follows:-

(a) to develop a technique for the micro-injection of a DNA solution into the embryos of *Ae.aegypti* so that the embryos remain viable and the DNA is incorporated into germ line tissue.

(b) to determine the effect of the antibiotic G418 on wild type *Ae.aegypti* larvae and to devise an assay for use in the selection of putative transformants.

(c) to study the fate of the introduced, exogenous DNA in adults arising from injected embryos and in subsequent generations.

(d) to embark on preliminary studies to determine whether the P element is expressed and functional in the mosquito *Ae.aegypti*.

CHAPTER 2

MATERIALS AND METHODS

2.1 BIOLOGICAL MATERIAL

Mosquito Strain	Date Colonized	Origin
<u>Ae.aegypti</u> strain		
Bangkok	1972	Bangkok, Thailand

Bacterial Strains (<u>E.coli</u>)	Genotype	Reference
MC1061	ara D139,Δ (ara, leu) 7697Δ lac X74, galu-, gal K-, hsr-, hsm+, str A	Casadaban and Cohen, 1980
DH5α	F-,φ80dlacZΔM15, Δ(lacZYA-argF)U169, recA1, end A1 hsdR17 (rk-,mk+), supE44, λ-, thi-1, gyrA, relA1	Hanahan, 1983
LE392	F-, hsd R514, (rk-, mk+) Sup E44, Sup F58, lacY1 gal K2, gal T22, met B1 hpR55,λ-	Maniatis et.al. 1982

2.2 MAINTENANCE OF MOSQUITOS

Adults and larvae were maintained at 27°C ±1°C, at 75% ±5% R.H. on a 12 hour light/dark cycle. Breeding stocks consisting of up to 500 individuals were kept in 30 x 30 x 30cm cages. Matings involving less than 30 individuals were carried out in 15 x 15cm perspex cylinders. For single matings, a putative transformed individual was placed in a 15 x 15cm perspex cylinder and 4 adults of the opposite sex added. In cages of this volume, the insemination rate for *Ae.aegypti* has previously been determined as 75 - 100% (Matthews, 1987. When storage space was a limiting factor, single mating was carried out in plastic cups 8.5cm x 11cm though the insemination rate in this volume had been shown to be considerably lower i.e. less than 50% (Matthews, 1987). Although the number of progeny obtained from crosses in this volume was adequate for the purposes of these experiments, this mating volume was avoided if possible.

Adults were supplied with a damp cotton wool pad along with a moist sugar cube to provide sustenance for males and a carbohydrate supplement for the females. Females were allowed to feed on an anaesthetized guinea pig four days post emergence, and twice a week subsequently. Four days after oviposition on moist filter papers, the egg papers were allowed to dry in the insectary and sealed in plastic bags for storage. Eggs were stimulated to hatch by immersion in a dilute solution of hay infusion, prepared by soaking dried grass in tap water for 24 hours at 27°C. Twenty four hours post hatch and daily thereafter, the hay infusion was replaced by fresh tap water and killed yeast added in the form of "Brewer's yeast" tablets. Metal trays 20 x 28cm were used to rear the larvae.

Details of media, buffers and solutions used in the following methods are given in the Appendix following Chapter 7.

2.3 ISOLATION OF MOSQUITO NUCLEIC ACIDS

Total genomic DNA from single mosquitos

DNA extraction was based on the method of Coen et.al. (1982) and yielded 1-2μg of total genomic DNA from a single adult mosquito. All centrifugation was carried out in an Eppendorf bench top centrifuge.

1. A single mosquito in a 1.5ml Eppendorf tube, was homogenized on ice using a glass rod, in 100μl of solution A.
2. 100μl of solution B were added and incubated on ice for 30 minutes at 65°C.
3. To this, 30μl of 8M potassium acetate were added and incubated on ice for a further 45 minutes.
4. The resulting precipitate was sedimented by centrifugation for 5 minutes and 2 volumes of absolute ethanol added to the supernatant before incubating at room temperature for 5 minutes.
5. The precipitate was sedimented by centrifugation for 10 minutes and the supernatant discarded.
6. The pellet was resuspended in 100μl of 0.1 x SSC, 0.2% DEPC and incubated at room temperature for 30 minutes.
7. 3 volumes of absolute ethanol were added and incubated at room temperature for a further 15 minutes before centrifuging for 10 minutes.
8. The resulting pellet was resuspended in 100μl of 200mM NaCl and 250μl absolute ethanol added.
9. After incubating at room temperature for 5 minutes, the DNA pellet was recovered by centrifugation for 10 minutes and the pellet washed in 800μl of 70% ethanol.

10. The DNA pellet was again recovered by centrifugation, dried under vacuum and resuspended in 16μl of T.E., pH 7.5, at 4°C, overnight.

N.B.

Live anaesthetized mosquitos may be used in this preparation. Alternatively, live mosquitos must be snap frozen and stored at -70°C prior to DNA extraction. Solution A is added without previous thawing and the mosquito homogenized immediately. DNA was extracted from individuals which had been stored at -70°C or snap frozen and then stored at -70°C, within 24 hours of their death, with only slight degradation of the DNA.

Large scale high molecular weight DNA extraction

This method is based on that of Bingham et.al. (1981) and yields 100-400ug of pure, high molecular weight genomic DNA from 100-400 fourth instar larvae.

1. Larvae were washed well with distilled water and filtered through fine gauze to remove any excess liquid.
2. Larvae were then ground in liquid nitrogen using a pre-cooled mortar and pestle.
3. The resulting powder was transferred to a sterile 10ml glass-glass homogenizer with 10mls TNESST and homogenized gently on ice until all the solid was in suspension. The homogenate was allowed to settle on ice, and the liquid phase decanted.
4. Nuclei were collected by centrifugation (7,000rpm in SS34 rotor, 7 minutes at 4°C) and the pellet resuspended in 10mls TNESST before repeating the centrifugation.
5. The pellet was resuspended in 5mls TNESST and 10% (w/v) Sarkosyl added to a final volume of 2%, mixing gently to lyse the nuclei.
6. 18.5g CsCl were added and the volume made up to 18.5mls with TNESST, 2% Sarkosyl. CsCl was dissolved by gently inverting the tubes.
7. The mixture was centrifuged at 40,000rpm (Sorvall T865 rotor) for 40 hours at 18°C.

8. The resulting gradient was fractionated in 0.5ml aliquots through a wide-gauge needle, from the base of the tube.

9. 1μl of each fraction was spotted onto 1% agarose gel containing ethidium bromide (0.5μg/ml) and, after 15 minutes, visualized over a U.V. light source.

10. DNA containing fractions were pooled and dialyzed extensively against TE buffer to remove CsCl and Sarkosyl.

11. The pooled fractions were extracted gently with an equal volume of TE saturated phenol and once with an equal volume of chloroform:isoamyl alcohol (24:1) to remove contaminating protein.

12. NaCl was added to a final concentration of 200mM, along with 2.5 volumes of absolute ethanol. The DNA was precipitated by snap-freezing at -70°C for 30 minutes or by incubating at -20°C overnight.

13. The DNA was recovered by centrifugation at 10,000rpm for 20 minutes at 4°C and the pellet washed in 70% ethanol to remove salt. After repeating the centrifugation, the DNA pellet was dried under vacuum and redissolved overnight at 4°C in T.E., to give a concentration of 200-400μg/ml.

N.B.

a. Vigorous shaking or pipetting through narrow tips results in shearing of the DNA.

b. The amount of starting material should not exceed the equivalent of 400 larvae to avoid high viscosity of solutions and overloading of the CsCl gradient.

c. Crude, total cytoplasmic RNA can be recovered by the addition of 2.5 volumes of absolute ethanol to the supernatant in step 4. This can be stored at -20°C until required.

Guanidinium thiocyanate extraction of total cellular RNA

This method is based on that used by Chirgwin et.al., 1979, adapted for use with adult mosquitos. 300 adults yielded 1-1.5mg total RNA.

1. Adults were ground in liquid nitrogen using a pre-cooled mortar and pestle.
2. The resulting powder was transferred to a baked glass-glass homogenizer with 5mls guanidinium thiocyanate solution and homogenized gently until all solid was in suspension. The homogenate was allowed to settle on ice and the liquid phase decanted.
3. The liquid phase was centrifuged at 2,500rpm for 10 minutes to remove any debris.
4. A CsCl gradient was prepared by placing 2mls 5.7M CsCl in 0.1M EDTA, in a 14ml polyallomer tube. 1.5ml each of 40%, 30% and 20% CsCl solutions in 0.1M EDTA, were layered onto this 5.7M CsCl cushion in decreasing order and the supernatant from step 4, layered on top of this prepared gradient.
5. RNA was pelleted by centrifugation (TST 41.14 rotor) at 35,000rpm for 14 hours at 14°C.
6. The supernatant was removed and the tube inverted to drain off any excess liquid before cutting off the bottom of the tube with a scalpel.
7. The glassy pellet was broken up using a micro-pipette tip and resuspended in 300µl of ddH_2O.
8. The volume was made up to 3mls with ddH_2O and sodium acetate added to a final concentration of 0.25M followed by 8mls of absolute ethanol.
9. RNA was precipitated by incubation at -20°C overnight.
10. The RNA was recovered by centrifugation at 35,000rpm (TST41.1 rotor) for 30 minutes at 4°C.
11. The pellet was resuspended in 200µl ddH_2O. All RNA samples were dispensed into 20µl aliquots and stored at -70°C prior to use.

N.B.

The amount of starting material should not exceed 150 - 200 adults per CsCl gradient, to avoid overloading.

Total RNA extraction from mosquito embryos

Two alternative methods were employed, both of which were scale down for use with small numbers (100-300) of embryos. The method described below is based on that of Chomczynski and Sacchi, 1986. Embryos were removed from the moist filter paper on which they were placed after micro-injection and placed directly into homogenization solution in a 1.5ml Eppendorf tube.

1. The embryos were homogenized on ice, using a glass rod, in 200µl solution D.
2. The following were added sequentially:- 20µl 2M sodium acetate pH4, 200µl phenol (water saturated) and 40µl chloroform:isoamyl alcohol (49:1), mixing thoroughly by inversion, with each addition and finally, shaking vigorously for 10 seconds before cooling on ice for 15 minutes.
3. The suspension was centrifuged at 11,000rpm (SS34 rotor) for 20 minutes at 4°C and the resulting aqueous phase removed (DNA and proteins should remain at the interface and in the phenol phase).
4. To this aqueous phase, 200µl of isopropanol was added and incubated at -20°C for 1 hour, to precipitate the RNA.
5. The RNA was recovered by centrifugation at 11,000rpm for 20 minutes at 4°C and the resulting pellet dissolved in 60µl of solution D.
6. To this, 60µl of isopropanol were added and incubated at -20°C for 1 hour.
7. RNA was again recovered by centrifugation, in an Eppendorf centrifuge for 10 minutes at 4°C
8. The RNA pellet was washed with 75% ethanol and centrifuged for 10 minutes at 4°C in the Eppendorf centrifuge.
9. The pellet was dried under vacuum and resuspended in 10µl 0.5% SDS at 65°C for 10 minutes.

The following method is based on that described by O'Hare et.al. (1983):-

1. Embryos were homogenized on ice in 100μl of solution E, using a glass rod.
2. The homogenate was then extracted several times with phenol:chloroform:isoamyl alcohol (50:49:1).
3. To the aqueous phase of the final extraction, sodium acetate was added to 0.25M followed by 2.5 volumes of absolute ethanol and incubated at -20°C overnight.
4. RNA was recovered by centrifugation for 10 minutes in an Eppendorf centrifuge at 4°C.
5. The pellet was washed in 70% ethanol and re-centrifuged as in step 4.
6. The final pellet was resuspended in 10μl ddH_2O.

N.B.

Both methods of RNA extraction resulted in RNA preparations contaminated with DNA. Before use, the RNA should therefore be treated with DNase to a final concentration of 50μg/ml for 30 minutes on ice.

Large scale plasmid preparation

This method yields 50-250μg of plasmid DNA per 250ml culture.

1. 250mls of L-broth, with an appropriate selective antibiotic, were inoculated with a single bacterial colony containing the required plasmid and grown overnight at 37°C with shaking in an orbital incubator at 250rpm.
2. The cells were pelleted in 250ml GSA bottles at 7,000rpm (GSA rotor) for 10 minutes and the supernatant discarded.
3. The pellet was drained and resuspended in 5mls of Plasmid Solution I and incubated at room temperature for 10 minutes.
4. 10mls of 0.2M NaOH, 1% SDS were added, shaken gently and incubated for 5-10 minutes on ice.
5. To this, 5mls of 3M potassium acetate, pH4.8, were added and vortexed thoroughly before incubating for 30 minutes on ice.
6. The mixture was centrifuged at 7,000rpm for 10 minutes at 4°C and the supernatant decanted.
7. To the supernatant, 12.5mls of isopropanol were added, mixed and incubated at room temperature for 5 minutes.
8. After centrifugation at 10,000rpm for 10 minutes at room temperature, the resulting pellet was resuspended in 13mls T.E..
9. To this, 13.7g of CsCl were added and dissolved, followed by 1.3mls ethidium bromide (10mg/ml).
10. Any debris and precipitated ethidium bromide was removed by centrifuging at 10,000rpm for 10 minutes at room temperature. The density of the supernatant must be 1.56g/ml.
11. The supernatant was then centrifuged at 45,000rpm (T865 fixed angle rotor) for 40 hours at 20°C.
12. By back-lighting the centrifuge tube with a U.V. light source, 2 visible bands were observed. The lower of the 2 bands was removed using a wide gauge syringe (this

band should contain supercoiled plasmid DNA-the upper band contains open-nicked plasmid and contaminating *E.coli* chromosomal DNA).

13. Ethidium bromide was removed from this fraction by extracting 3 times with CsCl saturated isoamyl alcohol, and the fraction dialyzed against 2 litres of T.E. buffer, to remove CsCl.
14. DNA was precipitated by adding sodium acetate to a concentration of 0.25M, 2 volumes of ethanol and incubating at -20°C overnight.
15. A DNA pellet was recovered by centrifuging for 10 minutes at 10,000rpm (SS34 rotor) at 4°C, and washed in 70% ethanol.
16. The final pellet was dried under vacuum and resuspended in T.E., pH7.5.

Low molecular weight DNA extraction from embryos (Hirt et.al., 1967)

1. Up to 100 embryos in a 1.5ml Eppendorf tube were homogenized on ice in 10 μl of Hayes buffer, using a glass rod.
2. 90μl of 0.6% SDS, 0.01M EDTA, pH7.5 was added and incubated at room temperature for 20 minutes.
3. To the viscous lysate, 25μl of 5M NaCl was added.
4. The sample was mixed slowly by inversion and incubated at 4°C for at least 8 hours.
5. SDS and protein were removed by centrifugation at 15,000rpm (SS34 rotor) for 30 minutes at 4°C.
6. The supernatant was removed and extracted twice with phenol/chloroform.
7. The aqueous phase was then extracted with chloroform:isoamyl alcohol 24:1) before precipitating with ethanol.
8. The final DNA pellet was resuspended in a minimum volume (10μl) of T.E., pH7.5.

2.4 AGAROSE GEL ELECTROPHORESIS

This method was used for the size separation of DNA fragments required for (i) screening of DNA via Southern blotting and hybridization techniques, and (ii) isolation of fragments for cloning or the preparation of radiolabelled probes.

Electrophoresis was performed through 0.9 or 1% agarose gels prepared in 1 x E buffer and 0.5ug/ml ethidium bromide. Samples were loaded into wells after addition of a 1/5 volume of Orange G stop solution. Electrophoresis was carried out at 70V for 60 - 90 minutes (minigels, 5 x 7.5cm) or 40V for 17 hours (large gels, 20 x 24.7cm) in 1 x E buffer with 0.5μg/ml ethidium bromide.

DNA was visualized using a U.V. light source (Ultra-Violet Products Inc. transilluminator). Sizing of fragments was by graphical comparison with Hind III digested phage DNA fragments which were included as markers on each gel. Gels were photographed over the U.V. transilluminator using a Polaroid MP4 land camera. The film used was 'Polapan 52' (400 ASA) instant film, or Ilford FP4 (125 ASA). Exposures were at f4.5 for 2 seconds, using a 'Wratten' filter.

Isolation of DNA from low-melting point agarose

1. A low melting point agarose gel was prepared in TBE buffer and 0.2μg/ml ethidium bromide.
2. Samples were loaded and run as above.
3. After visualization over U.V. light, the desired band was cut out of the gel and cut into small pieces in a 1.5ml Eppendorf tube.
4. The slivers of agarose were then covered with 1 - 5 volumes of 0.3M sodium acetate, pH7.0 and heated to 68°C for 15 minutes or until all the agarose melted.
5. An equal volume of phenol (previously warmed to 65°C), equilibrated in 0.3M sodium acetate was added, mixed, and the solution centrifuged at 10,000rpm (SS34 rotor) for

10 minutes at 30°C.

6. The aqueous phase was recovered, leaving the agarose at the interface, re-extracted with phenol and finally with chloroform:isoamyl alcohol (24:1).
7. Precipitation was by addition of 3 volumes of absolute ethanol without extra salt, and incubation at -20°C overnight.
8. The DNA pellet was recovered by centrifugation at 10,000rpm in an Eppendorf centrifuge, at 4°C.
9. After washing with 70% ethanol, the pellet was dried and resuspended in a minimum volume.

2.5 NUCLEIC ACID HYBRIDIZATION

DNA labelling using random oligonucleotide primers

40 - 60ng DNA were labelled using 30μCi α-32P-dCTP by 'Random primed' DNA labelling (Feinberg and Vogelstein, 1983) using a Boehringer kit and specifications. The specific activity of the resulting probe was normally in the region of 10^8 - 10^9 dpm/μg. Labelled DNA was collected by passing the reaction volume over a Sephadex G-50 column previously equilibrated with the elutant buffer, 6 x SSC. Samples containing the required labelled DNA were boiled for 5 minutes to denature DNA before adding to hybridization solution.

Hybridization

Unless otherwise stated, prehybridization and hybridization of filters was performed overnight at 42°C for 18 hours, in heat sealed polythene bags. 20 x 20cm filters were prehybridized in 20mls of hybridization solution and hybridized in 5mls. After hybridization, filters were rinsed twice in 2 x SSC, 0.1% SDS and then washed twice for 30 minutes in 2 x SSC, 0.1% SDS at 42°C. If necessary, filters were rewashed at higher stringencies after autoradiography, as indicated in the text or figure legends.

Autoradiography

Autoradiography was performed at -70°C in 'X-ograph' cassettes using 'Speed-X' intensifying screens and pre-flashed 'Fuji-RX' X-ray film.

Southern transfer of DNA to nitrocellulose

The basic technique of Southern (1975) was modified as described below.

DNA was denatured by soaking the agarose gel in 0.5M NaOH, 1.5M NaCl, with gentle shaking, for 30 minutes at room temperature. After rinsing briefly in ddH_2O, the gel was neutralized by soaking twice for 30 minutes in 0.5M Tris, 0.3M NaCl at pH 5.0. Blotting was carried out as follows:-

1. A 2cm thick block of paper tissues was soaked in 20 x SSC.
2. 2 Whatman 3MM filter papers, soaked in 2 x SSC, were laid on top of the tissues.
3. This block was wrapped in cling film, leaving an opening on the upper surface, corresponding to the size of the gel to be blotted.
4. The gel was placed on top of the Whatman filters and any trapped air removed by pressing gently with a gloved finger.
5. A nitrocellulose filter, soaked in 2 x SSC was laid carefully on top of the gel and again, any air bubbles carefully removed.
6. Two further Whatman 3MM filter papers, also soaked in 2 x SSC were laid on top of the nitrocellulose filter.
7. A block of dry tissues was placed on top of the arrangement and covered by a glass plate, secured by a weight.
8. Blotting was for a minimum of 4 hours at room temperature or overnight.

After blotting, the assembly was dismantled and the well slots of the gel marked on the nitrocellulose filter with a waterproof marker pen. The nitrocellulose was washed briefly for 5 minutes in 4 x SSC and air dried before baking for 2 hours at 80°C.

Dot blots

Total genomic DNA was denatured by boiling for 10 minutes in a final sample volume of 100μl in 5 x SSC. The DNA was cooled on ice for 5 minutes and blotted onto nitrocellulose, previously soaked in ddH_2O followed by 5 x SSC, using a dot blot manifold (Anderman and Co. Ltd.) connected to a suction pump. Each sample well was washed through with 400μl 5 x SSC. The nitrocellulose filter was then removed and rinsed in 5 x SSC, air dried and baked for 2 hours at 80°C.

2.6 CLONING INTO PLASMID VECTORS

De-phosphorylation of 5' hydroxyl groups

1. 1-10μg of linearized plasmid were incubated in 20μl calf intestinal alkaline phosphatase (CIAP) buffer plus 22 units CIAP at 37°C for 30 minutes.
2. CIAP was inactivated by adding EDTA to 25mM and heating to 65°C for 15 minutes.
3. After one phenol extraction followed by a chloroform extraction, plasmid DNA was recovered by ethanol precipitation.
4. The DNA pellet was vacuum dried and dissolved in 20μl T.E. buffer.

Ligation

A total of 1μg of insert and vector DNA was ligated in T4 ligase buffer plus 1 unit of T4 ligase, in a final volume of 20μl (optimum ratio of insert:vector DNA used in the ligation was determined empirically using test ratios of 1:1, 1:3 and 3:1)

2.7 TRANSFORMATION OF E.COLI (MC1061) WITH PLASMID DNA

The method used for high efficiency transformation was that of Hanahan (1985).

Preparation of competent cells

1. Three 2 diameter colonies from freshly streaked SOB agar plates were dispersed in 1ml SOB medium, by vortexing.
2. This was used to inoculate 30mls of SOB medium in a 1 litre conical flask, and the culture incubated at 37°C with shaking at 250rpm, until the cell density was 4 - 7 x 10^7 cells/ml.
3. The culture was transferred to sterile centrifuge tubes and chilled on ice for 10 - 15 minutes.
4. The cells were pell ated by centrifugation at 2,500rpm for 15 minutes at 4°C.
5. After draining the pellet thoroughly, the cells were resuspended in 10 mls of TFB by vortexing moderately and incubated on ice for 15 minutes.
6. Cells were pelleted as in step 4 and drained thoroughly before resuspending in 2.4mls of TFB.
7. DnD solution was added to 3.5% (v/v) and the cell suspension mixed immediately by swirling the tube. The tubes were then incubated on ice for 10 minutes.
8. A second aliquot of DnD solution was added as in step 8, to give a final concentration of 7% (v/v). The tubes were again incubated on ice, for 20 minutes.
9. 210μl aliquots were removed into sterile, chilled 1.5ml Eppendorf tubes.

Transformation procedure

1. DNA, in a volume of less than 20μl was added to an aliquot of prepared cells, swirling to mix, and the tube incubated on ice for 20 - 40 minutes.
2. The cells were heat shocked by placing the tubes in a 42°C water bath for 90 seconds before returning into ice for 2 minutes to quench the heat shock.
3. 800μl of SOC medium was added to each tube and incubated at 37°C with shaking at 250rpm for 60 minutes.

4. The cells were poured over nitrocellulose filters in a Buchner funnel and excess medium drawn off under vacuum.
5. The nitrocellulose filters were transferred to selective L-agar plates and grown overnight at 37°C.

Ordered arrays of bacterial colonies

1. Bacterial colonies were grown on nitrocellulose filters laid over appropriate selective L-agar plates at 37°C overnight.
2. Filters were marked with a waterfast marker pen for orientation.
3. The filters were placed, colony side up, on Whatman 3MM filter paper soaked in 1.5M NaCl/0.5M NaOH for 10 minutes, to lyse cells and denature DNA.
4. The filters were then transferred to Whatmans 3MM soaked in 3M NaCl, 0.5M Tris-HCl pH7.0, for 5 minutes, before removing to fresh 3MM paper soaked in the same solution.
5. Filters were baked at 80°C for 2 hours.
6. Prior to use, filters were soaked in 0.1M NaOH for 5 minutes with gentle shaking. Cellular debris was removed by rubbing the surface of the filters with a gloved hand.
7. After rinsing briefly in ddH_2O, filters were washed in 0.5M Tris-HCl pH7 for 15 minutes to neutralize. Filters were then ready for prehybridization.

CHAPTER 3

MICRO-INJECTION OF THE AEDES AEGYPTI EMBRYO

3.1 INTRODUCTION

The technique for micro-injection of the mosquito embryo was based on that used to introduce DNA into the developing embryo of *Drosophila melanogaster* (Germeraad, 1976, Spradling and Rubin, 1982). The *Drosophila* embryo is bounded by two envelopes, an inner transparent vitelline membrane, probably secreted by the egg itself and an outer opaque, white chorion. The chorion is rigid and can be removed by lightly stroking the embryo on double-sided sticky tape. The embryos, bounded only by their vitelline mebrane are aligned on a strip of double-sided sticky tape with their posterior tips extending off the tape and then desiccated to facilitate the introduction of a DNA solution. Desiccated embryos are immediately covered with halocarbon oil to prevent further desiccation during injection. A microcapillary tube, drawn to give a fine tip of a few micrometres is used as the injection needle. A 10ml syringe connected to the needle by air-filled plastic tubing is used to expel the DNA solution from the needle into the mounted embryo. An amount appropriate to the level of desiccation of the embryo is expelled and the needle rapidly withdrawn. The injected embryos are then placed in a moist chamber until the larvae hatch and are transferred to standard fly food at 25°C.

Theoretically, the introduced plasmid will be incorporated into the developing pole cells to integrate into the chromosomal DNA of the germ line. Integrated sequences may then be expressed throughout the somatic tissue of subsequent generations. Since the foreign DNA is not likely to be represented throughout the tissues of the organism in the G_0 generation - arising from injected embryos - only the next generation, the G_1 generation, and subsequent generations are assayed for expression of the transformed marker genes.

In developing the micro-injection system for the *Aedes aegypti* embryo, it was essential to compare and contrast its morphology and physiology with that of the *Drosophila melanogaster* embryo.

3.2 MORPHOLOGY AND PHYSIOLOGY OF THE AEDES AEGYPTI EMBRYO

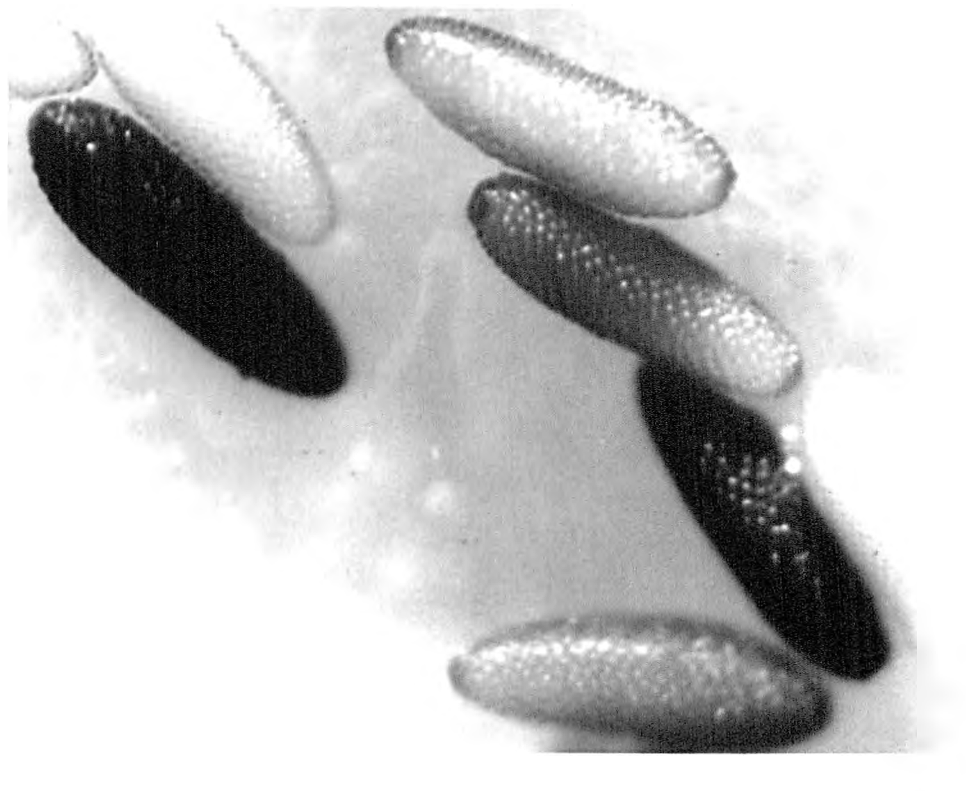
The dimensions of the mature *Ae.aegypti* embryo are 0.6mm x 0.17mm, making it slightly larger than that of *D.melanogaster* (0.42mm x 0.15mm). The width of the *Ae.aegypti* embryo is measured at the anterior end since the embryo is slightly wider at this point, tapering towards the posterior pole. The anterior pole of the embryo can be distinguished by the presence of the micropyle, a funnel shaped membrane with its open end protruding slightly beyond the surface of the embryo and serving as the point of entry of sperm during fertilisation.

The mature embryo is black in colour and covered by transparent globular bodies. The dorsal surface is convex with a flatter ventral surface. Oviposition is preferentially onto a moist surface rather than directly onto water, with the flatter ventral surface uppermost. In the laboratory, the embryos are readily laid on moist filter paper placed on a damp cotton wool pad.

The embryo is bounded by a translucent exochorion incorporating the globular bodies giving it a characteristic appearance. The fragile exochorion covers the more rigid endochorion which gives the embryo its shape and rigidity and is the principal mechanical protection for the egg. The exochorion is very easily damaged, particularly in the manipulation of the embryo for micro-injection. For this reason it can offer little protection to the embryo but plays an important role in anchoring them to the surface on which they are laid. Removal of the exochorion reveals the smooth, shiny endochorion. This endochorion is relatively soft and is a translucent white colour in the freshly oviposited embryo but darkens to a pale grey colour in approximately one hour after oviposition and to a dark grey/black colour over a period of two hours (Figure 3.1). Complete darkening to black takes four to five hours during which time it becomes progressively more rigid and brittle. This darkening is due to tanning of the protein making up the

Figure 3.1

Early (< 3 hours old) *Aedes aegypti* Bangkok embryos at various stages of development following oviposition.


0·1 mm

endochorion. Unlike the embryos of *Drosophila*, the vitelline membrane is not formed until 20 hours of development.

At oviposition, the embryo is up to 25% smaller than the mature embryo with no pronounced thickening at the anterior end. The increase in size of the embryo is due to its uptake of water over a period of 24 hours. During this period and for up to 72 hours the embryos are sensitive to desiccation. Even when the embryo has swollen to its maximum size and the endochorion fully darkened after 24 hours, the embryo will still collapse when removed from the moist ovipostion surface. The period up to 72 hours after oviposition is known as the conditioning period during which time larval development occurs. Following complete larval development at 72 - 96 hours the embryos become resistant to prolonged desiccation of up to 4 months though increasing the storage of the dried embryos beyond this may lead to a greater mortality.

If the embryos are allowed to collapse from desiccation prior to completion of the conditioning period they can be swollen to their original size by placing in water though the embryo contents will be disorganised and the embryo inviable. This phenomenon could lead to some confusion during the process of micro-injection in which the embryos are desiccated slightly as for *Drosophila* embryos. Over desiccated embryos may assume their original shape and appear viable following micro-injection when in fact their failure to hatch is due to procedures prior to the introduction of a DNA solution.

Following the conditioning period the moist filter paper serving as an oviposition surface can then be removed from the underlying damp cotton wool and the embryos dried. Alternatively, the embryos may be set to hatch directly. Water charged with organic matter has been found to exert a powerful stimulus on the hatching of eggs (Bacot, 1916). This is due to the reduction in dissolved oxygen in the water, as it is utilised by the organic matter. For this reason, a filtered infusion of grass proves to be an effective medium for stimulation of hatching (Christophers, 1960).

In the micro-injection of freshly oviposited *D.melanogaster* embryos, the DNA solution must be introduced into the embryo prior to pole cell formation so that it may become incorporated into these target cells. The optimum site of delivery of the DNA solution then is the region of the yolk adjacent to the periplasmic space, a region of clear cytoplasm at the posterior pole, since it is in this region of the yolk that pole cell formation occurs followed by their budding off into the periplasmic space itself at approximately 80 - 90 minutes of development. This target region for injection is clearly visible through the transparent vitelline membrane. The *Ae.aegypti* embryo has no periplasmic space. The pole cells begin to form at the posterior pole of the embryo at 3 hours. Pole cell formation is complete at 4 hours and by 5 hours they take up a position outside of the blastoderm. Although this is not visible through the opaque endochorion, initial orientation experiments were carried out by bleaching the endochorion with a solution of 0.3% (w/v) sodium chlorite, 0.2% (v/v) glacial acetic acid for 30 minutes (Trpis, 1970) at different stages of development. This does, however, render the embryos inviable and so cannot be applied in the micro-injection of the embryos.

To summarise then, the major, relevant differences between the embryos of *Ae.aegypti* and *D.melanogaster* are as follows:-

(i) Though the *Ae.aegypti* embryo is bounded by a rigid chorion, unlike *D.melanogaster* no vitelline membrane is present beneath it at this stage so that the chorion cannot be removed to facilitate micro-injection.

(ii) Although bounded by a rigid chorion, the embryo of *Ae.aegypti* is very sensitive to desiccation so that controlled desiccation to facilitate the introduction of a DNA solution is very difficult.

(iii) Pole cell formation begins at 3 hours in the embryos of *Ae.aegypti* but at 80 - 90 minutes in *D.melanogaster*

3.3 THE MICRO-INJECTION TECHNIQUE

Individual females from *Ae.aegypti* Bangkok strain were transferred 4 days post blood meal into glass tubes (3 x 8cm) with a circle of moist filter paper to serve as a surface for oviposition. Dark grey-black embryos approximately 90 - 120 minutes old are at the optimum age for injection. Earlier embryos will not survive micro-injection while later embryos become more difficult to inject due to the increased rigidity of the endochorion. The embryos were orientated on the oviposition paper with their posterior poles aligned. A strip of double-sided sticky tape, 3 x 10mm was attached to the edge of a coverslip to immobilise the embryos which were transferred to the tape by inverting the coverslip and picking up the embryos from the oviposition paper. Thorough drying of the oviposition paper prior to transfer of the embryos to the tape, is achieved by pressing a dry filter paper onto its edge. This prevents adherence of the embryos to the oviposition paper.

At this stage the embryos were very susceptible to desiccation and would collapse and become inviable within 1 - 2 minutes. Desiccation was monitored by eye under the dissecting microscope (x10 magnification). The exochorion was usually detached or damaged by the manipulation of the embryos so that only the endochorion was visible. In embryos where the exochorion was still present at least in part, the first sign of desiccation was the shrinking of the endochorion away from the exochorion giving the latter a silvery, translucent appearance. In embryos without the exochorion desiccation was first obvious when the endochorion lost its shiny appearance. Further desiccation results in one or both of the upper sides of the embryo collapsing so that the egg is eventually tri-radiate in section and, ultimately, flattened to a saucer shape. Adequate desiccation was achieved when the upper surface of the embryo began to bow inwards slightly. Several embryos became over desiccated before the majority of embryos reached a suitable stage of desiccation. These embryos were discarded to avoid confusion over viability of injected embryos. Insufficient drying led to excessive cytoplasmic leakage on micro-injection. In the *Drosophila* embryo with its chorion removed, desiccation is achieved by placing the mounted embryos in a petri-dish containing a desiccant. This was not necessary in the case of the *Ae.aegypti* embryo and 30 - 60 seconds in the atmosphere was sufficient for

desiccation. This time was significantly reduced to 15 - 30 seconds in other less humid laboratories making controlled desiccation more difficult to achieve.

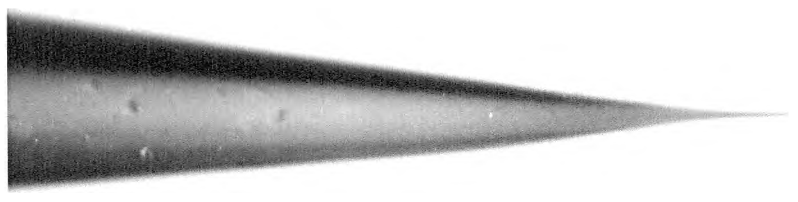
Having achieved a suitable degree of desiccation, the embryos were prevented from drying further by covering them with water saturated halocarbon oil (Series 700, KMZ Chemicals Ltd.). Various mineral oils have been found to be lethal to *Ae.aegypti* embryos (Powers and Headlee, 1939). This is apparently due to oxygen deprivation and is dependent on the viscosity of the oil. Water saturated halocarbon oil allows for oxygen and water uptake of the developing embryo. The oil covered eggs are kept in a petri-dish lined with moist filter paper prior to injection.

Capillary needles were loaded for injection with the DNA solution at a total concentration of either 500μg/ml at a ratio of 9:1, pUChsneo:pUChs$_\pi$(Δ2-3), in 5mM KCl, 0.1mM sodium phosphate pH6.8, or at a total concentration of 300μg/ml at a ratio of 5:1, pUChsneo:pUChs$_\pi$(Δ2-3). The injection needles, with a tip 300μm x 4 - 8μm (Figure 3.2) (pulled using a vertical needle puller, Scientific Research Instruments) were designed to have a short region of taper and a fine tip to prevent breakage of the needle when puncturing the rigid endochorion and tearing of the brittle endochorion itself during this procedure. A tip of 1 - 2 μm was ideal for puncturing the endochorion but tips of this diameter were very easily blocked by the contents of the embryo and by remnants of the exochorion on the outside of the embryo. A more practical tip diameter of approximately 4μm was routinely used though viable embryos often resulted from injection with a tip of up to 8μm. A bevelled tip did not appear to be necessary. The most satisfactory needles used were those drawn from capillary tubing with a glass filament fused to the inside surface (GC120F-10, Clark Electromedical Instruments).

The injection needles were connected by air filled plastic tubing to an injection system which uses two positive pressure levels - injection and holding - provided by a nitrogen gas cylinder and controlled by a foot pedal (Ansorge, 1982). This reduces blockage of the needle by eliminating suction at the tip when introduced into an insufficiently desiccated embryo and when the injection pressure is reduced before withdrawal of the needle tip.

Figure 3.2

A micro-injection needle used in the injection of *Ae.aegypti* Bangkok embryos.


30 0 μm

To ensure that DNA is introduced into the embryo prior to pole cell formation, all injections were performed within 2 hours of oviposition using the TDU500 micromanipulator (Research Instruments Ltd.) at x100 magnification. Figure 3.3 illustrates the arrangement of the mounted micro-injection needle with a raised stage (to ensure that the embryos are injected horizontally to prevent tearing of the endochorion) and an overhead light source. The volume of DNA solution injected was 160-180pl, corresponding to 1 - 5% of the embryo volume. This injected volume was controlled by adjusting the injection pressure and time and calculated by determining the volume of a spherical droplet ejected into an oil reservoir. Figure 3 illustrates microinjection of an embryo. After injection, the coverslip carrying the embryos was returned to the petri-dish lined with moist filter paper and placed in the insectary (27°C, 75 - 80% relative humidity) for 20 - 30 minutes. The embryos were then picked from the coverslip using fine forceps, placed onto a moist filter paper and returned to the insectaries for the four day conditioning period to allow for larval development. The injected embryos were stimulated to hatch in a dilute solution of hay infusion.

3.4 PERCENTAGE SURVIVAL OF INJECTED EMBRYOS

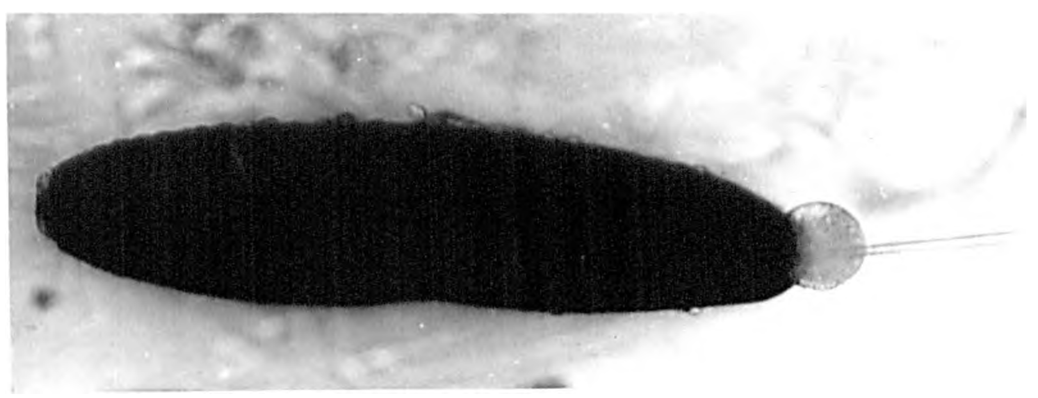
Table 3A shows the percentage survival of injected individuals at different stages of development. These post injection survival rates are comparable to those obtained routinely with *Drosophila* embryos. The G_0 progeny - adults arising from the injected embryos - were pooled and crossed to virgin *Ae.aegypti* Bangkok individuals at a ratio of 1:4, G_0:Bangkok non-injected individuals. Progeny arising from this cross were designated the G_1 generation. Once the G_1 generation was established, genomic DNA from the G_0 parents was prepared.

TABLE 3A SURVIVAL OF INJECTED AEDES AEGYPTI EMBRYOS

EMBRYOS INJECTED	HATCHED	ADULTS	% EGGS TO LARVAE	% LARVAE TO ADULTS	% EGGS TO ADULTS
63	15		24		
125	44	50	35	70	16
120	12		10		
180	32	21	18	66	12
204	51	24	25	47	12
69	6		9		
101	27	23	27	40	16
197	27		14		
188	25				
283	88				
130	8	166	22	69	15
316	101				
206	20				

Figure 3

Micro-injection of the *Aedes aegypti* embryo. A small amount of cytoplasm may be lost from the embryo on injection, without loss of viability.



0·1 mm

Figure 3.3

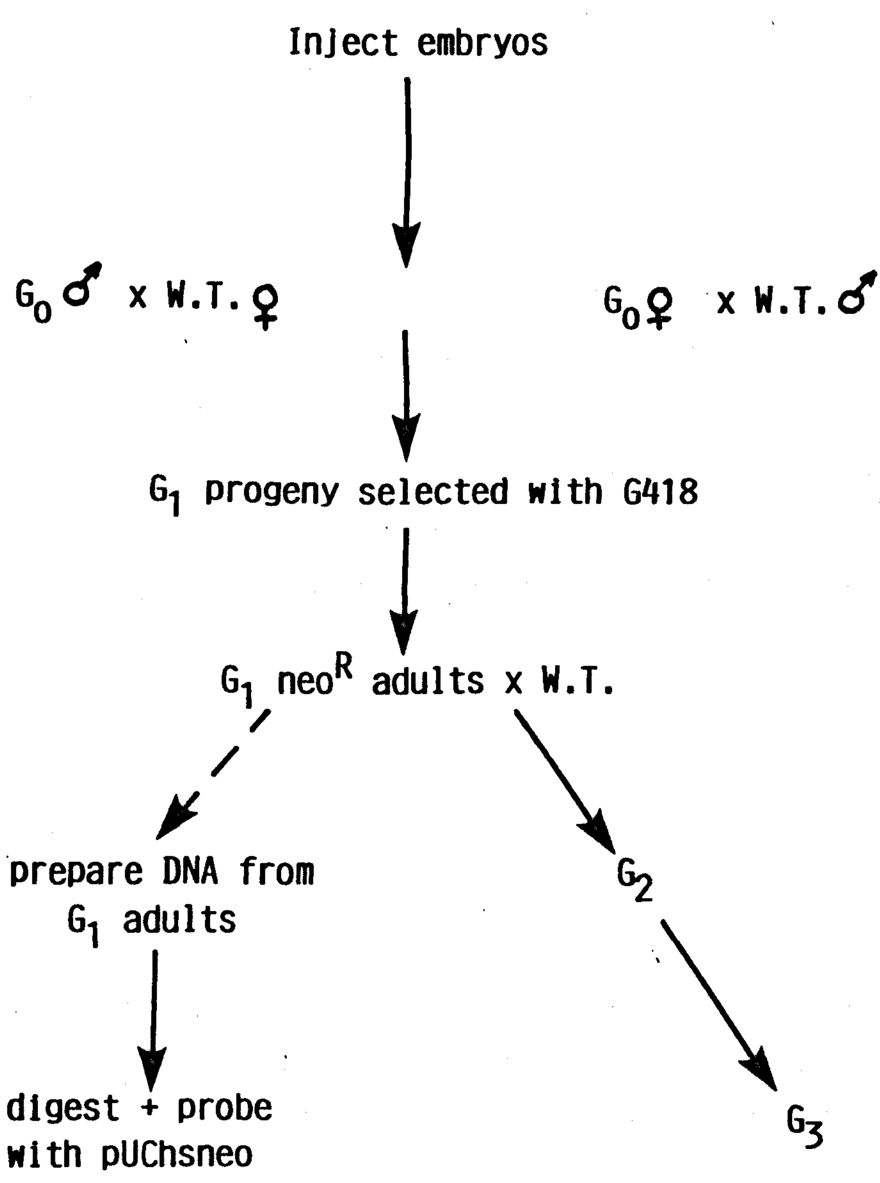
Microscope stage and mounted micro-injection needle. The stage is effectively elevated using an immobilized stack of microscope slides to ensure that embryos are injected horizontally, to prevent tearing of the endochorion.

a
b
c

Figure 3.4

Summary of the experimental design employed in transformation experiments with *Ae.aegypti* Bangkok.

EXPERIMENTAL DESIGN



3.5 DETERMINATION OF LETHAL G418 CONCENTRATIONS

In an initial experiment to determine the optimum concentration of G418 required to kill non-transformed individuals, 100 *Ae.aegypti* Bangkok strain larvae, 0-24 hours old, were assayed in 300mls of water in metal trays, 25cm x 30cm, with G418 concentrations of 200ng/ml, 100ng/ml, 50ng/ml, 25ng/ml and 0ng/ml. For each concentration of G418 the percentage adult survival was similar to that obtained in the 0ng/ml control (70%), indicating that G418 concentrations within this range are not selective with first and early second instar larvae. The assay was repeated using 100 and 157 larvae, 2 and 4 hours old respectively, with a G418 concentration of 200ng/ml. In these assays the percentage survival at day 6 was 100% with pupation occurring at day 8. Christophers (1960) gives 6-8 days as the average time of larval development to pupation with this period extended under adverse conditions. Clearly then, a G418 concentration of 200ng/ml is also not selective, and in fact, appears to have no effect on early first instar larvae. Allowing a period of 24 hours after setting embryos to hatch and prior to assay would ensure that the majority of viable embryos were hatched - an important factor when assaying small numbers of putative transformants. Consequently, subsequent assays were performed on larvae from embryos set to hatch 24 hours previously.

The original range of G418 concentrations was chosen on the basis of an economical and practical use of the G418 though it became evident that higher concentrations of the antibiotic must be used. To study the effects of these higher concentrations, 25 larvae were placed in 30mls of dilute hay infusion (water:hay infusion, 4:1) in a series of 9cm petri-dishes with G418 concentrations of 2mg/ml, 1mg/ml, 500ug/ml, 250ug/ml, 125ug/ml and 0ug/ml. Larvae were either removed from the G418 solution after 48 hours and placed in flat plastic trays, 14 x 10 x 2.5cm, with fresh water and killed yeast, or left in the petri-dishes with the G418 solutions. The assay was repeated four times and the results for the two treatments are summarised in Table 3B and Figures 3.5 and 3.6.

In the light of these results, considering the size and motility as well as the number of survivors, a G418 concentration of 1mg/ml was determined as the optimum concentration for the assay

TABLE 3B PERCENTAGE SURVIVAL OF AEDES AEGYPTI BANGKOK LARVAE AFTER TREATMENT WITH G418

% SURVIVAL

G418 CONC.	24 HOURS	removed from G418 48 HOURS	72 HOURS	144 HOURS
2 mg/ml	100 ++++	24 +	0	0
1 mg/ml	100 ++++	36 +	0	0
500 ug/ml	100 ++++	32 +	24 +	12 +
250 ug/ml	100 ++++	100 +++	96 +++	56 +++
125 ug/ml	100 ++++	100 +++	92 +++	64 +++
0 ug/ml	100 ++++	100 ++++	100 +++	100 +++

% SURVIVAL

G418 CONC.	24 HOURS	48 HOURS	72 HOURS	144 HOURS
2 mg/ml	100 ++++	32 +	0	0
1 mg/ml	100 ++++	48 +	8 +	0
500 ug/ml	100 ++++	32 +	0	0
250 ug/ml	100 ++++	100 +++	56 ++	4 +
125 ug/ml	100 ++++	100 +++	56 ++	0
0 ug/ml	100 ++++	100 +++	100 ++	100 ++

+ denotes larval development and motility relative to untreated controls in a 300ml volume

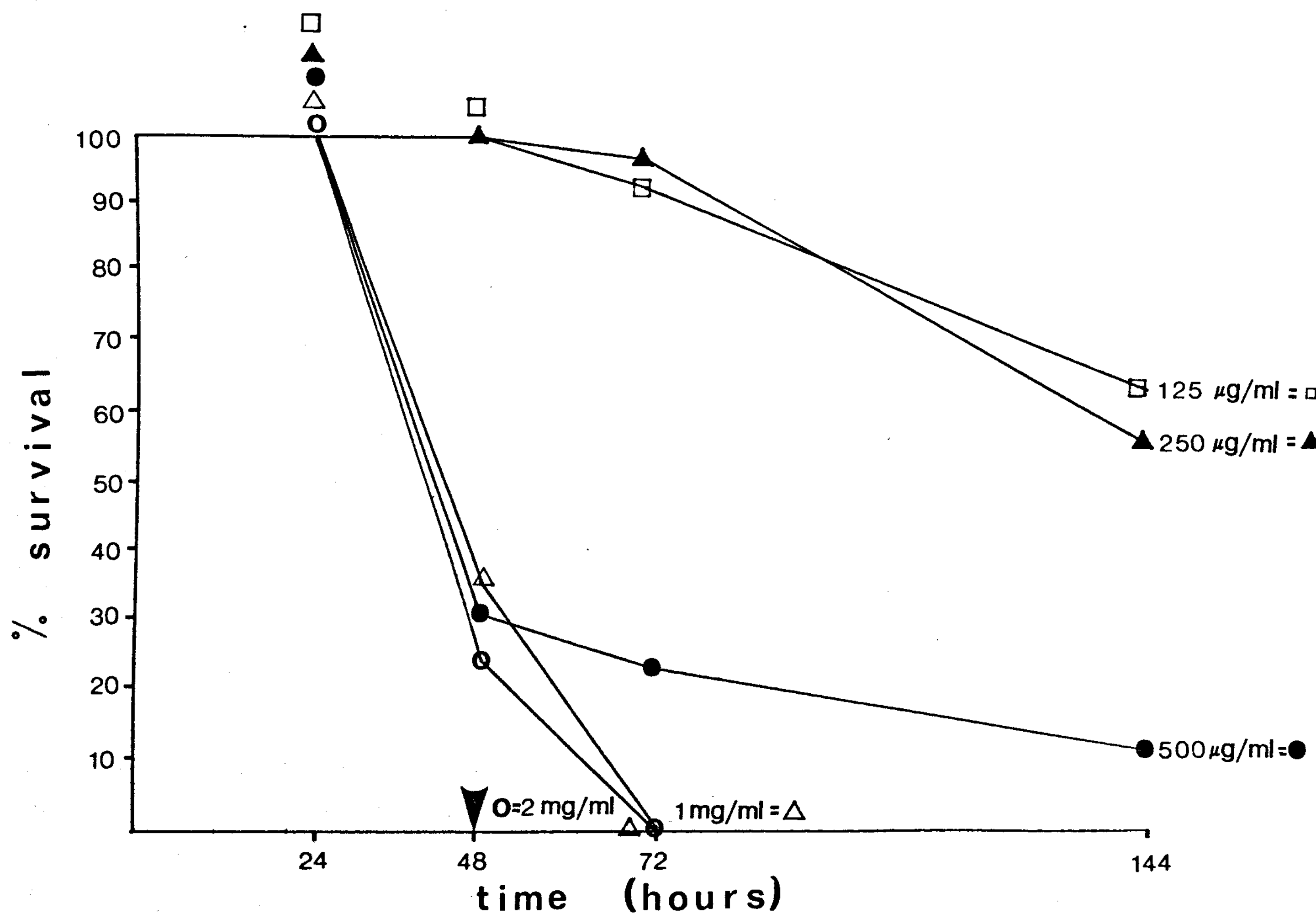
of putative transformants. Steller and Pirrotta (1985) report that large numbers of resistant *Drosophila* larvae do not consume or inactivate G418 in food suggesting that the number of larvae per unit concentration of G418 is not critical though it is obvious from the results obtained in this assay of mosquito larvae that prolonged overcrowding leads to retarded development of even the control larvae indicating that the number of larvae per unit volume of G418 is important. All surviving larvae were, therefore, transferred into larger trays with fresh water and yeast as soon as 98 - 99% morbidity of the control 1mg/ml larvae was achieved. Killed yeast was added, in addition to the G418, in all subsequent assays to serve as a food source to prevent the retarded development of survivors.

In a further series of experiments a G418 concentration of 200ng/ml in a 300ml volume was found to be sufficient to kill 99% of newly emerged *Anopheles stevensii* Ind S larvae. Miller et. al. (1987) also achieved 99% mortality with larvae of the G3 strain of *Anopheles gambiae*, using the same G418 concentration and volume. This would suggest a generic difference between the aedine and anopheline larvae in their response to G418. However, McGrane et. al. (1988) found that a G418 concentration of only 500ng/ml was required to kill one day old *Aedes triseriatus* larvae, a concentration 2000 times lower than that required for *Ae.aegypti* Bangkok larvae. In insecticide resistance tests with organochlorine, organophosphate and pyrethroid insecticides, *Ae.aegypti* Bangkok larvae repeatedly show resistance of an order of magnitude greater than another strain of *Ae.aegypti* - the London strain. This London strain, and other strains of *Ae.aegypti* would need to be assayed with G418 before any relationship could be distinguished between the relatively high G418 concentration required to achieve 99% mortality and these insecticide resistance properties.

G_1 and subsequent progeny were assayed for G418 resistance with 1mg/ml G418. The results of individual G418 assays will be considered in subsequent chapters along with the corresponding putative transformants.

Figure 3.5

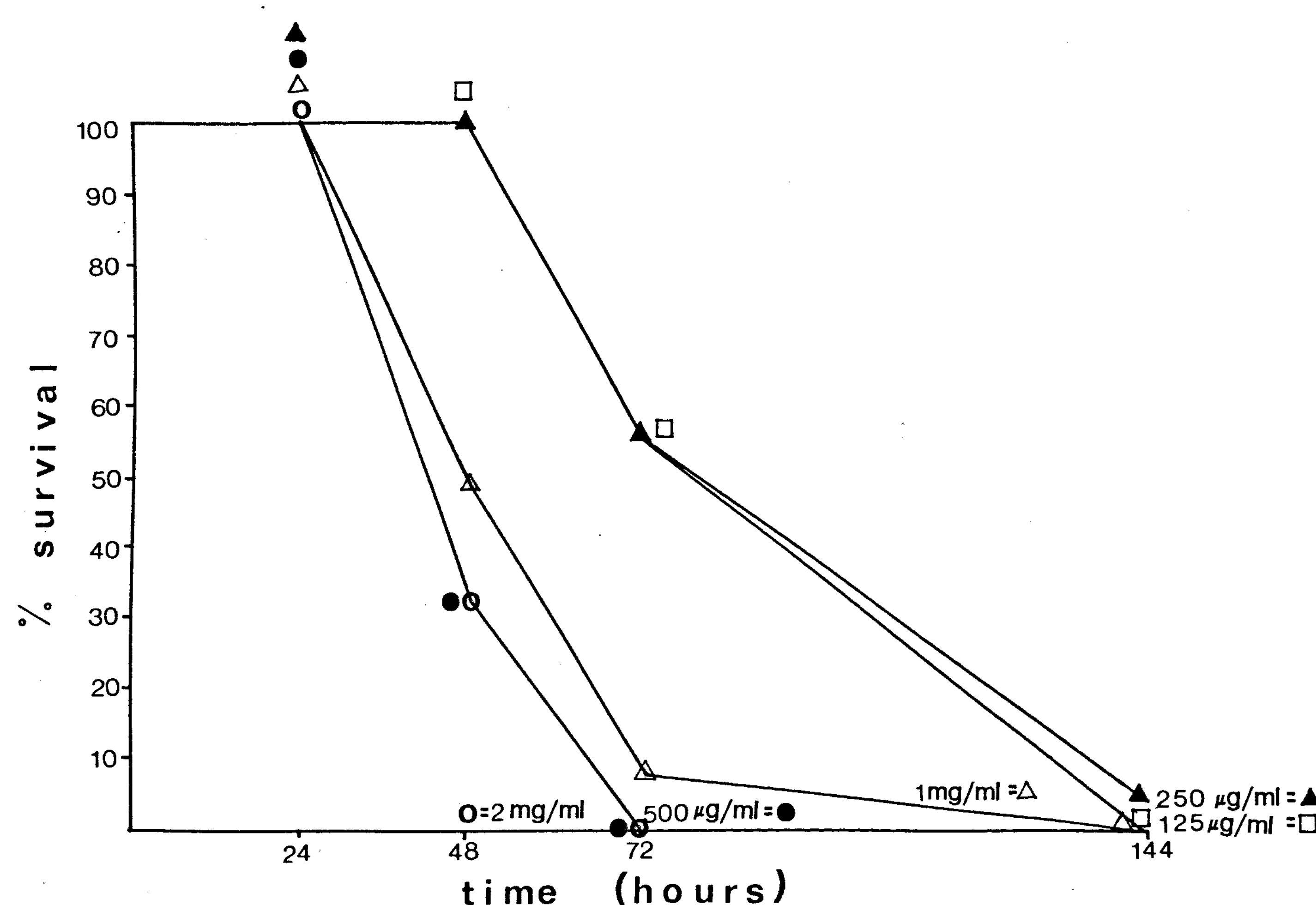
Graph of percentage survival of *Ae.aegypti* Bangkok first and early second instar larvae, following treatment with G418. All larvae were removed from the G418 solution after 48 hours.



100
90
80
70
60
50
40
30
20
10
% survival
24
48
72
144
time (hours)
125 µg/ml = □
250 µg/ml = ▲
500 µg/ml = ●
O=2 mg/ml
1 mg/ml = △

Figure 3.6

Graph of percentage survival of *Aedes aegypti* Bangkok first and early second instar larvae, following treatment with G418. Larvae were not removed from the G418 solution.



100
90
80
70
60
50
40
30
20
10
% survival
24
48
72
144
time (hours)
O=2 mg/ml
500 µg/ml =●
1mg/ml =△
250 µg/ml =▲
125µg/ml =□

3.6 SUMMARY

A DNA solution was introduced into the embryos of the *Ae.aegypti* Bangkok mosquito and survival rates comparable to those obtained in micro-injection of *Drosophila* embryos were achieved. Wild type *Ae.aegypti* Bangkok first and early second instar larvae were found to be sensitive to a relatively high concentration of the antibiotic G418 so that the *neo* gene coding for bacterial resistance to G418, may be effective as a marker gene in mosquito transformation i.e. putative transformants would be isolated on the basis of their resistance to G418.

CHAPTER 4

INTEGRATION OF P ELEMENT DERIVED SEQUENCES INTO THE MOSQUITO GENOME

4:1 INTRODUCTION

Having established the micro-injection technique to introduce foreign DNA sequences into the embryos of *Ae. aegypti* Bangkok, the DNA content of G_0 and subsequent generation flies was examined and their progeny assayed with G418 to detect the expression of integrated sequences.

In the G_0 generation, expression of the foreign DNA introduced by micro-injection is not expected throughout the somatic tissue of the adult. In theory it will only be expressed in the target germline cells into which it is incorporated. However, in *Drosophila* transformation experiments using a phenotypic marker such as the *rosy* gene, partial restoration of the wild type phenotype in a *rosy*$^-$ mutant is often detected in G_0 flies (Rubin and Spradling, 1982). This is thought to be due to expression of non-integrated *rosy* sequences i.e. the *rosy* gene carried on the intact vector plasmid which, although not integrated into the genome, is expressed and represented in the total DNA isolated from the organism. This observation led to an analysis of the total DNA isolated from G_0 mosquitos. The foreign DNA sequences detected in total G_0 DNA and in the total DNA isolated from subsequent G_1 and G_2 generation flies is considered in this chapter.

4:2 PLASMID DNA USED FOR TRANSFORMATION

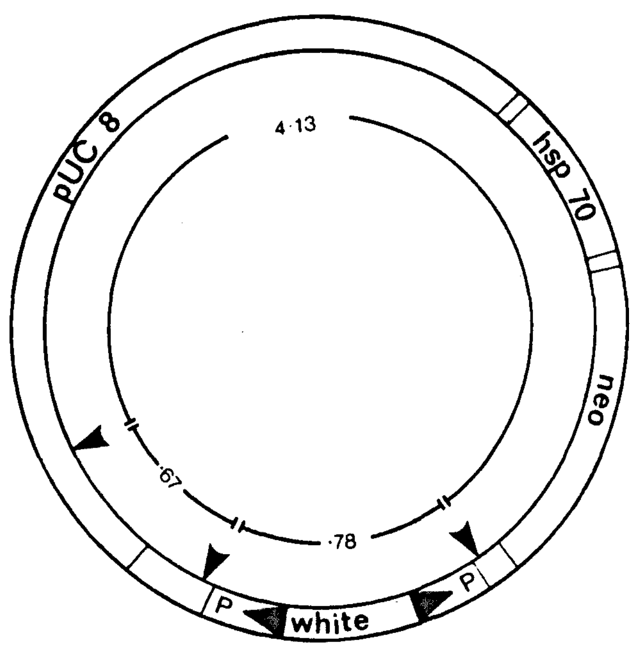
As for transformation experiments with *Drosophila*, the DNA solution to be introduced into the embryo was made up of two plasmids, a vector plasmid carrying a dominant selectable marker gene, and a helper element supplying the transposase function but unable to integrate into the genome. Figure 4.1 shows the vector plasmid - pUChsneo (Steller and Pirotta, 1985) - carrying the bacterial neomycin resistance gene which confers resistance to the antibiotic G418, under the control of a *Drosophila* heat shock promoter, hsp70. A multiple cloning site from pUC8 also lies within the inverted repeats of the P element to facilitate cloning of DNA into the vector. The P sequences are joined by 500bp of DNA from the *white* locus of *Drosophila*.

The second P element construct - pUChsπ(Δ2-3) (figure 4.2) - acts as a helper element, providing the transposase activity required for effective transposition of the vector element (Laski *et.al.*, 1986). It has one defective inverted repeat so that it is unable to integrate into the host genome and has the intron between ORF2 and ORF3 of the intact 2.9kb P element spliced out to overcome the tissue specificity of transposase production encountered in *Drosophila*.

In the G418 assay for putative transformants, subjecting the larvae to a 37°C heat shock prior to the assay, should activate the hsp-70 on the pUChsneo plasmid. This will increase the level of expression of the *neo* gene in transformants, to ensure that an adequate level of its phosphotransferase product is produced, to render the transformant resistant to the G418. In the experiments discussed in this chapter, the larvae were heat shocked for one hour at 37°C in a humid oven and returned to the insectaries for a 2 hour recovery period prior to treatment with G418. In subsequent experiments, the length, timing and temperature of the heat shock were altered in an attempt to determine optimum conditions. This will be discussed in section 5:2.

Figure 4.1

The pUChsneo plasmid. This P element vector carries the bacterial neomycin gene which confers resistance to G418, under the control of a *Drosophila* heat shock promoter, hsp 70. A multiple cloning site lies within the inverted terminal repeats of the P element and the P sequences are joined by 500bp of DNA from the *white* locus of *Drosophila melanogaster*. The arrows indicate the Hind III restriction sites and fragment sizes are given in kilobases. The regions covered by the probes used in figures 4.4, 4.5, 4.6, and 4.7 are indicated A - E. Probe B is a 1.5kb Sal I fragment isolated from plasmid pCs156, which covers the pUChsneo sequences derived from the *white* locus of *Drosophila*. The remaining probes (A, C, D and E) represent sequences isolated from the pUChsneo plasmid.



white pUC8 hsp70 neo

A

B

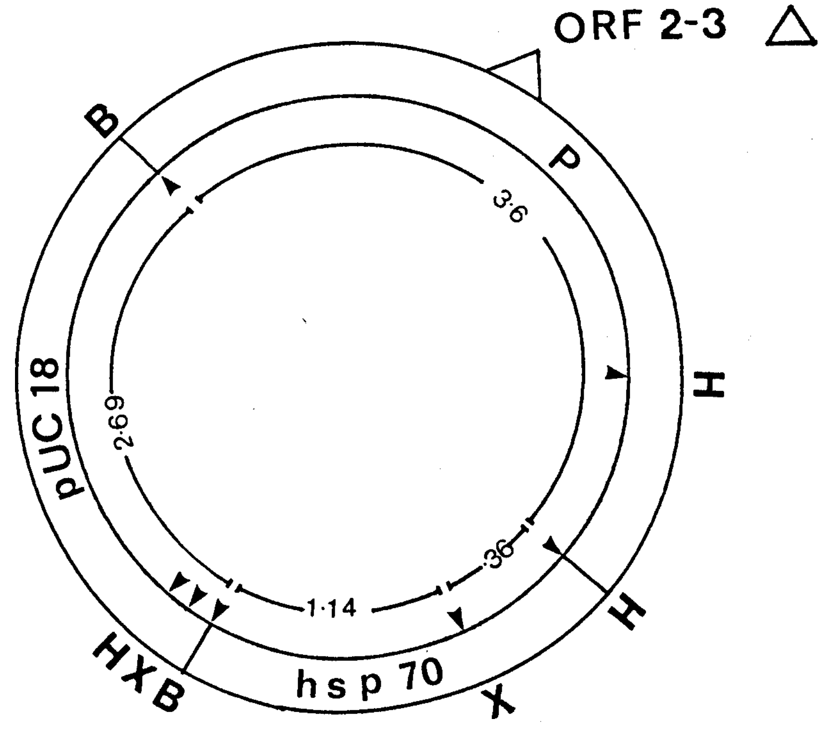
C

D

E

Figure 4.2

The pUChsπ(Δ2-3) plasmid. This plasmid was kindly donated by F. Laski and was constructed by combining the 3.6kb Hind III partial - Bam HI fragment from Pc[ry] 2-3 (Laski *et.al.*, 1986), carrying the P element coding sequence (and lacking the ORF 2 - 3 intron, with the 1.5kb Bam HI - Hind III fragment from pNHP (Rio and Rubin, 1985). The vector is pUC18. The arrows indicate Bam HI (B), Hind III (H) and Xba I (X) restriction sites and fragment sizes are given in kilobases.



ORF 2-3
B
P
H
H
X
HXB
pUC 18
hsp 70
3·6
2·69
1·14
·36

4:3 DETECTION OF pUChsneo SEQUENCES IN G_0 GENOMIC DNA

Genomic DNA was prepared from 24 individual G_0 flies, denatured and dot blotted onto nitrocellulose using an S&S minifold. The filter was then hybridized with the pUChsneo probe, washed in 0.1 x SSC, 0.1% SDS at 65°C and autoradiographed. The probe detected pUChsneo sequences in 3 of the 24 individuals (data not shown). Consequently, genomic DNA from further G_0 individuals was digested with the restriction enzyme Hind III and analysed by Southern blotting using the pUChsneo plasmid as a probe. In 5 - 10% of G_0 flies studied in this way, the probe detected three bands corresponding to the sizes expected for a Hind III digest of the free pUChsneo plasmid (figure 4.3). This confirms that the pUChsneo plasmid can be incorporated into the developing embryo and, in these cases, had remained intact in the adult.

In the genomic DNA of a single G_0 fly analysed in this way, 4 bands were detected (figure 4.4A), 3 of which correspond to the sizes expected for a Hind III digest of a precise P mediated integration event i.e. the 4.13kb fragment along with 2 fragments joined to host mosquito genomic DNA at the P element terminii. The fourth and smallest band of 3.2kb was also found in several other G_0 individuals and in total *Ae.aegypti* Bangkok DNA isolated from non-injected individuals.

4:4 G_1 AND G_2 GENERATION GENOMIC DNA ANALYSIS

The pUChsneo probe hybridized to 4 bands in the Hind III digested total DNA from a G_1 individual (Figure 4.5A) and 2 bands in that of a G_2 individual (Figure 4.6A). In the G_2 banding pattern, the 2 bands are of identical size to the largest and smallest (6.6kb and 2.85kb respectively) of the 4 bands observed in the G_1 individual. The 2.85kb band was also detected in other G_1 and G_2 individuals and in total *Ae.aegypti* Bangkok DNA isolated from non-injected individuals (Figure 4.7A), as for the 3.2kb band detected in the G_0 individual (section 4.3). This band will be discussed in section 4:11 and in more detail in 5:5. Both the G_1 and G_2 flies were originally thought to arise from one G_0 x wild type Bangkok strain cross, suggesting a single

Kb
4·13
0·78
0·67

Figure 4.4

Southern transfer of Hind III digested genomic DNA from a G_0 adult, probed with the labelled pUChsneo plasmid (A), and probes B - E, as denoted in figure 4.1. Lane F shows the ethidium bromide stained gel prior to Southern transfer. Following hybridization, filters A - D were washed twice, for 30 minutes, in 2 x SSC, 0.1% SDS at either 65°C (A), room temperature (B), or at 42°C (C and D). Lane E was washed in 6 x SSC at room temperature. Autoradiography was at -70°C for either 2 weeks (A and C), 4 weeks (B) or 60 hours (E). Hybridization of the probe in lane E was difficult to visualize with longer exposures due to a low level of homology throughout the genomic DNA and non-specific binding over the whole filter. This also accounts for the relatively short exposures of filters shown in figures 4.5E, 4.6E and 4.7E.

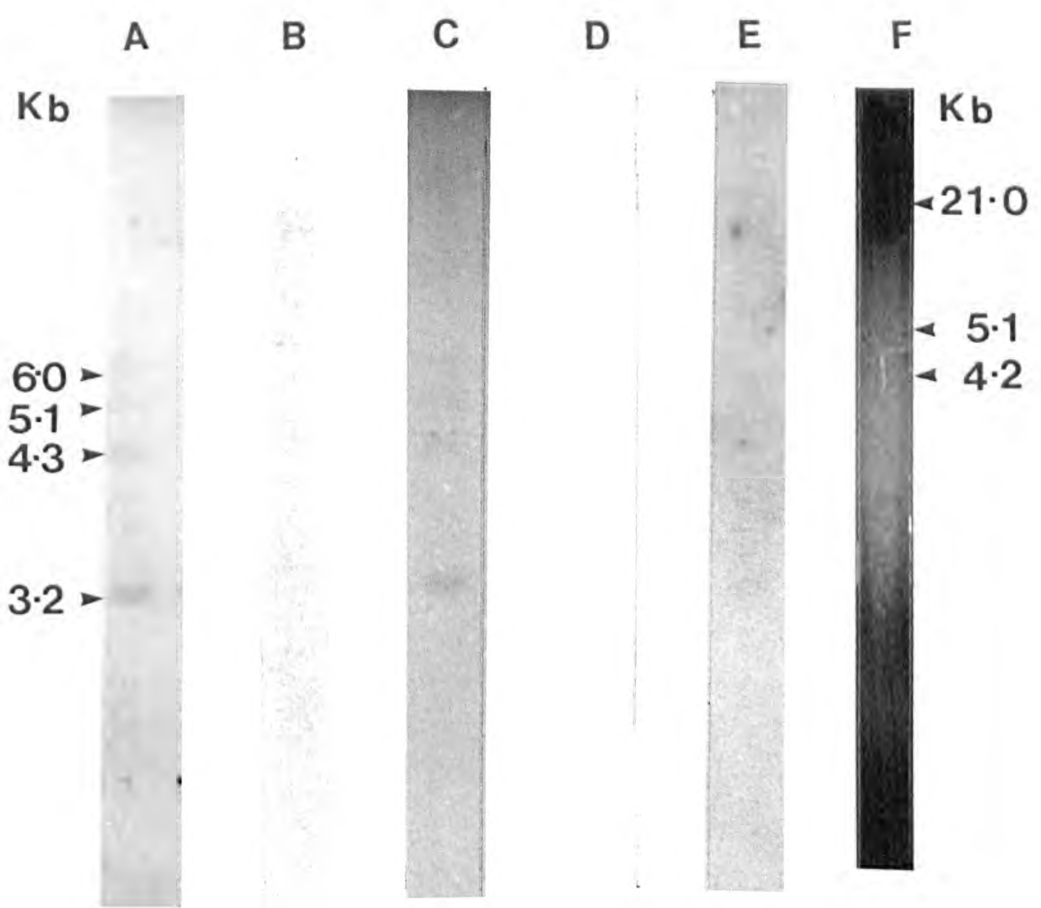

A
B
C
D
E
F
Kb
6·0
5·1
4·3
3·2
Kb
21·0
5·1
4·2

Figure 4.5

Southern transfer of Hind III digested genomic DNA from a G_1 transformed adult (number 8), probed with the labelled pUChsneo plasmid (A) and probes B - E as denoted in figure 4.1. Lane F shows the ethidium bromide stained gel prior to Southern transfer. Following hybridization, filters were washed twice, for 30 minutes, in 2 x SSC, 0.1% SDS at either 42°C (A, C, D) or room temperature (B). Lane E was washed in 6 x SSC at room temperature. Autoradiography was at -70°C for either 4 weeks (A, B, D), 2 weeks (C) or 60 hours (E).

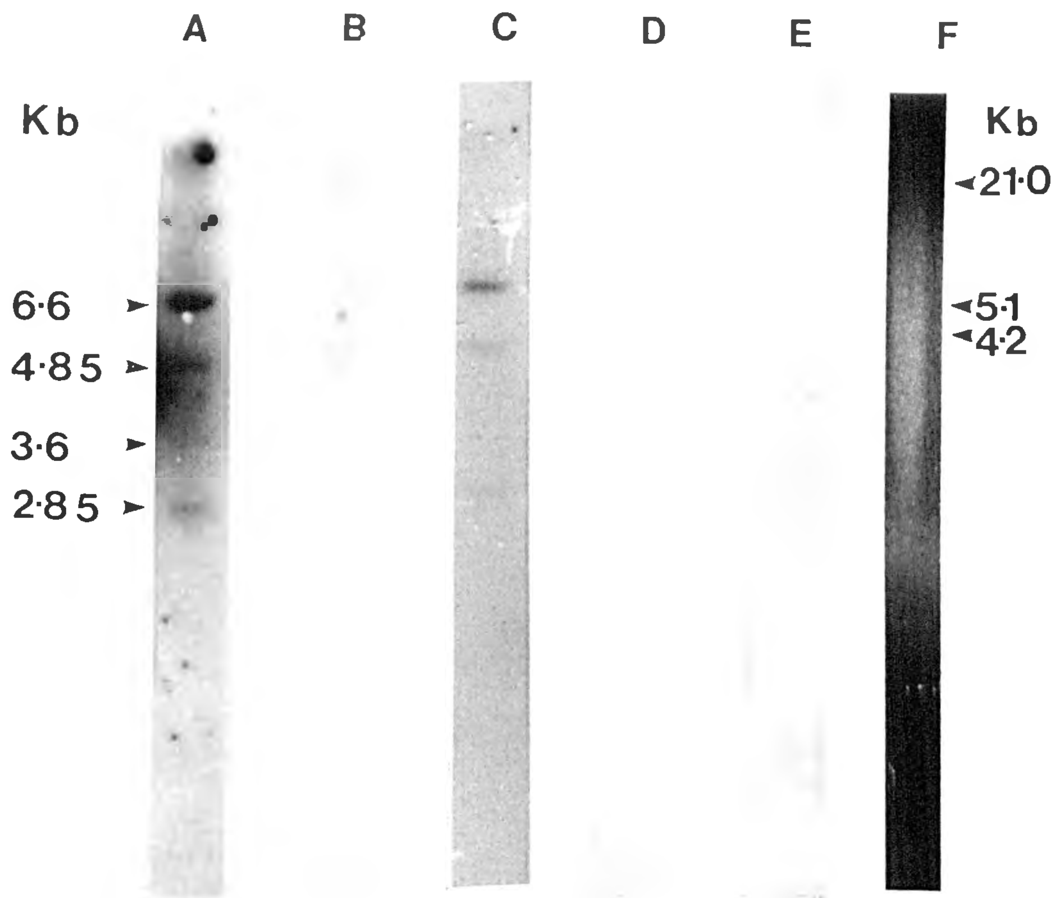

A
B
C
D
E
F
Kb
6·6
4·85
3·6
2·85
Kb
21·0
5·1
4·2

Figure 4.6

Southern transfer of Hind III digested genomic DNA from a G_2 transformed adult (number 31), probed with the labelled pUChsneo plasmid (A) and probes B - E as denoted in figure 4.1. Lane F shows the ethidium bromide stained gel prior to Southern transfer. Following hybridization, filters were washed twice, for 30 minutes, in 2 x SSC, 0.1% SDS at either 42°C (A, C, D) or room temperature (B). Lane E was washed in 6 x SSC at room temperature. Autoradiography was at -70°C for either 1 week (A), 4 weeks (B, D), 2 weeks (C) or 60 hours (E).

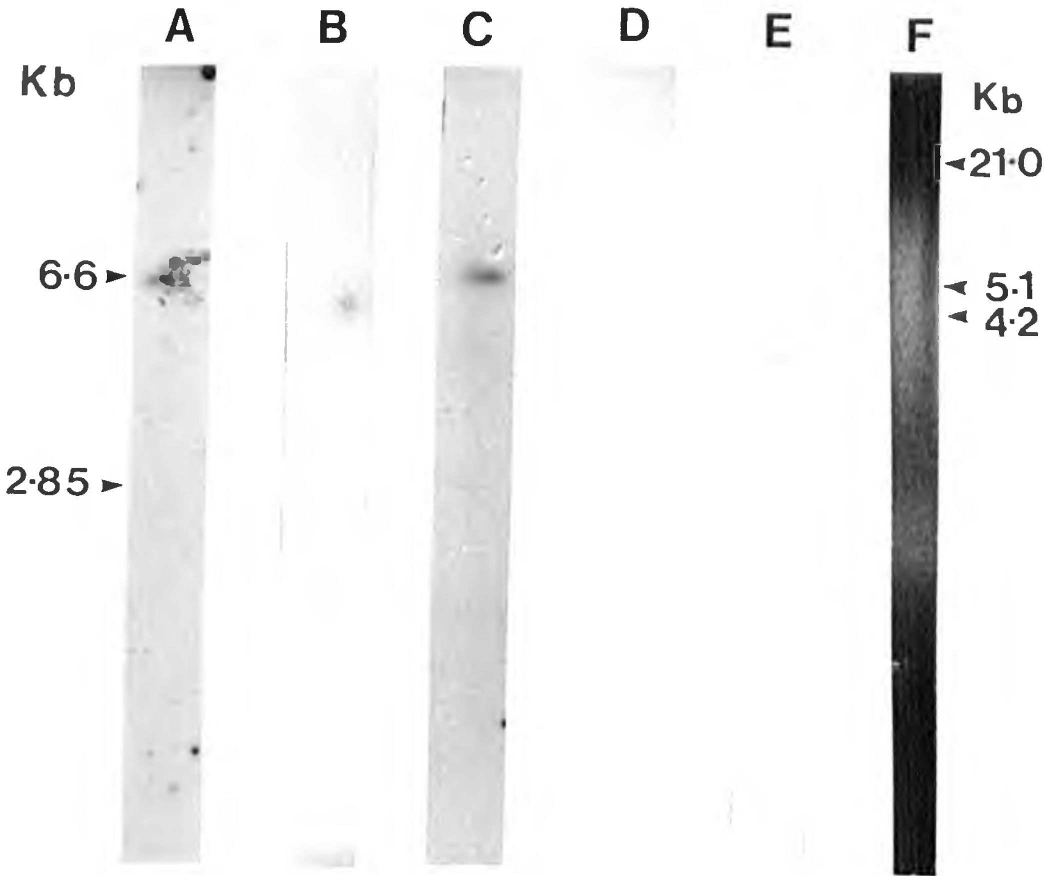
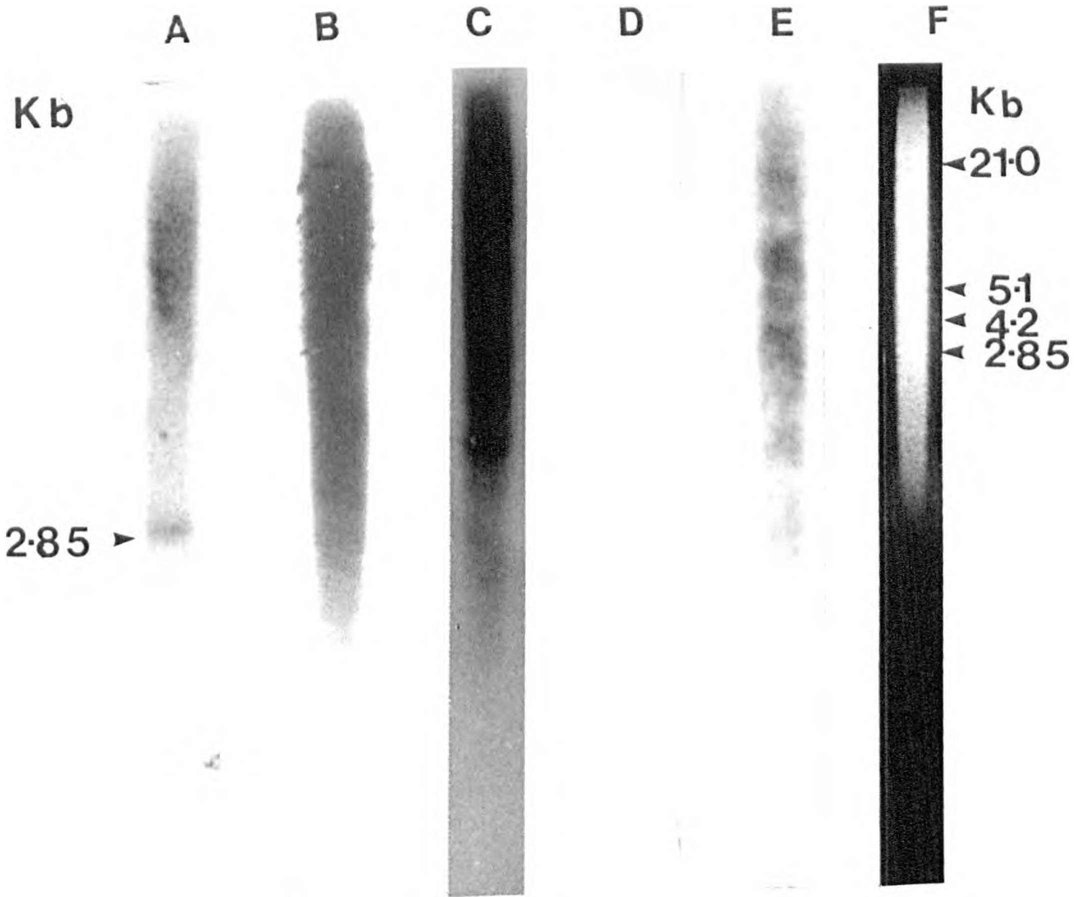

A
B
C
D
E
F
Kb
6·6
2·85
Kb
21·0
5·1
4·2

Figure 4.7

Southern transfer of Hind III digested genomic DNA from *Ae.aegypti* Bangkok non-transformed individuals, probed with the labelled pUChsneo plasmid (A) and probes B - E as denoted in figure 4.1. Lane F shows the ethidium bromide stained gel prior to Southern transfer. Following hybridization, filters A - D were washed twice, for 30 minutes, in 2 x SSC, 0.1% SDS at either 42°C (A, C, D) or room temperature (B). Lane E was washed in 6 x SSC at room temperature. Autoradiography was at -70°C for either 1 week (A), 4 weeks (B, D), 2 weeks (C) or 60 hours (E). The band of 2.85kb is thought to represent identical sequences to the band of 3.2kb detected in a G_0 individual and in other non-transformed individuals in separate experiments. This is discussed in section 4.11.


A
B
C
D
E
F
Kb
2·85
Kb
21·0
5·1
4·2
2·85

integration event in one G_0 parent through 2 generations. However, on further investigation, though the G_1 and G_2 flies originated from the same batch of injected embryos, the G_1 fly was found to have a male G_0 parent while the G_2 fly arose from a female G_0 parent. G_0 males and females were crossed separately to wild type Bangkok strain flies so that the G_1 and G_2 flies apparently carrying identical integrated sequences, could not be related. Possible interpretations of these banding patterns are advanced in the discussion (section 4:11).

4:5 FURTHER ANALYSIS OF THE G_0, G_1, AND G_2 INTEGRATION EVENTS

In the case of the G_0, G_1, and G_2 integration events (figures 4.4A, 4.5A and 4.6A), probing with the 500bp of the *white* locus of *Drosophila* joining the P element sequences in the pUChsneo plasmid would be expected to distinguish a P mediated transposition event from a random integration into the host genome. P transposition involves the integration of only the intact sequences flanked by the 31bp inverted repeats of the P element (O'Hare and Rubin, 1983) and not the external *white* locus region.

The appropriate filters were treated with 0.1M NaOH for 5 minutes at room temperature to remove the previous pUChsneo probe, neutralised with two, 15 minute washes with 0.1M Tris/Cl pH 7.5 at room temperature and reprobed with a fragment of the pCS156 plasmid (Sang *et.al.*, 1984) (kindly donated by Dr.David Finnegan). This 1.5kb Sal I fragment from the coding region of the *white* locus of *Drosophila* includes the 500bp of *white* sequence present in the pUChsneo plasmid. No hybridization was detected by this probe in the case of the G_0, G_1, and G_2 integration events, even after prolonged exposure times (Figures 4.4B, 4.5B and 4.6B). This suggests that the integration events were independant of the *white* region of the pUChsneo plasmid and therefore, possibly mediated by P element transposition rather than random chromosomal integration. There was some homology between the probe and the total genomic DNA from non-transformed individuals though there was apparently no specific hybridization to the band of approximately 3kb (Figure 4.7B).

In an attempt to identify the region of the pUChsneo plasmid hybridizing to the genomic DNA, different regions of the pUChsneo plasmid were isolated and labelled to reprobe the appropriate filters. Isolated Hind III fragments of pUChsneo were labelled using the method of Feinberg and Vogelstein (1984).

Hind III digested pUChsneo DNA was electrophoresed through a 1% low melting point agarose gel for one hour at 70V. The 3 resulting fragments were then visualized using a U.V. light source and each fragment excised from the gel in a slice of agarose. Each slice was placed in a separate, pre-weighed 1.5ml Eppendorf tube and weighed. Double distilled water was added to a volume of 1.5ml H_2O/g of agarose slice. The Eppendorf tube was then placed in a boiling water bath for 7 minutes to melt the agarose and denature the DNA, and the resulting solution aliquoted and stored at -20°C. To label the DNA, an aliquot was removed, boiled for 3 minutes, and placed at 37°C for 10 minutes before using in a standard random primed labelling reaction at 37°C. 50ng of DNA were used for each labelling reaction and average specific activities of 5×10^8 dpm/μg were achieved.

4:6 PROBING WITH THE 4.13KB HIND III FRAGMENT OF pUChsneo

The 4.13kb Hind III fragment covers 152bp of the 3' P element sequence, all of the promoter, hsp-70, the bacterial neomycin resistance gene and 2.5kb of the pUC8 sequence. This fragment detected all of the 4 bands in the G_0 integration event, as for the intact pUChsneo probe (Figure 4.4C). This does not correspond to the results expected in the event of a P mediated transposition event. If this was the case, the 4.13kb band should be detected but the two larger bands representing either 40bp or 240bp of pUChsneo joined to genomic DNA, are not included in this 4.13kb fragment and so should not hybridize to it. The smallest band of 3.2kb is also detected by the 4.13kb fragment probe as are the bands of the same size in other G_0's and non-transformed *Ae.aegypti* Bangkok total genomic DNA (Figure 4.7C). The fact that this 3.2kb band is detected in control, non-transformed flies suggests that it represents a region of the *Ae.aegypti* Bangkok genome which is homologous to the 4.13kb fragment of the pUChsneo

probe.

All of the 4 bands in the G_1 integration event were also detected by the 4.13kb pUChsneo HindIII fragment probe, though the 3.6kb fragment was very faint (Figure 4.5C). On probing with the 4.13kb fragment probe, the intensity of the 6.6kb band was reduced 3 times (determined by densitometry) so that a corresponding decrease in the intensity of the 3.6kb band was understandable. Though the filter probed with the pUChsneo plasmid followed by the 4.13kb fragment was hybridized with probes of similar specific activity and washed under identical conditions, the latter, probed with the 4.13kb fragment, was exposed to film for 2 weeks and not 4 weeks as for the filter probed with the pUChsneo probe. This could account for the reduction in band intensity.

In the G_2 integration event, the 6.6kb and 2.85kb bands detected by the intact pUChsneo probe were detected by the 4.13kb fragment probe (Figure 4.6C). Hybridization of the 2.85kb band in this and in non-transformed *Ae.aegypti* Bangkok total genomic DNA, again suggests a region of homology of the Bangkok genome to the 4.13kb fragment of the pUChsneo probe, as for the 3.2kb band in the G_0 integration event.

In summary then, all of the fragments detected by the pUChsneo probe in each of the G_0, G_1, and G_2 integration events, appear to represent sequences within the 4.13kb fragment of the pUChsneo plasmid.

4:7 PROBING WITH THE 0.67KB HIND III FRAGMENT OF pUChsneo

The 0.67kb Hind III fragment covers 500bp of the 5' P element sequence and the remaining 165bp of the pUC8 sequence. This probe did not hybridize to any of the bands resulting from the G_0, G_1, or G_2 integrations (Figures 4.4D, 4.5D, and 4.6D), even after prolonged exposures of up to 4 weeks. There was some evidence of limited hybridization to *Ae.aegypti* Bangkok total genomic DNA, though it did not detect the bands of 2.85kb or 3.2kb which hybridized to the intact pUChsneo plasmid and the 4.13kb probe (Figure 4.7D).

4:8 PROBING WITH A 31bp OLIGONUCLEOTIDE TO DETECT THE P ELEMENT INVERTED REPEATS

If the integration events are due to precise, P mediated transposition, then the 31bp inverted repeats, essential for P transposition, must be represented in the integrated sequences.

0.5μg of a 31bp oligonucleotide, which should detect the P element inverted repeats, was labelled by polynucleotide kinase end labelling with 30μCi 32P ATP and 1μl of polynucleotide kinase enzyme (9u/μl). The reaction was carried out in 50mmol/l Tris/Cl, 10mmol/l MgCl2, 5mmol/l dithiothreitol, pH 7, in a total volume of 10μl for 30 minutes at 37°C and the labelled oligonucleotide separated from free nucleotides by running through a Sephadex G-50 column equilibrated with 6 x SSC. The filters shown in Figures 4.4, 4.5 and 4.6 were stripped as in section 4:5 and hybridized to the oligonucleotide probe in a total volume of 10mls, 6 x SSPE, 5 x Denhardts solution, 0.1% SDS and 100μg/ml yeast tRNA, at 37°C for 18 hours.

Unfortunately, specific hybridization of this probe was difficult to visualize in the case of the G_0 integration and the smaller, less intense bands in the G_1 and G_2 integration (Figures 4.4E, 4.5E and 4.6E). There was apparently a low level of homology to *Ae.aegypti* Bangkok total genomic DNA from both transformed and non-transformed individuals and non-specific hybridization throughout the filter. However, it appeared that the larger bands of 6.6kb in the G_1 and G_2 integrations were not detected by this probe.

4:9 G418 ASSAYS OF PROGENY RELATED TO THE G_1 AND G_2 INDIVIDUALS CARRYING pUChsneo SEQUENCES

Unfortunately, the G_1 generation female mosquito, carrying pUChsneo sequences (labelled mosquito 'number 8') laid very few embryos before its death. The 17 larvae which hatched

from these embryos were assayed with G418 though none survived this treatment.

The G_2 generation male mosquito - 'number 31' - arose from the G_1 mosquito number 14. 6.4% of the G_1 progeny of mosquito number 14 survived the G418 assay giving rise to G_2 mosquitos 21, 22, 23, 24 and 31. The Hind III digested total genomic DNA of mosquitos 14, 21 and 23 was also probed with the pUChsneo probe but no hybridization was detected (results not shown). 1.9% of the G_2 progeny of mosquito number 22 and 2.3% of the G_2 progeny of mosquito number 31 survived the G418 assay giving rise to G_3 mosquitos 38 and 43 respectively. None of the G_4 progeny of mosquitos number 38 and 43 survived the G418 selection, and no hybridization was detected on probing the Hind III digested total genomic DNA of mosquitos 38 and 43 with pUChsneo. The results of these G418 assays are summarized in Table 4A. Table 4B summarizes the banding patterns detected by the individual probes in the relevant mosquitos. These results suggest that sequences giving rise to a degree of G418 resistance may be inherited through the line arising from mosquito number 14, but not by all progeny.

4:10 RECOVERY OF INTEGRATED SEQUENCES FROM TOTAL GENOMIC DNA

The only conclusive way to determine the precise nature of an integration event is to recover the integrated pUChsneo sequences and their flanking genomic DNA from the total genomic DNA so that they may be amplified and restriction enzyme mapping carried out followed by DNA sequencing. The sequences could be recovered from the total genomic DNA by preparing a genomic DNA library from the total genomic DNA isolated from mosquitos number 8 and 31. The resulting recombinant phage or plasmid clone could then be amplified in an E.coli host. However, due to the amount of DNA usually required in the construction of a genomic DNA library and the amount of DNA available, this was not the method of choice in this work. The method employed was that of plasmid rescue whereby the genomic DNA was digested with a restriction enzyme and then religated in dilute solution (10ng/μl) so that the ends of the restriction enzyme digest fragments will self-ligate, forming open, circular DNA. Any of the resulting circles possessing an origin of replication and an antibiotic resistance gene can be

TABLE 4A SUMMARY OF G418 ASSAYS OF PROGENY RELATED TO THE G_1 AND G_2 INDIVIDUALS CARRYING pUChsneo SEQUENCES

PARENT MOSQUITO	NUMBER ASSAYED	PROGENY % SURVIVAL	SURVIVING PROGENY MOSQUITOS
no. 8 G1	17	0	-
14 G1	78	6.4	no. 21
			22
			23
			24
			31
21 G2	19	0	-
22 G2	75	1.9	38
24 G2	51	0	-
31 G2	59	2.3	43
38 G3	68	0	-
43 G3	87	0	-

TABLE 4B SUMMARY OF BANDING PATTERNS DETECTED IN TRANSFORMED INDIVIDUALS AND THEIR PROGENY

	BAND SIZES (Kb) HYBRIDIZING TO PROBES A - D			
PARENT MOSQUITO	PROBE A	PROBE B	PROBE C	PROBE D
8:G1	6.6	-	6.6	-
	4.85		4.85	
	3.6		3.6	
	2.85		2.85	
14:G1	-	-	-	-
21:G2	-	-	-	-
23:G2	-	-	-	-
31:G2	6.6	-	6.6	-
	2.85	-	2.85	
38:G3	-	not probed	not probed	not probed
43:G3	-	not probed	not probed	not probed

Relative band intensities are denoted by hatched, stippled or open boxes where:-

Relative band intensities within a single track were determined visually. Relative band intensities of the 6.6kb bands were determined by densitometry.

amplified in *E.coli* host cells and selected by plating on media with a suitable antibiotic. The restriction enzyme of choice for the initial digestion of the genomic DNA should cut outside of the integrated sequences and result in a high percentage of fragments of less than 5kb, to facilitate their transformation into the host bacterial cell. Bgl II and Xba I are both suitable enzymes for this purpose. However, in an experiment to rescue the integrated sequences in the genome of mosquito number 8, Hind III was used in error to digest the total genomic DNA. If the integration events were P element mediated, then the junctions of the pUChsneo sequences with the genomic DNA and the origin of replication would lie on different restriction enzyme fragments and so would not necessarily be co-rescued. Following transformation of the *E.coli* host MC1061 using the method of Hanahan (1985), no transformants were recovered from the selective ampicillin media. This could be due to a number of reasons including (i) the possibility that the pUChsneo sequences integrated into the genome do not include the origin of replication or the ampicillin resistance gene from the pUC8 region of pUChsneo; (ii) the transformation efficiency is too low to recover a single copy integration event. This may be improved by using electroporation to increase the efficiency of DNA uptake; (iii) restriction of the mosquito genomic DNA on introduction into the *E.coli* host. Use of a restriction$^-$ host such as DH5α may overcome this; (iv) the amount of DNA available for the plasmid rescue experiment (<500ng) may be too low, though in theory, this should be sufficient.

4:11 DISCUSSION

The putative G_0 germline integration event was unexpected in that it is only likely to be represented in the DNA of a relatively small number of cells and so should be difficult to detect. However, a somatic integration event, with sequences integrated into the chromosomal DNA of a more abundant cell type, may have occurred which could explain this result. If this integration was a precise P mediated event then, although this is not satisfactory in terms of the genetic modification of the mosquito in successive generations, it would provide evidence that the pUChsneo:pUChsπ(Δ2-3) transposition system may be operational within the mosquito genome. Although this integration event apparently occurred independently of the *white* region of DNA,

as expected in a P mediated transposition event, probing with other specific regions of the pUChsneo plasmid does not confirm this.

The internal 0.67kb Hind III fragment is absent, though this is perhaps not surprising given the small amount of DNA available for loading (<500ng), the relative inefficiency of transfer of small fragments and the long exposure times required before even the higher molecular weight bands can be detected. However, hybridization of the 3 highest molecular weight bands to the 4.13kb Hind III fragment probe is more difficult to explain. If the banding pattern represents a single, random integration of sequences within the 4.13kb fragment, a single band would be expected, representing the integrated sequences flanked by genomic DNA. If this is the case, then the multiple bands could represent partial digests of the genomic DNA. This would seem unlikely since the digestion of the DNA was carried out using excess enzyme for 3 hours and, following electrophoresis through a 0.9% gel and visualization using a U.V. light source, there was no apparent concentration of high molecular weight DNA as expected in a partial digest (Figure 4.4F). The 3.2kb band is detected in some other G_0 and G_1 putative transformed mosquitos showing no other banding pattern, and also in non-transformed control *Ae.aegypti* Bangkok DNA, as for the 2.85kb band in the G_1 and G_2 integrations. If the 3.2kb band and the 2.85kb band do represent sequences of homology in the Bangkok genome then it is likely that they represent identical sequences and that the slight difference in their sizes could be due to irregularities in electrophoresis such as uneven gel thickness. These bands are discussed in section 5:5 in the light of results presented in chapter 5.

A final consideration was whether the banding pattern in the G_0 fly represented regions of the free pUChsneo plasmid, independent of genomic DNA. However, the sizes of the 2 highest molecular weight fragments and their failure to hybridize to the 0.67kb fragment, the *white* locus region and the P inverted repeats, confirms that this is not the case.

In the case of the G_1 and G_2 integration events, the banding pattern is unlikely to correspond to that of a Hind III digest of the free pUChsneo plasmid since it is expected that any of the pUChsneo injected into the G_0 embryo should be degraded at this stage or would be diluted

out in subsequent generations. After probing the integrated DNA with specific regions of the pUChsneo plasmid, all of the fragments were found to hybridize only to the 4.13kb region, as for the G_0 integration event.

Hind III digestion of total genomic DNA with a single random integration event would give rise to a single band detected by the pUChsneo probe. This corresponds to the pattern obtained for the G_2 integration (having speculatively accounted for the 2.85kb band) but not for the G_1 integration. An alternative explanation of the G_1 integration event is that the 6.6kb band represents a partial digest of the lower molecular weight bands or that the 3 fragments represent multiple integrations of the 4.13kb fragment sequences. As in the case of the G_0 integration, partial digestion seems unlikely considering the digestion conditions and the distribution of the electrophoresed DNA (Figures 4.5F and 4.6F). However, the difference in intensity of the bands observed in the G_1 integration event could suggest partial digestion, since the pUChsneo probe appears to hybridize more strongly to the highest molecular weight fragment of 6.6kb than to the lower molecular weight fragments. This could though, be due to the nature of the random primed labelling reaction which leads to the hybridization of short, labelled fragments along the length of a homologous fragment, so that a higher proportion of radioactive label would be bound to a high molecular weight fragment than to a smaller fragment.

An alternative explanation for the appearance of multiple bands in the G_1 generation integration event is that a tandem array of a particular pUChsneo sequence is integrated into the genome. In transformation of D.melanogaster, Rubin and Spradling (1983) reported the integration of a tetramer, carrying 4 copies of the original recombinant P element present in the injected vector plasmid. Such a tetramer plasmid could be generated by recombination between molecules of the plasmid after injection into an embryo. The multimers could then integrate into the genome. Alternatively, a region of the pUChsneo plasmid could integrate randomly or by homologous recombination, followed by succesive events of homologous recombination between the integrated sequences and free pUChsneo plasmid. In order to detect multiple bands in a Hind III digest of a tandem repeat, the integrated sequence must include a Hind III site, in which case, some bands should be detected by probes containing either pUC8 sequences or P element

sequences. The possibility of integrated tandem repeats will also be considered in the discussion of the integration events described in sections 5:3 and 5.4.

If the 4.13kb fragment probe was in fact contaminated by pUChsneo sequences flanking this region i.e. it was not purified properly from the remainder of the plasmid, then of course, the results would be misleading. Probing a Hind III digest of pUChsneo with these Hind III fragment probes would have confirmed their purity though this was not done. However, in view of the fact that the 0.67kb fragment, the *white* sequence and the P inverted repeats do not hybridize to the same fragments as the 4.13kb fragment, then it seems unlikely that the fragments used as probes were contaminated by flanking pUChsneo sequences. The integrated 4.13kb sequences could include very small regions of flanking pUChsneo sequences which may not be detected by hybridization with the 0.67kb probe and a P element probe. Sequencing of the junction between integrated pUChsneo sequences and the genomic DNA would be necessary to determine this.

It would appear then that in each of the 3 cases - G_0, G_1 and G_2 - integration of sequences from the 4.13kb fragment of the pUChsneo plasmid has occurred with the exclusion of the remaining plasmid DNA. In view of the hybridization of non-transformed *Ae.aegypti* Bangkok total genomic DNA exclusively to the 4.13kb fragment, it was originally proposed that these integration events could be the result of homologous recombination between these regions of the mosquito genome and the pUChsneo plasmid. In the G_1 and G_2 generation, if the 6.6kb fragment does represent a complete digest of a single integration of the 4.13kb sequences then the identical sizes of the resulting fragments in the two unrelated mosquitos would suggest a precise, preferential site of integration, possibly mediated by this region of homology. However, the nature of this 3kb band is discussed further in section 5:5.

To test the hypothesis that the G_0, G_1 and G_2 integration events (figures 4.4, 4.5 and 4.6) are due to homologous recombination and are not P element mediated, it would be useful to perform the following experiments:-

(i) to introduce the pUChsneo vector plasmid into the mosquito embryo in the absence of the helper pUChsπ(Δ2-3) element which would normally provide the trans-acting transposase function. If the frequency of integration events remains similar to that obtained in the experiments reported in this chapter (1 in 125 embryos injected) it would suggest that the integration events are the result of non P element mediated recombination events. A lower frequency of integration would suggest that the pUChsπ(Δ2-3) plasmid plays a role in integration of pUChsneo sequences;

(ii) to investigate the nature of the recombination events i.e. to determine whether thay are random events or due to homology between the pUChsneo plasmid and the host genome, linear pUChsneo fragments could be introduced into the mosquito embryo. Orr-Weaver *et.al.* (1981) demonstrated in yeast, that by using linear rather than circular DNA molecules, the frequency of recombination between introduced plasmids and homologous host sequences can sometimes be increased. Following introduction of linear pUChsneo sequences into the mosquito embryo, a frequency of integration of those sequences which is higher than that observed in the introduction of the circular pUChsneo plasmid would suggest that homologous recombination may be occurring.

Using this strategy Rubin and Spradling (1983) report no evidence for homologous recombination of the P element with the D.melanogaster genome. Their attempts to transfer *rosy* genes carried on linear fragments were unsuccessful, regardless of whether the *rosy* gene was within a P element or not.

In transformation experiments with *Drosophila*, using the *rosy* gene as a dominant, selectable marker, 0.4 - 39% of G_1 flies showed the wild type *rosy*$^+$ phenotype (Rubin and Spradling, 1982). However, in the experiments described in this chapter, an average of only 2.3% of G_1 progeny survived the G418 assay compared to an average of 2.18% wild type survival in the same assays i.e. an increase in survival of only 0.12%. This suggests either a very low rate of transformation in the G_0 generation, instability of any integrated sequences from the G_0 to G_1 generations or failure of the G418 assay to select transformants. Since individual survivors of the assay do not necessarily carry detectable pUChsneo sequences (e.g. mosquitos 14, 21, 23,

38 and 43), it appears that G418 treatment is not accurate in its selection of transformants. Transformed progeny, expressing the *neo* gene at levels too low to render them resistant to the G418, could also have gone undetected.

If a G_1 or subsequent generation mosquito carries a single stable integration of pUChsneo sequences which includes the promoter, hsp-70, and the *neo* gene, approximately 50% of its progeny should display a resistance to G418. This prediction is based on normal Mendelian inheritance of the integration event but ignores aberrations such as position effects. A double integration event, involving both homologues in a diploid pole cell nucleus would result in up to 100% of the progeny displaying G418 resistance. Although the G_1 parent (mosquito number 14) of the G_2 mosquito (number 31) carrying pUChsneo sequences does give rise to apparently resistant progeny, their percentage survival falls far short of the expected 50%, and of the 5 selected progeny, only one appeared to carry integrated sequences. Similarly, the G_1 parent (mosquito number 14), itself selected on the basis of its G418 resistance, was also apparently devoid of integrated sequences, as were subsequent G_3 progeny (mosquitos number 38 and 43) from this line.

In these assays a relatively small number of potential transformants were subjected to selective media and the accurate interpretation of any results would require much larger sample sizes. Unfortunately these particular assays involved all of the progeny resulting from the relevant mosquitos. In future work the number of progeny was increased by replacing dead, male, wild-type flies with fresh male flies, in crosses, to ensure fertilization, and by repeating crosses of putative male transformants with fresh, wild-type females.

The loss of integrated DNA from one generation to the next is easily explained in terms of an unstable integration event so that foreign DNA is excised pre-meiotically from its germline chromosomal location and ultimately degraded. Post-meiotic excision of the integrated DNA would result in transformed progeny but at a lower frequency than predicted. However, the apparent loss or absence of integrated sequences from one generation (e.g. from mosquito number 14), which then reappear in the subsequent generation (e.g. G_2 mosquito number 31) is more complex. Such an event would have to involve the preferential loss of the integrated

sequence from a large proportion of nuclei in the early embryo with the exception of at least one nucleus giving rise to pole cells. The resulting adult could then carry copies of the integrated sequence in some germline cells and possibly in a very low proportion of somatic cells, depending on their origin from nuclei retaining the sequence. The relatively low number of copies of the integrated DNA would thus be very difficult to detect in the analysis of total genomic DNA. Of course, if the total genomic DNA of the G_1 parent (mosquito number 14) was degraded then any integrated sequences would also not be detected but in this case there was no apparent concentration of low molecular weight DNA following electrophoresis of the Hind III digestion and the amount loaded on the gel was similar to that loaded for the G_1 and G_2 flies in which integrated sequences were detected.

These three integration events - G_0, G_1 and G_2 - provide an insight into the behaviour of foreign DNA introduced into the developing embryo of the mosquito. In the light of these results, further transformants were sought so that any pattern in the nature of these events could be determined. Ideally, greater numbers of progeny from further generations needed to be studied so that any partial loss and reappearance of sequences, if indeed this occurs, could be distinguished from anomalies in the G418 assay. This work is reported in Chapter 5.

It is already apparent that the G418 resistance is not an ideal selectable marker for the identification of transformants. A much better selectable marker would involve a morphological mutation such as the *rosy* eye phenotype employed in *Drosophila* transformation experiments. This would have the added advantage of enabling partial marker expression to be detected. As discussed in Chapter 1.5 a suitable alternative marker gene was not available for use in *Ae.aegypti* so that the G418 resistance gene was the only available method of selection for further transformants. Preliminary work on the isolation of a marker gene from the mosquito genome is reported in chapter 5 (section 5:6).

144 of 155 individuals selected on the basis of their survival of the G418 assay, do not carry detectable pUChsneo sequences. This suggests that the integrated sequences detected in the remaining 11 individuals, may not be the cause of their G418 resistance. The observed

non-mendelian inheritance of resistance also supports this suggestion. However, in experiments with D.melanogaster, non-transformed individuals have also demonstrated "resistance" to G418. The basis of this resistance is not understood but could be a consequence of variation in uptake and utilization of G418, detoxification or metabolism of the drug or breakdown of G418 by associated micro-organisms. The role of the integrated sequences in determining resistance to G418 in putative transformants could be confirmed by assaying for phosphotransferase activity. However, the limited availability of material due to the low frequency of recovery of such transformants, means that this experiment would be impracticable at this stage.

The occasional occurrence of bands of approximately 3kb in mass DNA preparations of non-transformed individuals warrants further investigation. Additional bands of greater than 3kb and analagous to those observed in the putative transformants (Figures 4.4, 4.5 and 4.6) could be detected in non-transformed individuals. If these occur at a low frequency, a mass DNA preparation would mask their presence. Sequences homologous to the pUChsneo plasmid were detected in an average of 1 in 14 individuals selected on the basis of their resistance to G418. Based on the hypothesis that the bands observed in Figures 4.4, 4.5 and 4.6 are not due to transformation, there is a 95% probability that similar sequences would be detected in at least 1 in 40 non-transformed individuals. Genomic DNA from a minimum of 40 non-transformed individuals should therefore be screened with the pUChsneo plasmid to test this hypothesis.

Plasmid DNA arising from associated micro-organsims could be contributing the hybridizing bands seen in figures 4.4, 4.5, 4.6 and 4.7. To investigate this possibility, undigested DNA could be probed with the pUChsneo probe. Plasmid DNA would be detected as relatively low molecular weight DNA whilst integrated sequences would be located in the mass of high molecular weight chromosomal DNA. The fact that all of the bands in the putative transformants hybridize to the 4.13kb fragment of the pUChsneo probe, containing the pUC sequences, is consistent with them being derived from unrelated plasmid. However, as previously discussed, a random integration of pUChsneo sequences could result in the incorporation of pUC DNA into the genome which would then be detected in the analysis of DNA preparations. The amount of plasmid DNA which might be expected to arise from associated micro-organisms is unlikely

to account for all of the bands of the intensity observed in the putative transformants. The less intense bands, including those of approximately 3kb, detected in non-transformed and transformed individuals, are more likely to be due to plasmid DNA arising from associated micro-organisms. Reprobing filters with an actin probe would enable the relative amounts of DNA loaded from each individual to be determined and so would facilitate a quantitive analysis of the band intensities observed in putative transformants. This would be useful in estimating the relative copy number of integrated sequences.

Differences in methylation patterns of eukaryotic and prokaryotic DNA may be exploited to determine the nature of the observed bands. Insect DNA is methylated at a relatively low level in comparison with prokaryotic DNA (Rae and Steele, 1979, Adams et.al., 1979). The restriction enzyme Hpa II recognizes the base sequence CCGG only if this sequence is not methylated. Msp I also recognizes the base sequence CCGG, but in its methylated form. The banding pattern detected by the pUChsneo plasmid in Msp I or Hpa II digested genomic DNA would therefore vary with the relative level of DNA methylation. The presence and extent of such a difference would enable the detected DNA to be identified as prokaryotic (and therefore possibly arising from associated micro-organisms) or eukaryotic and therefore, presumably arising from the mosquito.

CHAPTER 5

ANALYSIS OF INTEGRATED SEQUENCES THROUGH TO THE G_4 GENERATION

5:1 INTRODUCTION

In view of the 3 integration events discussed in chapter 4, further transformants were sought and two transformed flies were detected. The DNA isolated from these individuals was not analysed as extensively as the G_0, G_1 and G_2 integration events previously discussed i.e. by reprobing with various regions of the pUChsneo plasmid, in an attempt to locate the region of this plasmid hybridizing to the integrated sequences. Instead, effort was focused on using the intact pUChsneo plasmid to probe larger numbers of progeny from the transformed G_1 parents through successive generations. This provided an insight into the fate of foreign DNA sequences integrated into the mosquito genome.

5:2 OPTIMIZATION OF THE G418 ASSAY

The total numbers of embryos injected over the course of the experiments discussed in this chapter are summarized in Table 5A. The 3 integration events discussed in chapter 4 arose from injected embryos at a frequency of approximately 1 in 125 embryos injected. Only 2 integration events were detected in the adults of cages 2 - 7 i.e. a frequency of only 1 in 2231 embryos injected. However, only one larva from cages 2-6 survived treatment with the G418 giving rise to a putative transformed adult (Table 5B). This low recovery of putative transformants is due in part to the attempts made to optimize the G418 assay for G_1 progeny of these cages, to make it simple, rapid and economical. These attempts included increasing the assay volume, increasing the length and frequency of the heat shock and increasing the length of time that

TABLE 5A SUMMARY OF EMBRYOS INJECTED IN CAGES 2-7

Cage Number	Embryos Injected	Larvae Hatch-ed	Adults	% Eggs to Larvae	% Larvae to Adults	% Eggs to Adults
2	426	88	45	20.7	51.1	10.6
3	367	60	23	16.3	38.3	6.3
4	232	42	24	18.1	57.1	10.3
5	1284	276	167	21.5	60.5	13
6	1152	67	37	5.8	55.2	3.2
7	1002	122	48	12.2	39.3	4.8
Total	4463	655	344	14.7	52.5	7.7

With the exception of embryos giving rise to cage 6, all embryos were injected with pUChsneo:pUChs (Δ2-3) at a ratio of 9:1 and a total concentration of 500μg/ml. Embryos giving rise to cage 6 were injected with pUChsneo:pUChsπ(Δ2-3) at a ratio of 5:1 and a total concentration of 300μg/ml.

The total number of G_0 and wild type mosquitos in each cage (30 x 30 x 30cm) ranged from 45 - 388.

TABLE 5B SUMMARY OF G_1 PROGENY ASSAYED FROM CAGES 2 - 7

	Cage Number	G_1 Progeny Assayed	G_1 Survivors Survivors	Total % G_1 Survival	Average % G_1 Survival*
	2	71	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	5	3634	1	0.03	0.03
	6	1175	0	0	0
	7	3366	24	0.71	2.04
Total		450	4	0.89	0.88

* % G_1 survival averaged from all assays

the larvae were left in the G418 solution. Increasing the length, frequency or temperature of the heat shock resulted in increased mortality in the control larvae which were not treated with G418. Increasing the assay volume resulted in 100% mortality of both treated control larvae and treated putative transformants. These assays were carried out using tap water which was not allowed to equilibrate to room temperature prior to addition of the larvae. The mortality of the larvae may have been due to the low temperature of the water or to its dissolved contents. Subsequent assays were performed using tap water which was allowed to equilibrate to room temperature for 2 hours or more, prior to use.

Large numbers of G_1 progeny from cages 2 and 3 did not hatch. This may have been due to premature drying of the embryos, prior to completion of the conditioning period. Bleaching of the endochorion of these embryos, using the method of Trpis (1970), revealed larvae in various stages of development. G_1 progeny from cage 4 were omitted from G418 assays in error. All G418 larvae from cage 7 were heat shocked in a humid oven at 37°C for 1 hour and returned to the insectaries for 2 hours prior to the addition of G418 as for the G_1 progeny from cage 1 and as described in section 4:2. G_1 embryos were not heat shocked. All other aspects of the assays of cage 7 progeny were performed as in section 3:5.

The embryos giving rise to the adults in cage 6 were injected with the pUChsneo and pUChsπ(Δ 2-3) plasmids at a ratio of 5:1 and at a total concentration of 300μg/ml. Although no putative transformants were recovered from this cage following the G418 assay of G_1 larvae, it would be incorrect to attribute this to the lower concentration and altered ratio of the 2 injected plasmids, since the standard G418 assay described in sections 3:5 and 4.2 was altered, as discussed, in the assay of cage 6 progeny.

A more accurate frequency of detection of integration events would be determined by considering only the progeny arising from cage 7. In this case the frequency of detection of integrated sequences is 1 in 501 embryos injected.

5:3 G_1 GENERATION GENOMIC DNA ANALYSIS

The pUChsneo probe hybridized to 6 bands in the HindIII digested total genomic DNA from a G_1 individual (number 50) (figure 5.1). A major difference between these bands and those of the transformed G_1 discussed in chapter 4, is the range of sizes of the bands. The bands in this case represent DNA fragments ranging from 26kb to 4.3kb, while those in the previously discussed G_1 fly ranged from only 6.6kb to 2.85kb. Another striking difference is the absence of any bands in the region of approximately 3kb since in chapter 4 it was proposed that a band of this size represented a region of homology of the Bangkok genome to the pUChsneo probe. This 3kb band was also absent from other G_0, G_1 and G_2 flies on the same filter. In the second G_1 individual (number 67) the pUChsneo probe detected a single band of 2.7kb (figure 5.2). This could represent the putative region of homology of pUChsneo to the Bangkok genome or it could represent an integration of pUChsneo sequences. In section 5:7 proposals as to the nature of these integration events will be put forward.

The G_1 mosquito numbered '50', arose from a G_0 male parent while the G_1 mosquito numbered '67' arose from a G_0 female parent so the 2 mosquitos could not be related.

5:4 GENOMIC DNA ANALYSIS AND G418 SELECTION OF PROGENY OF SUBSEQUENT GENERATIONS

A single G_2 generation mosquito was derived from G_1 mosquito number 50. Only 29 of the progeny of this mosquito (number 72) were assayed with G418 (Table 5C). However, since the G418 assay does not appear to be a completely successful method of selection, allowing the survival of some non-transformed mosquitos, and possibly eliminating transformed progeny, the remainder of the G_3 progeny from mosquito 72 were not assayed but were crossed to wild type Bangkok strain mosquitos. No further mosquitos were assayed with G418 throughout subsequent generations of this line. G_4 and G_5 generations were established and total genomic DNA isolated from the G_3 and G_4 mosquitos. Unfortunately, the G_2 mosquito, number 72, was

TABLE 5C SUMMARY OF G418 ASSAYS OF PROGENY RELATED TO THE G1 INDIVIDUALS CARRYING pUChsneo SEQUENCES

PARENT MOSQUITO	NUMBER ASSAYED	PROGENY % SURVIVAL	SURVIVING PROGENY MOSQUITOS
50:G1	35	2.9	72:G3
67:G1	303	2.3	99
			100
			101
			102
			106
			109
			131
			132
			133
			134
			135
			136
			137
			138
			148
72:G2	29	24.1	110
			111
			112
			113
			114
			142
			143

All numbered mosquitos were survivors of G418 assays. The remainder of the progeny from mosquito number 72 were not selected with G418 but crossed to wild type flies to give rise to the G_4 generation. These G_4 progeny were also not selected with G418.

lost during manipulation for oviposition, so that its genomic DNA could not be analysed. However, the G_3 generation was already established at this stage. On probing Hind III digested genomic DNA from G_3 progeny arising from mosquito number 72, 4 banding patterns were observed (Figures 5.3, (1) and (2), and 5.4, (1) and (2)). The loss of the G_2 mosquito in this line was particularly unfortunate since it would have been extremely valuable to note the sizes of Hind III fragments carrying integrated sequences, in this intermediate generation. No bands have yet been detected in genomic DNA isolated from G_4 generation flies of this line.

The 3kb band detected in the G_3 flies may represent the putative region of homology between the pUChsneo probe and the *Ae.aegypti* Bangkok genome, which was proposed in section 4:11 though its absence from other individual flies on the same filter makes this seem unlikely. However, the 4kb band detected in 3 G_3 individuals was observed in control Bangkok genomic DNA and in other flies from different lines indicating non-specific homology of the probe with respect to individual flies. This 4kb band was not detected in any DNA preparations loaded on the same filter, other than those isolated from *Ae.aegypti* Bangkok flies. These 4kb and 3kb bands will be considered further in section 5:5.

All G_2 progeny from G_1 parent number 67 were subjected to treatment with G418 and 15 were selected on the basis of their survival of the G418 assay (Table 5C). When the G_3 generation was established, total genomic DNA was isolated from the G_2 parents. Unfortunately, time did not permit the analysis of the G_3 generation progeny. The Hind III digested total genomic DNA from 13 of the G_2 progeny selected by the G418 assay, were probed with the labelled pUChsneo plasmid. Of these 13 progeny, the pUChsneo probe hybridized to DNA from 12 individuals. In 9 of these the probe hybridized to a single 4kb band as in the DNA from G_3 individuals arising from mosquito number 72. The 3 remaining banding patterns detected are shown in Figure 5.5 and 5.6.

These results indicate that the integration of pUChsneo sequences into the genome of G_1 adults is apparently unstable in subsequent generations of both of the lines studied. Possible mechanisms for the rearrangement of the genome in both of these lines, will be considered in

Figure 5.1

Southern transfer of Hind III digested genomic DNA from a G_1 transformed adult (number 50), probed with the labelled pUChsneo plasmid (1) and a 1.5kb Sal I fragment of plasmid pCs156 (2). Lane 3 shows the ethidium bromide stained gel prior to Southern transfer. Following hybridization, filters (1 and 2) were washed twice, for 30 minutes, in 2 x SSC, 0.1% SDS at either 65°C (1) or at 42°C (2). Autoradiography was at -70°C for either 4 weeks (1) or 8 days (2). Hybridization of the probe was also not detected in lane 2 after an exposure of 4 weeks (results not shown).

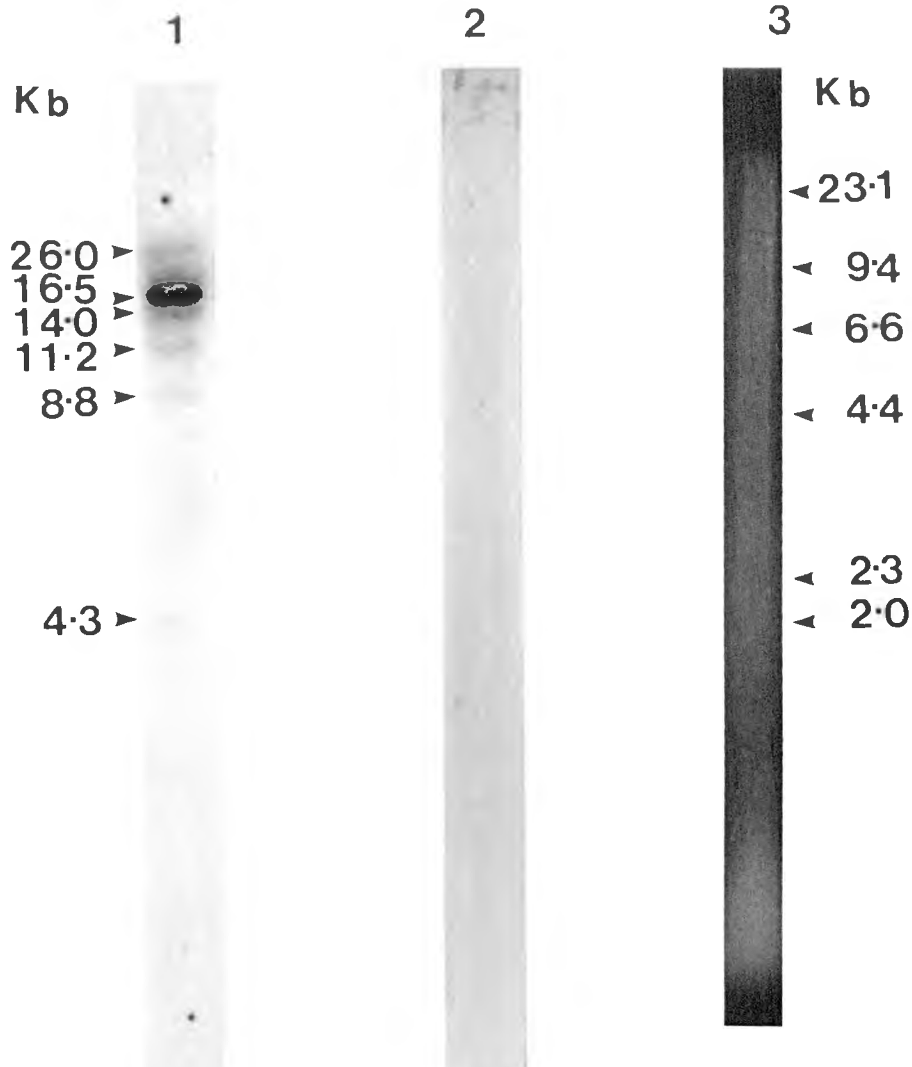

1
2
3
Kb
26·0
16·5
14·0
11·2
8·8
4·3
Kb
23·1
9·4
6·6
4·4
2·3
2·0

Figure 5.2

Southern transfer of Hind III digested genomic DNA from a G_1 transformed adult (number 67), probed with the labelled pUChsneo plasmid (1) and a 1.5kb Sal I fragment of plasmid pCs156 (2). Lane 3 shows the ethidium bromide stained gel prior to Southern transfer. Following hybridization, filters (1 and 2) were washed twice, for 30 minutes, in 2 x SSC, 0.1% SDS at 42°C and autoradiography was at -70°C for 4 weeks.

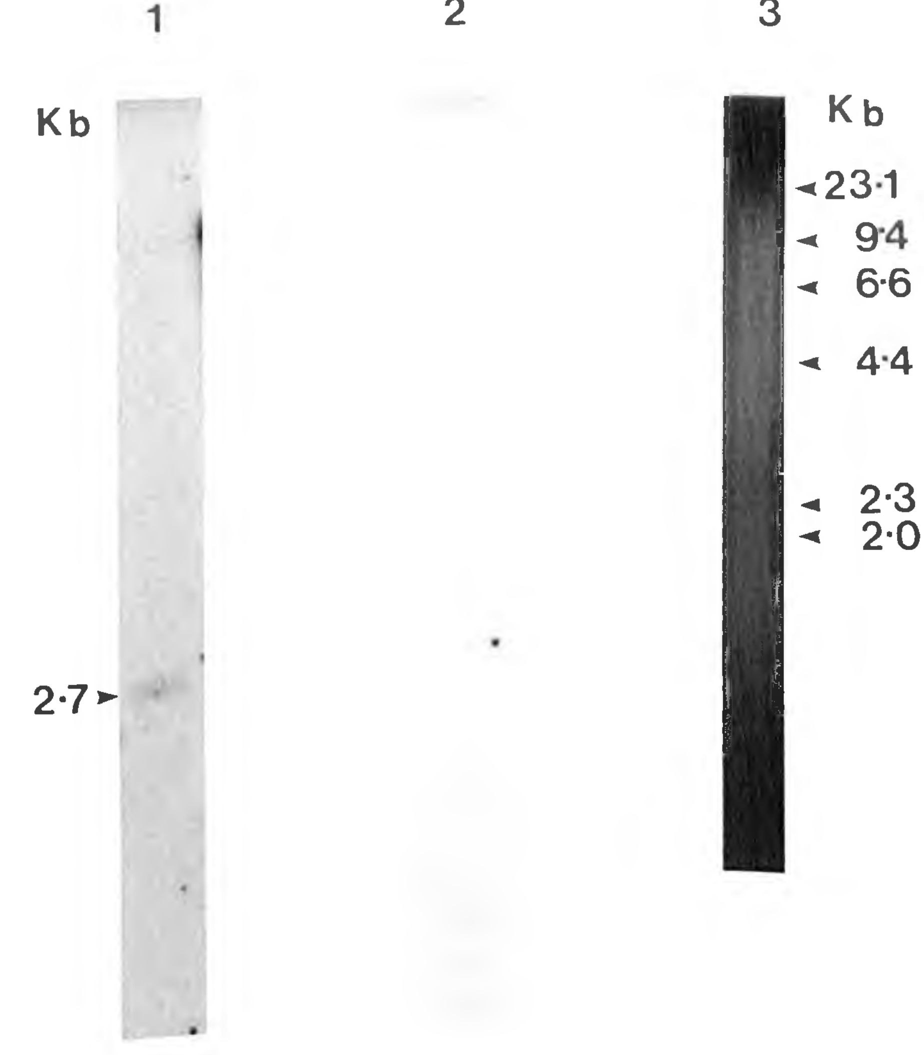

1
2
3
Kb
2·7
Kb
23·1
9·4
6·6
4·4
2·3
2·0

Figure 5.3

Southern transfer of Hind III digested genomic DNA from G_3 transformed adults descended from G1 mosquito number 50, probed with the labelled pUChsneo plasmid (1 and 2). Lanes 1i and 2i represent the corresponding ethidium bromide stained gels prior to Southern transfer. Following hybridization, filters were washed twice, for 30 minutes, in 2 x SSC, 0.1% SDS at 65°C. Autoradiography was at -70°C for 1 week.

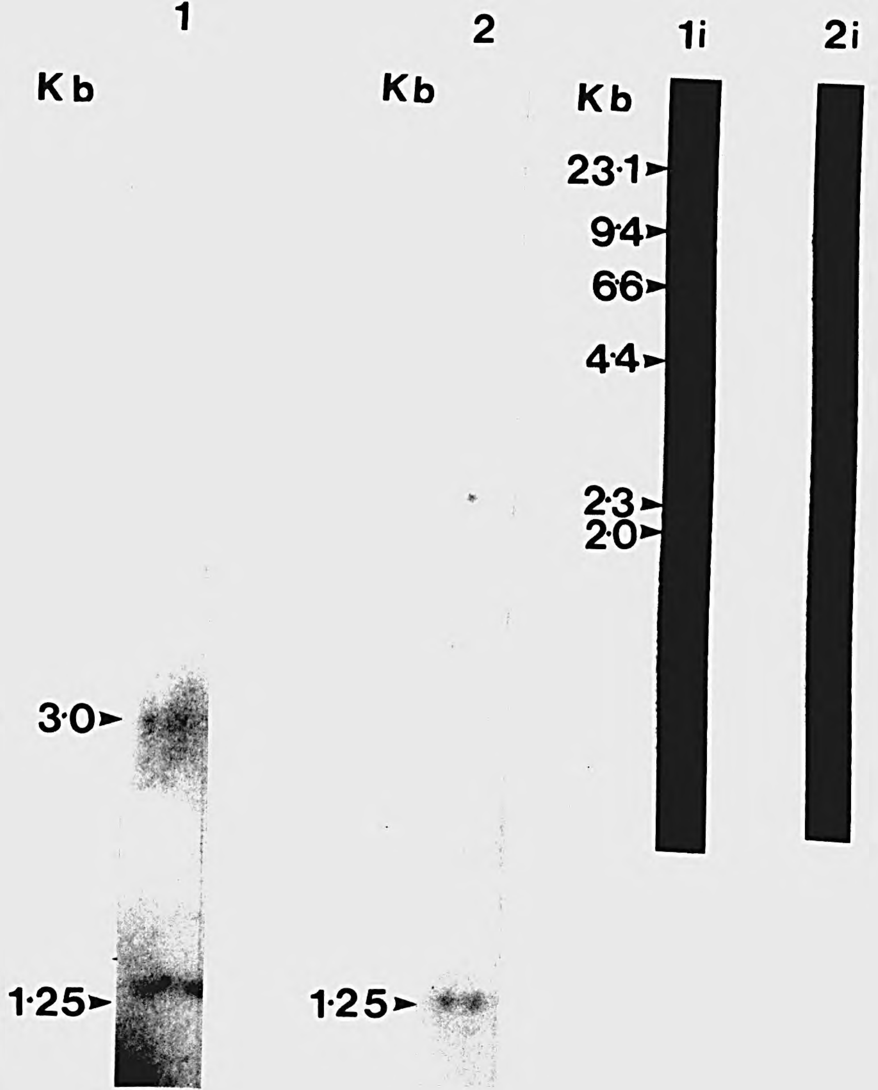
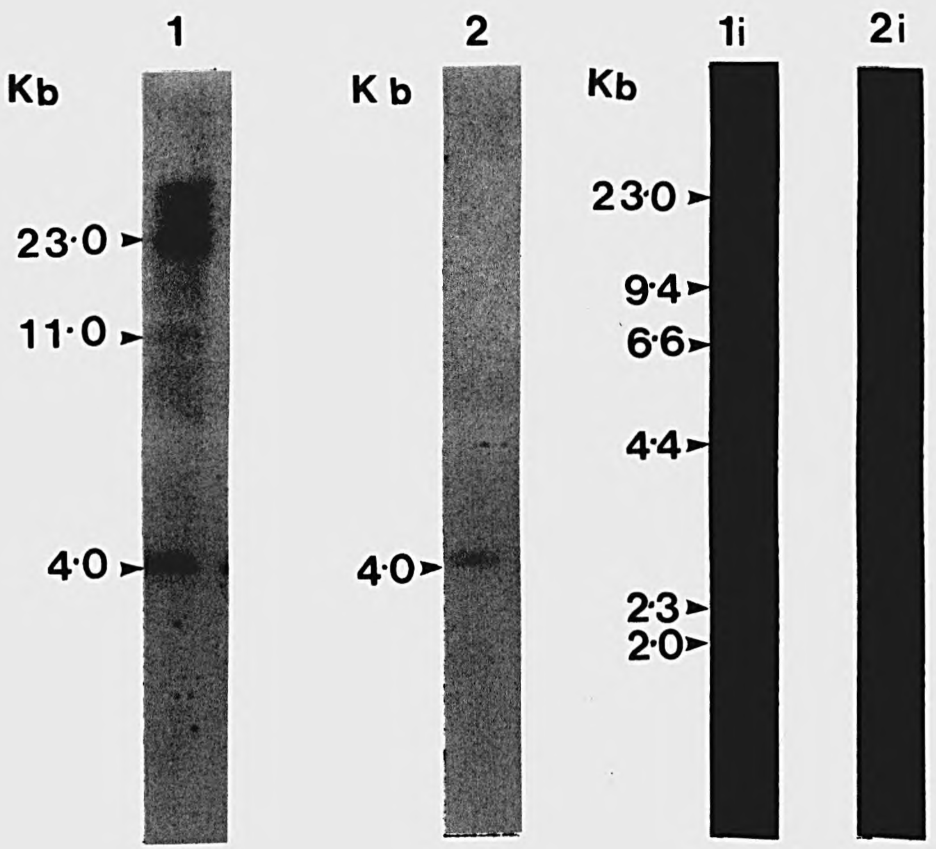

1
2
1i
2i
Kb
Kb
Kb
23·1
9·4
6·6
4·4
2·3
2·0
3·0
1·25
1·25

Figure 5.4

Southern transfer of Hind III digested genomic DNA from G_3 transformed adults descended from G_1 mosquito number 50, probed with the labelled pUChsneo plasmid (1 and 2). Lanes 1i and 2i represent the corresponding ethidium bromide stained gels prior to Southern transfer. Following hybridization, filters were washed twice for 30 minutes in 2 x SSC, 0.1% SDS at 72°C. Autoradiography was at -70°C for 2 weeks.



1
2
1i
2i
Kb
23·0
11·0
4·0
K b
4·0
Kb
23·0
9·4
6·6
4·4
2·3
2·0

Figure 5.5

Southern transfer of Hind III digested genomic DNA from a G_2 transformed adult descended from G_1 mosquito number 67, probed with the labelled pUChsneo plasmid (1). Lane 1i represents the corresponding ethidium bromide stained gel prior to Southern transfer. Following hybridization, the filter was washed twice, for 30 minutes, in 2 x SSC, 0.1% SDS at 42°C. Autoradiography was at -70°C for 2 weeks.

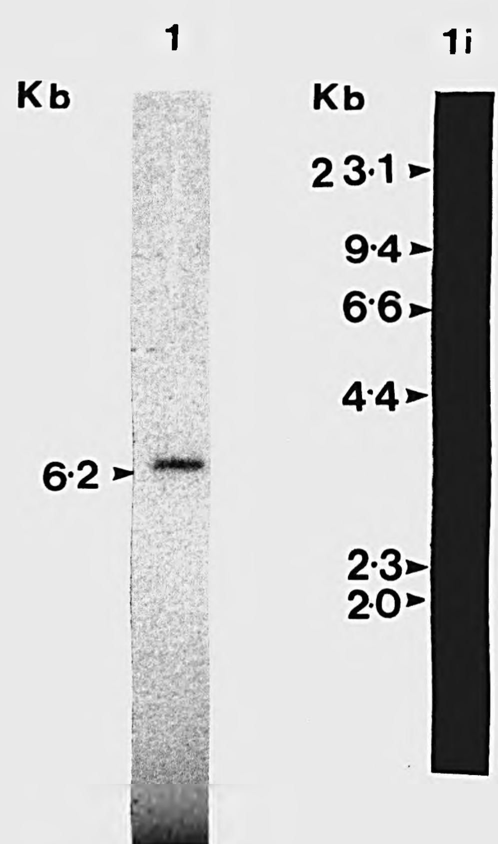
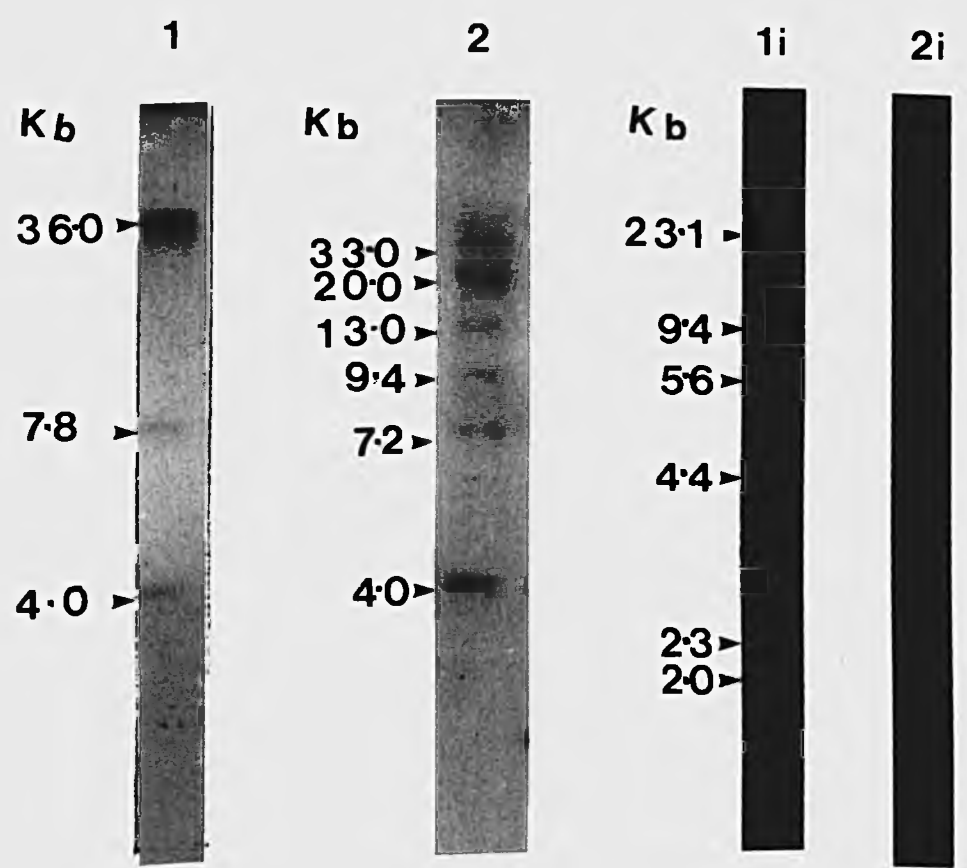

1
Kb
6·2
1i
Kb
23·1
9·4
6·6
4·4
2·3
2·0

Figure 5.6

Southern transfer of Hind III digested genomic DNA from G_2 transformed adults descended from G_1 mosquito number 67, probed with the labelled pUChsneo plasmid (1 and 2). Lanes 1i and 2i represent the corresponding ethidium bromide stained gels prior to Southern transfer. Following hybridization, filters were washed twice, for 30 minutes, in 2 x SSC, 0.1% SDS at 72°C. Autoradiography was at -70°C for 2 weeks.



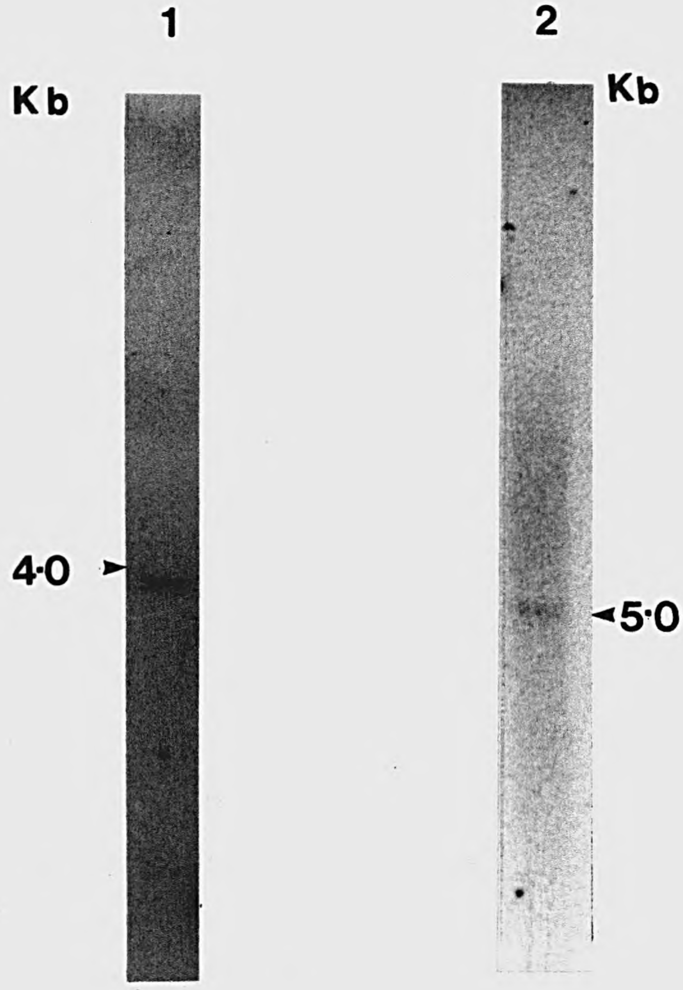
1
2
1i
2i
Kb
36·0
7·8
4·0
Kb
33·0
20·0
13·0
9·4
7·2
4·0
Kb
23·1
9·4
5·6
4·4
2·3
2·0

Figure 5.7

Southern transfer of Hind III digested (1) and Eco RI (2) digested genomic DNA from non-transformed *Ae.aegypti* Bangkok larvae (2) and adults (1) probed with the labelled pUChsneo plasmid. Following hybridization, the filters were washed twice, for 30 minutes, in 2 x SSC, 0.1% SDS at 42°C (2) or 72°C (1) and autoradiography was at -70°C for 9 days (2) or 2 weeks (1).

Figure 5.7

Southern transfer of Hind III digested (1) and Eco RI (2) digested genomic DNA from non-transformed *Ae.aegypti* Bangkok larvae (2) and adults (1) probed with the labelled pUChsneo plasmid. Following hybridization, the filters were washed twice, for 30 minutes, in 2 x SSC, 0.1% SDS at 42°C (2) or 72°C (1) and autoradiography was at -70°C for 9 days (2) or 2 weeks (1).


1
2
Kb
Kb
4·0
5·0

section 5:7.

5:5 FURTHER ANALYSIS OF THE PUTATIVE REGION OF HOMOLOGY BETWEEN THE pUChsneo PROBE AND THE AEDES AEGYPTI BANGKOK GENOME

In section 4:11, it was proposed that a region of the *Ae.aegypti* Bangkok genome, found on a Hind III fragment of approximately 3kb, was homologous to the pUChsneo probe. This proposal was based on the detection of bands of approximately 3kb in transformed and non-transformed flies. However, in the series of experiments discussed in this chapter, a 3kb band was detected only in 2 G_3 progeny in the line arising from mosquito number 50 and not in non-transformed Bangkok DNA. It was speculated that the 2.7kb fragment in the G_1 mosquito 67, corresponds to this region of homology, but the increase in the size of the band detected by the pUChsneo probe in subsequent G_2 generation flies, suggests either that a genomic rearrangement event leads to the relocation of the region of homology onto a larger Hind III fragment, or that this is not the case and the 2.7kb band does in fact represent integrated pUChsneo sequences. Hybridization of the pUChsneo probe to a 4kb band in Hind III digested genomic DNA isolated from non-transformed individuals (Figure 5.7 (1)), casts doubt on the theory of a region of homology since the size of such a region may be expected to remain constant in all Hind III digested genomic DNA isolated from *Ae.aegypti* Bangkok. Similarly, a region of homology should be detected in equal loadings of all transformed and non-transformed flies. Clearly, this is not the case, suggesting that the Hind III fragments of approximately 3kb (Figures 4.4, 4.5 and 4.6) or 4kb (Figures 5.4 and 5.6) are artefacts of particular experiments. Further evidence for the region of homology was obtained though, when a band of 5kb was detected by the pUChsneo probe in Bangkok total genomic DNA digested with Eco RI (Figure 5.7 (2)).

An attempt was made to isolate any putative region of homology from the Bangkok genome by screening an Eco RI Bangkok total genomic DNA library in a λgtwes vector (prepard by J.M. Crampton) with the pUChsneo probe. Original attempts were hampered by cross hybridization of the pUChsneo probe to all of the library clones screened. Previous screening of a Bangkok

library with a number of cloned DNA's in pUC vectors showed no problem of hybridization between the pUC plasmids and λgtwes (J.M. Crampton unpublished data), so that the cross hybridization of the pUChsneo plasmid was thought to be unlikely to be due to its pUC8 region. In an attempt to block hybridization of the pUChsneo plasmid to regions of the gtwes vector DNA, subsequent hybridizations were performed using unlabelled λgtwes DNA at a concentration of 5μg/ml, along with the radiolabelled pUChsneo probe. Again, the probe apparently hybridized to all of the library clones. To overcome this problem, the library was rescreened using an Xho I/Pst I restriction enzyme fragment of the pUChsneo plasmid as a probe, along with 5 μg/ml unlabelled λgtwes vector DNA. This fragment of the pUChsneo plasmid represents 276bp of the hsp-70 region. The probe was prepared and labelled and the library screened in this instance, by Mr. Iain Comley of this laboratory. 3 clones were detected in this primary screening, suggesting that the hsp-70 region may be responsible for the homology of the pUChsneo probe to *Ae.aegypti* Bangkok total genomic DNA. Secondary screening and sequencing of detected clones must be carried out to confirm this result. Other regions of the pUChsneo probe should also be isolated for use as probes to detect any homology to the remainder of the plasmid. Having identified homologous regions of pUChsneo, they could be used to probe genomic DNA isolated from other *Ae.aegypti* strains to determine any strain specificity of the homology. However, a region of genomic DNA homologous to the probe would be expected to hybridize to this probe at all times when probing under identical conditions but this is not the case in the experiments performed as part of this thesis. This indicates that the putative region of homology represented by the hsp-70 sequences is in itself not a satisfactory explanation for the bands of approximately 3kb and 4kb, found in non-transformed individuals. Alternative explanations for the existence of the 3 and 4 kb bands were considered.

Repetitive sequences of genomic DNA carrying Hind III sites may be visible as distinct bands in Hind III digested DNA stained with ethidium bromide and viewed over U.V. light, due to the relatively high density of identical size fragments representing this region. Any non-specific binding of the probe to genomic DNA may effectively be concentrated at this position and so not fully removed by a medium stringency wash (2 x SSC, 42°C). However, none of the bands

observed in ethidium bromide stained digests of Bangkok genomic DNA, and thought to represent repetitive sequences, were equivalent to 3kb or 4kb. Binding of the pUChsneo probe to the 3 and 4kb bands was still apparent after washes at 2 x SSC, 65°C (3kb) and at 2 x SSC, 72°C (4kb) suggesting that non-specific binding to repetitive sequences is also not a satisfactory explanation for the detection of these bands in non-transformed individuals. The bands detected by the pUChsneo probe are yet to be satisfactorily explained.

5:6 ISOLATION OF MOSQUITO GENOMIC SEQUENCES HOMOLOGOUS TO THE WHITE LOCUS OF DROSOPHILA

Since the *neo* gene coding for G418 resistance is not an ideal selectable marker in transformation experiments with *Ae.aegypti* Bangkok, alternative marker genes were sought. A phenotypic marker was considered to be most suitable, enabling partial expression to be detected, and so giving some insight into the nature of the integration event. For example, integration of an incomplete DNA vector element may result in reduced expression of the marker. Similarly, integration of the intact vector element may only lead to incomplete expression in the event of its integration at an unfortuitous site in the genome. Interpretation of data from DNA analysis of integration events may be advanced by this consideration of the nature of expression of a phenotypic marker.

Although the biochemical eye pigment pathway in mosquitos has not been well characterized, it is thought to involve pathways similar to those in *Drosophila*, so that a wild type eye colour gene isolated from *Drosophila* may complement a corresponding eye colour mutant in the mosquito. In contemplating experiments of this type, the problem arises in distinguishing failure to complement the mutant phenotype, from failure of the introduced DNA to integrate into the mosquito genome, both of which would be manifested in the maintenance of the mutant phenotype. In transformation experiments using *D.melanogaster*, expression of the phenotypic marker gene, *rosy*, is often detected in G_0 adults (section 4:1). On the basis of this and since non-integrated vector plasmid has been detected in the genomic DNA of G_0 adults, it would be

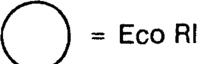
interesting to introduce functional marker genes into the genome of corresponding mutants of aedine mosquitos by micro-injection. Expression of either the integrated or non-integrated gene may restore the wild type phenotype in G_0 adults. The *white* eye locus from *Drosophila* may be a suitable candidate for this experiment.

The plasmid pW8 (Klemenz *et.al*, 1987) was originally thought to be ideal for introduction into the mosquito embryo. This plasmid carries a truncated version of the *white* gene linked to the promoter hsp-70, is readily expressed in *Drosophila* and is a good selectable marker for transformation. However, in transformation experiments with *D.melanogaster*, expression of the *white* gene was not detected in G_0 adults, unlike the *rosy* gene in similar experiments. A *white* eye mutant strain, *Aedes cooki*, was chosen as a candidate for the complementation experiment. Unfortunately, due to the weak nature of the particular stock available, insufficient embryos were obtained to attempt this experiment.

In an alternative approach to this problem, a region of the *white* locus in *Drosophila* was used in an attempt to isolate the homologous eye colour locus from the *Ae.aegypti* Bangkok genome. A clone had previously been isolated from a genomic DNA library of *Ae.aegypti* Bangkok (P.Eggleston, unpublished results), on the basis of its homology to the plasmid pCs155, representing a 0.8kb Sal I fragment from the coding region of the *white* locus of *Drosophila* (Sang *et.al.*, 1984). This clone, pBW1, was mapped using restriction enzyme digests, as part of this thesis and the resulting map of the genomic DNA insert is shown in Figure 5.8. Sequence data from this and overlapping clones from the genomic DNA library should be obtained and compared to the *white* locus sequence of *Drosophila* to determine whether this region of mosquito genomic DNA does in fact code for a step in the biochemical eye pigment pathway. Since work presented in this thesis has now established the technology for introduction of DNA into the mosquito genome, complementation analysis, involving introduction of the putative mosquito *white* sequences into the genome of eye colour mutants, and independent of their expression in G_0 adults, may be a viable prospect.

Figure 5.8

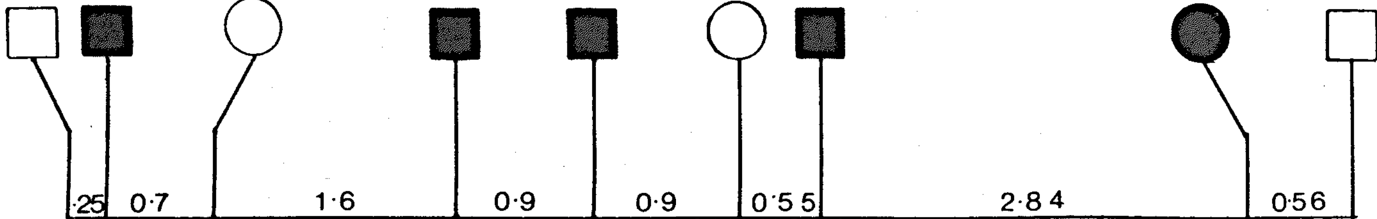
Restriction enzyme map of *Ae.aegypti* genomic DNA sequences found on a gtwes genomic library clone, selected on the basis of its homology to a 0.8kb Sal I fragment from the *white* locus of *Drosophila*.



= Pst I

= Hind III

= Hinc II



·25
0·7
1·6
0·9
0·9
0·5 5
2·8 4
0·56
1·0 Kb

5:7 DISCUSSION

The G_1 integration event in mosquito number 50 is difficult to interpret on the basis of the banding pattern observed using the intact pUChsneo plasmid as a probe. As for the G_1 integration event discussed in chapter 4, the banding pattern in this mosquito is unlikely to be due to a partial digestion of the genomic DNA since the DNA was digested as described in section 4:11 and no concentration of high molecular weight DNA was observed following electrophoresis of the digested DNA (Figure 5.1 (3)).

The size of the bands detected by the pUChsneo probe in mosquito number 50, do not correspond to those expected for a P element mediated transposition, though the *white* locus sequences are apparently excluded. Further analysis of this integration event is essential to determine its precise nature. Preliminary investigation of this event could include reprobing the Hind III digest in Figure 5.1 (1) with different regions of the pUChsneo probe, as in chapter 4. However, the only conclusive way to determine the precise nature of the event would be to recover the integrated pUChsneo sequences and their flanking genomic DNA from the total genomic DNA for sequence analysis. Following the failure of the plasmid rescue method reported in section 4:10, alternative methods were considered for the recovery or amplification of the integrated sequences. In the plasmid rescue experiment, absence of an origin of replication on the 'rescued' plasmid, would effectively lead to loss of the DNA on transformation into *E.coli*. To overcome this problem, the circular DNA could be amplified in vitro using the polymerase chain reaction (PCR) (Mullis and Faloona, 1987). However, PCR requires 2 oligonucleotides complementary to opposite ends and strands of the sequence to be amplified, which act as primers for the PCR reaction. In the amplification of pUChsneo sequences carried on a restriction enzyme digest fragment of the genome, a problem arises in the construction of these 2 oligonucleotides since the genomic DNA terminals of the fragment would be unknown. However, since the fragment would be circularized in the ligation step of the plasmid rescue, oligonucleotides complementary to the pUChsneo sequences could be constructed. The reaction would then be primed to start within the pUChsneo integrated sequences and continue through the flanking genomic sequences.

Mullis and Faloona (1987) successfully amplified DNA segments from 24-100bp in length, but found that amplification of sequences greater than 200bp led to considerable production of DNA fragments other than that intended, due to mispriming events whereby the 3' end of one of the primers interacts with a region of partial homology within the region to be amplified. Considering the range of sizes of the Hind III fragments detected by the pUChsneo probe in the genomic DNA of mosquito number 50 (26 - 4.3kb) and the size of the pUChsneo plasmid itself, it seems unlikely that PCR would prove to be such a valuable tool in the amplification of integrated pUChsneo sequences. The two enzymes of choice to cut the genomic DNA, Bgl II or Xba I (section 4:10) both cut genomic DNA less frequently than Hind III so that digestion with one of these 2 enzymes is likely to produce genomic fragments carrying the integrated pUChsneo sequences, which are larger than those obtained with a Hind III digest. Amplification of such large fragments could lead to the production of misleading by-products with a low specificity.

Once the sequence of the integrated DNA in the G_1 generation has been determined, recovery and analysis of integrated sequences in subsequent generations should elucidate the nature of genome rearrangements involving this foreign DNA. As discussed in section 4:10 and in this chapter, integration events and their flanking genomic sequences in G_1 or subsequent generations have not been recovered from total genomic DNA at this stage but possible mechanisms of rearrangement which could lead to the observed banding patterns, have been considered.

Initially, the effect of introduction of P sequences along with a functional transposase gene into the mosquito embryo was considered, in terms of their effect on the mosquito genome. In *Drosophila*, P elements cause mutations by means of excision and insertion events and chromosomal rearrangements, (Engels and Preston, 1981). A single, autonomous P element introduced into the *Drosophila* embryo by micro-injection and integrated into an M strain genome remains in its active state i.e. it will transpose and excise from its original integrated position (Karess and Rubin, 1984). The advantage of the 'vector-helper' system used in these

mosquito transformation experiments is that the integrated P vector element (pUChsneo) is non-autonomous and requires the functional transposase activity in trans from the helper element (pUChsπ[Δ2-3]). The defective inverted repeats of the helper element ensure that it is not integrated into the genome itself so that any integrations of the non-autonomous P element should remain stable in subsequent generations as the helper element is effectively diluted out. For this reason, any integration of the pUChsneo P element sequences into the mosquito genome, should not be mobilized by P transposition in subsequent generations.

A further consideration in the introduction of a functional transposase into the mosquito embryo, is its effect on any endogenous mobile elements in the mosquito genome. A number of reports have been published of cross mobilization of other transposable elements (*mdg 1, mdg 2, mdg 3,copia* and *FB* elements) in the genome of *Drosophila*, in response to P-M hybrid dysgenesis (e.g. Gerasimova *et.al.*, 1984, Lewis and Brookfield, 1987, Biemont *et.al.*, 1987). However, a more recent report by Eggleston *et.al.* (1988) states that P-M hybrid dysgenesis does not increase the transpositional activity of other families of transposable elements in *D.melanogaster*. On the basis of this, it seems unlikely that transposase function could mobilize endogenous mosquito transposable elements. However, if this did in fact occur initial genomic rearrangements should be stabilized on removal of the *trans* acting helper element in subsequent generations so that in analysis of only $>G_1$ generations such mobilization would remain undetected.

In the absence of P mediated genomic rearrangements, what are the mechanisms which could result in the observed banding patterns? The mobilization of *copia* elements reported by Gerasimova *et.al.*, (1984) and by Biemont *et.al.*, (1987) was proposed to be the result of transposition i.e homology independent insertion of transposon sequences into a target site. However, in response to this idea, Boeke (1988) pointed out that the transposition of the *copia* element is a replicative process and appearance of a new copy does not require the loss of the donor copy so that the excisions observed may be due to a homology dependent recombination process involving the LTR's of the element. Such LTR-LTR recombination is frequent in yeast *Ty* elements and has also been observed with *copia* (Zachar *et.al.*, 1985). Recombination

between any pair of direct repeats may delete the material between them and leave a single copy of the originally repeated sequence in the genome (Figure 5.9a). Recombination between inverted repeats would result in the inversion of the intervening sequences. If pUChsneo sequences were integrated into the genome in tandem arrays, or as multiple copies on a single chromosome, these direct repeat sequences could serve as recombination sites leading to the loss of either intervening tandem repeats or intervening chromosomal sequences (Figure 5.9b). Recombination between the inverted repeats of integrated pUChsneo P sequences could result in inversion of the intervening pUChsneo sequences leading to loss of the asymmetrical Hind III sites. It seems unlikely that the P element inverted repeats are involved in the integration events detected in this work though any pUChsneo sequences integrated more than once and in reverse orientation on the same chromosome, could act as inverted repeats, leading to inversion.

In the G_3 generation of the line arising from mosquito number 50, the reduction in size of the Hind III fragments hybridizing to the pUChsneo probe could be explained by excisions due to recombination events between multiple direct repeats of integrated sequences throughout the genome or between tandem arrays of an integrated sequence. Only one Hind III fragment hybridizes to the pUChsneo probe in the genomic DNA from G_1 mosquito number 67. This suggests either a single integration of a pUChsneo sequence, a series of tandem repeats on a single Hind III fragment or a series of integration events in a region of repetitive DNA carrying a Hind III site at regular intervals. The increase in size of the Hind III fragment detected by the pUChsneo probe in the genomic DNA of G_2 mosquitos from this line, suggests either an amplification of a sequence within the Hind III fragment, the relocation of the original integrated sequence onto a larger Hind III fragment or loss of a Hind III site. Such amplification may occur as a result of unequal crossing over due to the mis-alignment of tandem repeats resulting in a reciprocal addition and deletion of the repeated sequence (Figure 5.9c). However, this mechanism relies on more than one copy of a recombinogenic sequence per molecule which seems unlikely in the case of the genome of mosquito number 67, given that only one Hind III fragment is detected by the pUChsneo probe. Hind III sites could be lost by inversion of intervening regions between inverted repeat sequences, which could also account for the

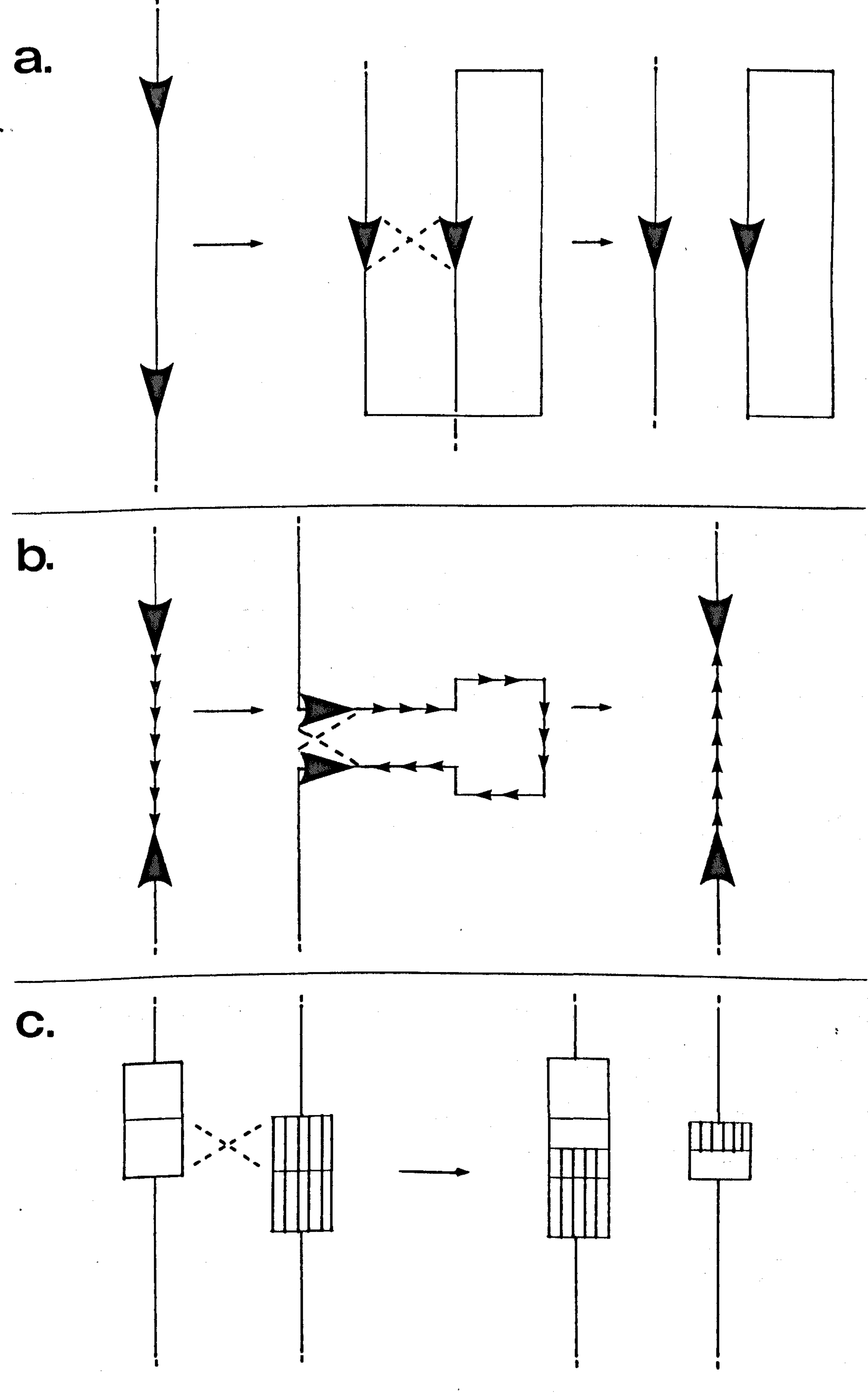
Figure 5.9

Possible mechanisms of genomic rearrangement which could account for the banding patterns observed in figures 5.1, 5.2, 5.3, 5.4, 5.5 and 5.6.

a. Intramolecular recombination between two direct repeat sequences resulting in excision of the intervening region.

b. Intramolecular recombination between two inverted repeat sequences resulting in inversion of the intervening region.

c. Intermolecular recombination between two sets of mis-aligned tandem repeats, resulting in reciprocal duplication and deletion of the repeat sequences. Intermolecular mis-alignment of direct repeats with intervening genomic sequences would result in reciprocal duplication and deletion involving the genomic sequences, and hence may have more severe effects in terms of mutagenesis.


a.
b.
c.

increase in size of the Hind III fragment detected in the G_2 generation.

Of course, any rearrangements of the integrated sequences within the genome may occur independently of the nature of the foreign DNA, rather, they may be consequences of recombination and rearrangement of endogenous repetitive sequences in the mosquito genome. Such mutations give rise to variation in DNA content and play a major role in determining the structure and sequence composition of complex eukaryotic genomes and their evolutionary success. Finally, the possibility cannot be overlooked that integrated sequences and endogenous repetitive sequences interact to produce chromosomal rearrangements akin to those arising from introduction of P element sequences into a locus where a P element already exists, at or near the new site of integration (Engels and Preston, 1981).

Clearly then, no accurate interpretations of the observed results can be made without more detailed sequence analysis of the transformed genomes, though there is no direct evidence that the integration events are mediated by the P element sequences on the pUChsneo plasmid, or that subsequent mobilization of the integrated sequences is P mediated. Determination of the precise sequences and the genomic location of the initial integration events could reveal a preferential region of integration, mediated by homology between pUChsneo and the genome of *Ae.aegypti*. Such a region may prove to be useful in the construction of alternative transformation vectors. Integration of pUChsneo sequences into the genome in a totally random fashion, which are then mobilized as a consequence of endogenous genome rearrangement would make the possibility of using the pUChsneo plasmid as a transformation vector, seem unlikely. However, mutations arising from rearrangements of the integrated pUChsneo sequences and as a direct consequence of their presence in the genome, may prove to be more useful. If preferential sites of integration were determined and repeated patterns of sequence rearrangements observed, the fate of integrated sequences could potentially be predicted and the ability to effect specific mutations utilized in insect control programmes.

Use of a transformation vector in the germline manipulation of the genome requires that the integrated sequence be stable in the genome so that genome rearrangements induced by the

integration of pUChsneo sequences must be controlled in some way. Characterization of any useful mutations induced by the integration of pUChsneo sequences must therefore, be followed by the development of a mechanism whereby the action of the integrated sequences is limited in subsequent generations. The instability of the integrated sequences detected in these experiments indicates that P element transposition as a basis for transformation in *Ae.aegypti* is currently questionable.

Perhaps a more realistic, short term prospect for the use of integrations of the pUChsneo plasmid would be in 'transposon tagging' (section 1:6). Selectable mutations, arising as a result of pUChsneo integration could be analyzed at the level of their genomic DNA. The mutated gene or part of the gene could be recovered from a library representing the mutant genome, on the basis of its homology to the pUChsneo plasmid. Genes isolated in this way could be used as markers in subsequent transformation experiments or as targets for genome manipulation employed in control programmes.

CHAPTER 6

ROLE OF THE P ENCODED TRANSPOSASE PROTEIN IN INTEGRATION OF pUChsneo SEQUENCES

6:1 INTRODUCTION

A total of 5 integration events have been detected (Figures 4.4, 4.5, 4.6, 5.1 and 5.2) in this work, though no direct evidence that any of these events were P mediated has yet been reported. This chapter considers attempts made to obtain such evidence.

P transposition in the genome of *Drosophila melanogaster* is dependent on the activity of the functional transposase protein. To determine whether this transposase protein played a role in the integration of pUChsneo sequences into the mosquito genome, the expression of the transposase protein in the mosquito embryo was investigated and an assay for a known function of the transposase protein was carried out. If a functional transposase is produced from the coding region of the pUChsπ(Δ2-3) plasmid in the mosquito cell, then it should be possible to identify primary, and possibly processed, transcripts from total RNA isolated from embryos injected with pUChsπ(Δ2-3). Any block in the transcription, processing or translation of the transposase message would prevent the production of the functional transposase protein.

Mutations involving the insertion of a P element may revert to the wild type phenotype by precise excision of the P element sequence itself plus one of the 8bp duplications, thus restoring the original wild type sequence (O'Hare and Rubin, 1983). This excision event is dependent on transposase activity. Using an assay for this P element function, devised by Rio *et. al.* (1986) for use in transformed *Drosophila* tissue culture cells, the ability of any transposase protein produced from the pUChsπ(Δ2-3) plasmid, to excise the P element from a known DNA sequence,

can be measured in the mosquito cell.

RESULTS

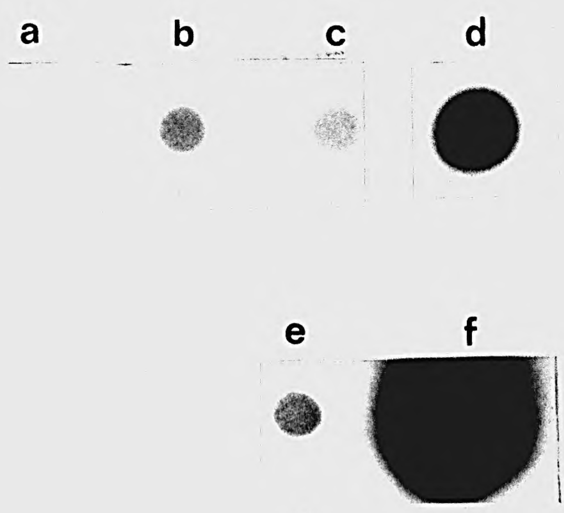
6:2 ISOLATION OF RNA FROM EMBRYOS INJECTED WITH THE pUChsπ(2-3) PLASMID

Ae.aegypti Bangkok embryos were injected (as in section 3:3) with the pUChsπ(Δ2-3) plasmid at a concentration of 500μg/ml in injection buffer. The embryos were returned to the insectaries for 18 hours after removing from halocarbon oil and placing on moist filter paper. The embryos were then heat shocked in a humid incubator for 1 hour at 37°C before returning to the insectaries for a further 2 hours (The transcriptional activity of the heat shock promoter, hsp 70, has been demonstrated in the mosquito *Anopheles gambiae* (Miller *et.al.*, 1987) and in *Anastrepha suspensa* of the family Tephritidae (O'Brochta and Handler, 1988), though not yet in the Aedine mosquito.)

Total RNA was isolated from the injected embryo using the methods of either O'Hare *et. al.* (1983) or Chomczynski and Sacchi (1986). Due to the low yield of RNA from these embryos, Northern analysis was not performed and dot blot analysis was the method of choice in determining the presence or absence of pUChsπ(Δ2-3) transcripts. Unfortunately, all RNA preparations were thought to be contaminated with DNA so that the original pUChsπ(Δ2-3) plasmid injected may have been recovered along with the RNA and results obtained were misleading (Figure 6.1). As a result of this, no firm conclusions could be drawn as to whether the P element sequences of the pUChsπ(Δ2-3) plasmid are transcribed in the mosquito embryo.

Figure 6.1

DNase treated RNA isolated from *Ae.aegypti* Bangkok embryos previously injected with the pUChsπ(Δ2-3) plasmid. The labelled pUChsπ(Δ2-3) plasmid was used as a probe. b and c represent DNase treated RNA isolated from 188 and 100 embryos respectively. a represents total RNA isolated from *Ae.aegypti* Bangkok adults. d corresponds to untreated RNA isolated from 170 embryos injected with the pUChsπ(Δ2-3) plasmid. 4.35ng of pUChsπ(Δ2-3) plasmid were treated with DNase and are shown against 4.35ng of untreated control plasmid (e and f respectively). This quantity of plasmid was calculated as the maximum amount recoverable from 100 embryos injected with DNA at a concentration of 0.435mg/ml, assuming an injection volume of 100pl per embryo.


a
b
c
d
e
f

6:3 <u>IN VITRO</u> TRANSCRIPTION OF THE P ELEMENT CODING REGION TO INVESTIGATE TRANSCRIPT PROCESSING

To investigate the mechanism of processing of transposase transcripts in the mosquito cell, *in vitro* transcription could be used to produce trancripts which may be introduced into the developing embryo, recovered and analysed by means of RNase protection analysis. This method involves the production of a full length transposase transcript by *in vitro* transcription from an SP6 or T7 `phage promoter, followed by injection of this transcript into the mosquito embryo. Total RNA recovered from these embryos could be hybridized to labelled, complementary, full length transcripts, also produced by *in vitro* transcription, and then treated with RNase in conditions such that any single stranded RNA is degraded. The denatured protected fragments could then be analysed by polyacrylamide gel electrophoresis and autoradiography. Splicing of transcripts introduced into the mosquito embryo would result in the formation of loops when these transcripts are hybridized to complementary transcripts. The unprotected sequences making up the loop structure will be degraded on treatment with RNase. Denaturation of the RNA hybrids would therefore result in labelled fragments representing the spliced complementary strand, the sizes of which would correspond to the number and magnitude of splicing events occurring in the transcript originally introduced into the mosquito embryo.

In an attempt to obtain full length transcripts of the transposase coding region by *in vitro* transcription from the `phage T7 promoter, a 3.9kb BamHI-XbaI fragment from pUChsπ(2-3), carrying the P element transposase coding region lacking the third intron, was cloned into the plasmid pSPT19 (obtained from Boehringer). This plasmid carries both SP6 and T7 promoters, diametrically opposed and separated by a multiple cloning site. 0.5μg of the recombinant plasmid was linearized by digestion with the restriction endonuclease Bam HI and ethanol precipitated. The pellet was resuspended in 9.5μl of T7 transcription incubation buffer (40mmol/l Tris/Cl, 6mmol/l MgCl2, 10mmol/l DDT, 4mmol/l spermidine and 0.4mmol/l each of ATP, GTP, CTP and UTP, at pH 7.2). 0.5μl of T7 RNA polymerase (10u/μl) was added to the reaction and

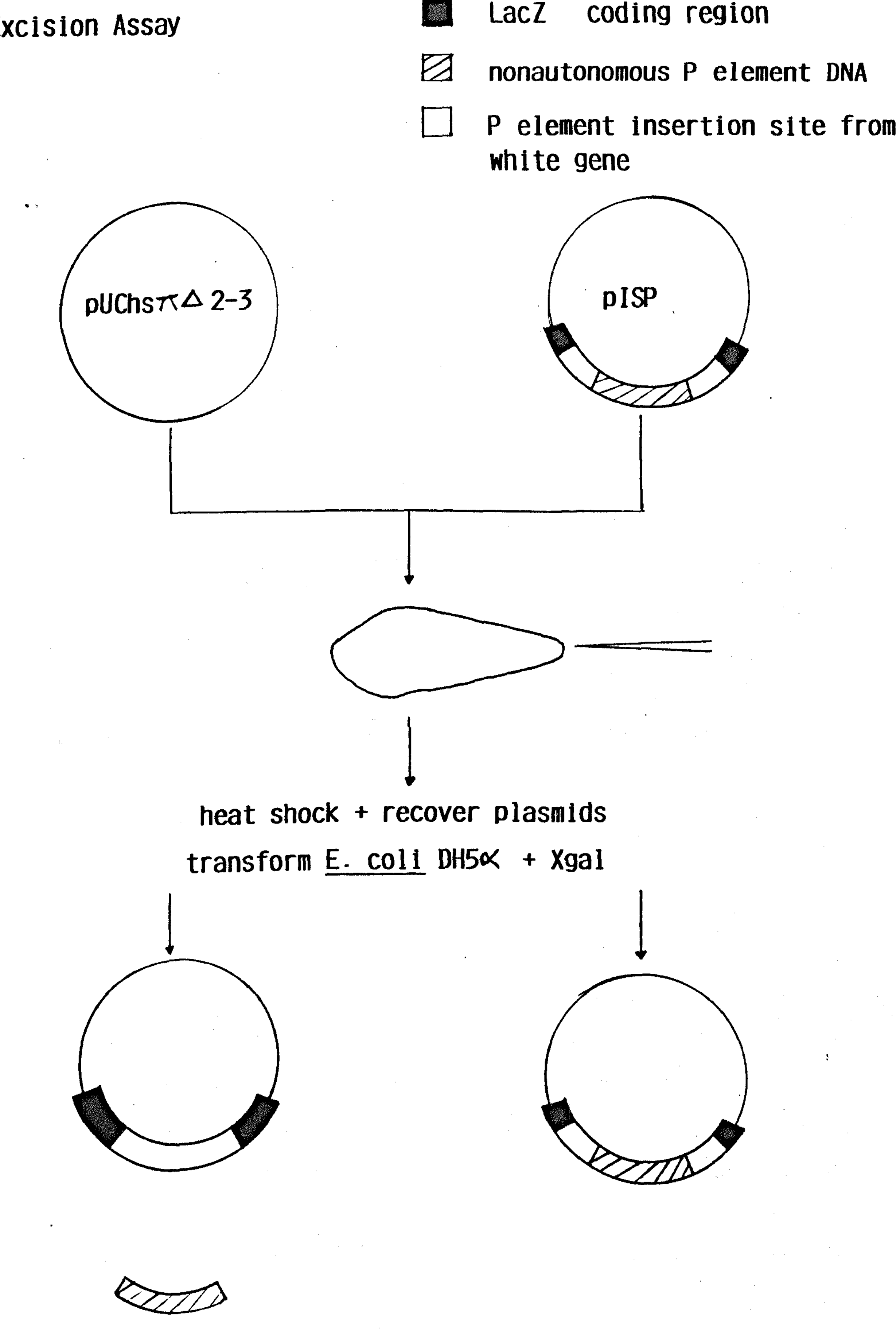
incubated for a further 30 minutes at 37°C. SDS was added to a concentration of 1% (w/v) and the resulting transcripts run on a 0.9% agarose gel with no ethidium bromide but with 0.1% SDS in both the agarose and the running buffer. Following electrophoresis at 50V for 90 minutes, the gel was washed twice for 30 minutes in 1 x E buffer, stained in 0.5μg/ml ethidium bromide for 30 minutes and visualized over a U.V. light source. The transcripts were visible as a smear between 1.5 and 0.9kb and not in the region of 3.9kb as expected for full length transcripts. Few transcripts were obtained so that this result was not reproducible photographically. The possible cause of this failure to produce full length transcripts is considered in section 6:5 and an alternative strategy to investigate transcript processing is proposed.

6:4 P ELEMENT EXCISION ASSAY

Determination of transposase dependent, precise P element excision from a marker gene would indicate whether or not a functional transposase was present and able to mediate this event. Failure to precisely excise the P sequences would suggest either that the transposase protein was not present or that it was unable to perform the excision function. The assay used to detect excision of the P sequences (illustrated in Figure 6.2) is based on the fact that P elements can excise not only from genomic DNA but also from plasmids introduced into *Drosophila* (Rio *et. al.*, 1986) and mammalian (Rio *et.al.*, 1988) cell lines. The excision indicator plasmid consists of a 47bp fragment from the *white* locus of *Drosophila* inserted into the α-fragment portion of the *E.coli* β-galactosidase gene of pUC8 but retaining the lacZα-encoded peptide complementing function of that plasmid. A 0.6kb fragment carrying a non-autonomous P element was inserted into the 47bp *white* sequence thereby creating either an 8bp duplication of the white target site (pISP-2) or an 8bp duplication with a 2bp mismatch depending on the orientation of the inserted P sequence (Rio *et.al.*, 1986). Since the inserted 0.6kb sequence now disrupts the β-gal open reading frame, these pISP and pISP-2 plasmids fail to restore the lac^{+} phenotype when introduced into lac^{-} *E.coli*. Precise excision of the 0.6kb P sequence would, however, allow lacZα complementation.

Figure 6.2

Protocol employed in the pISP assay for the excision function of the transposase protein. The two plasmids were co-introduced into the mosquito embryo. After 18 hours, the embryos were heat shocked and low molecular weight DNA isolated from them after a further 2 hours. This DNA was used to transform *E.coli* DH5α and lacZ^{+} transformants scored.



Excision Assay
LacZ coding region
nonautonomous P element DNA
P element insertion site from white gene
pUChsπΔ 2-3
pISP
heat shock + recover plasmids
transform E. coli DH5α + Xgal
lacZ+
LacZ−

If either the pISP or pISP-2 plasmid were co-introduced into a cell with the pUChsπ(Δ2-3) plasmid, any functional transposase protein expressed by the pUChsπ(Δ2-3) plasmid could mediate the precise excision of the P sequences from pISP or pISP-2, restoring the β-gal open reading frame. Plasmid DNA recovered from the cells can be used to transform a lac⁻ *E.coli* host and the lac⁺ phenotype can be selected on X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) containing indicator plates. O'Brochta and Handler (1988) performed this assay in drosophilid and non-drosophilid species and the experiments reported here were based on their methods.

The pISP and pUChsπ(Δ2-3) plasmids were co-injected into the mosquito embryo as in section 3:3, each at a concentration of 500μg/ml. After injection and removal from halocarbon oil onto moist filter paper, the embryos were returned to the insectaries for 18 hours. After a 1 hour heat shock at 37°C in a humid oven, the embryos were again returned to the insectaries for a 2 hour recovery period. Low molecular weight DNA was then recovered from the injected embryos as described by Hirt (1967), using 100μl of Hirt lysis buffer to extract DNA from approximately 100 injected embryos. Low molecular weight DNA was extracted with phenol/chloroform and ethanol precipitated with 5μg of tRNA to act as carrier. The DNA was then resuspended in 6mM Tris pH 7.4, 0.1mM EDTA and 10mM NaCl before using to transform lacZ⁻ *E.coli* hosts, DH5α.

All of the DNA isolated from up to 250 embryos was used to transform 200μl of competent DH5α cells, originally prepared according to the method of Hanahan (1983) but subsequently purchased from Bethesda Research Laboratories. Transformants were plated on LB plates containing 75μg/ml ampicillin and 50μg/ml X-gal. Precise excision of the P element from the pISP plasmid was determined by the growth of blue lacZ⁺ colonies indicating restoration of the β-gal open reading frame. pUC12 and pBR322 plasmids were used to transform *E.coli* DH5α to serve as positive and negative controls respectively, in plating on X-gal containing media. The numbers of ampicillin resistant tranformants obtained for each batch of embryos injected are shown in Table 6A. Of these transformants, none were of the lacZ⁺ phenotype suggesting that either the transposase protein is not expressed by pUChsπ(Δ2-3) or that it is expressed but is not functional in the mosquito cell.

The transformation efficiency of the DH5α cells used, was calculated as 1.6 x $10^7/\mu g$ with the pBR322 and pUC12 plasmids. However, the number of transformants recovered on transformation with the low molecular weight DNA isolated from the embryos, apparently falls far short of that expected for this calculated transformation efficiency (Table 6A). The amount of low molecular weight DNA recovered per embryo was calculated on the basis of the maximum and minimum volumes of DNA introduced into the embryos by micro-injection. These volumes were taken as 160pl - 800pl, representing 1 - 5% of the embryo volume. The DNA solution introduced was at a final concentration of 1mg/ml so that the expected yield of DNA should be approximately 0.16 - 0.8ng per embryo. A transformation efficiency of 1.6 x $10^7\mu g$ should therefore result in the recovery of 2560 - 12800 colonies per embryo. The average number of colonies recovered per embryo is 23. This shortfall may be due to inaccurate determination of the precise volumes injected into individual embryos or to loss and degradation of low molecular weight DNA during isolation. If the isolated low molecular weight DNA was contaminated with genomic DNA, this could compete with plasmids for sites of entry into the *E.coli* host cell, reducing the efficiency of plasmid transformation. To investigate this possibility, the low molecular weight plasmid DNA, isolated from embryos, should be titrated in subsequent transformations. Increased efficiency of transformation with lower concentrations of transforming DNA would suggest that this is indeed the case.

To confirm that the transformants did carry the pISP plasmid, the DNA of the transformed colonies was probed with the labelled pISP plasmid. 96 colonies were selected randomly and used to inoculate 200μl of L-broth and ampicillin (75μg/ml) at 37°C, and grown overnight. Using a sterile replicating tool, samples from the 200μl cultures were spotted onto sterile nitrocellulose filters placed on selective plates (LB with 75μg/ml ampicillin). The plates were then incubated at 37°C overnight. DNA from the resulting colonies was fixed onto the nitrocellulose filter and hybridized with the pISP probe. The pISP probe detected homologous DNA in 91 of the 96 colonies screened suggesting that the ampicillin resistance of the remaining 5% of transformants was due to the uptake of the pUChsπ(Δ2-3) plasmid. To determine the actual number of pISP molecules recovered, the number of transformants was therefore corrected in each case by

multiplying by 0.95 to take into account the transformants representing the pUChsπ(Δ2-3) plasmid (Table 6A). O'Brochta and Handler (1988) found that the pUChsπ(Δ2-3) plasmid represented 10% of the recovered plasmids following co-injection of equal amounts and concentrations of pISP and pUChsπ(Δ2-3). This relatively low efficiency of recovery of pUChsπ(Δ 2-3) may be due to the larger size of this plasmid. O'Brochta and Handler also found that after injection of only pUChsπ(Δ2-3), one in 10^3 transformants were lacZ$^+$, a reversion which they attributed to recombination events. This suggests that any lacZ$^+$ transformants could represent reversions of the pUChsπ(Δ2-3) plasmid and not excisions of the P element from pISP. Since no lacZ$^+$ transformants were detected in the experiments carried out as part of this thesis, such reversions did not appear to be a problem though to eliminate the possibility of this occurring, the pACYChsΔ2-3 plasmid (O'Brochta and Handler, 1988) could be used in place of pUChsπ(Δ 2-3). pACYChsΔ2-3 does not contain a lacZα peptide coding region or a β-lactamase gene so that all transformants grown on ampicillin containing selective media, should carry only copies of pISP.

6:5 DISCUSSION

It would be very interesting to determine whether or not the P element on pUChsπ(Δ2-3) is transcribed when introduced into the mosquito genome since failure to express the transposase coding region would show conclusively that the integration of pUChsneo sequences into the mosquito genome must occur independently of the *Drosophila* transposase protein.

When O'Brochta and Handler (1988) injected the pUChsπ(Δ2-3) plasmid into embryos of *Anastrepha suspensa*, a tephritid species outside of the genus *Drosophila*, P element homologous RNA species of 3.0, 1.5 and 1.0kb were detected in total RNA preparations from these embryos. These transcripts were not detected in uninjected embryos. Identical transcripts were detected in total RNA isolated from embryos of the *D.melanogaster* strain Canton S (M strain), injected with pUChsπ(Δ2-3). The transcript sizes therefore appear to be consistent from both *A.suspensa* and *D.melanogaster* Canton S injected embryos, and the 3.0kb transcript is

TABLE 6A TRANSFORMANTS RECOVERED FROM LOW MOLECULAR WEIGHT DNA ISOLATED FROM <u>AEDES AEGYPTI</u> EMBRYOS INJECTED WITH pISP AND pUChsπ(Δ2-3)

	EMBRYOS INJECTED	TRANSFORMANTS	pISP MOLECULES RECOVERED
	75	2175	2066
	169	3230	3069
	160	920	874
	150	4570	4342
	65	2115	2009
Total	**554**	**13010**	**12360**

pISP molecules recovered = total number of transformants x 0.95

consistent with the RNA species of this size detected in polyA$^+$ RNA isolated from the line carrying a single autonomous P element in Karess and Rubin's work (1984), and the minor transcript of this size found in natural P strains (section 1:4).

It would appear then that the transposase coding region can be transcribed from the promoter hsp-70 in tephritids, suggesting that P mediated transposition could occur in this species, although further results reported by O'Brochta and Handler (1988) do not support this theory.

Confirmation of P element transcription in the mosquito would be a useful first step in determining the mechanisms involved in integration of pUChsneo sequences into the mosquito genome. Further attempts should therefore be made to this end and may involve pooling total RNA preparations from individual batches of embryos - previous attempts have involved the isolation of total RNA from batches of up to 250 embryos injected with the pUChsπ(Δ2-3) plasmid - to increase the amount of RNA available for Northern analysis. O'Brochta and Handler (1988) also report the occasional recovery of plasmid DNA in RNA preparations from injected embryos using the method of Chomczynski and Sacchi (1986) though this plasmid DNA would easily be separated from P-homologous RNA transcripts in Northern analysis. Failure to detect any P-homologous transcripts in total RNA isolated from mosquito embryos would suggest that the transposase coding region is not transcribed. However, low levels of expression may not be detected and it may be difficult to distinguish between such a level of expression and a true negative result. Reprobing with a ribosomal probe (Gale and Crampton, 1989) should detect transcripts in the total RNA preparation, indicating that the negative result is not due to any error in the Northern blotting technique.

If the transposase coding region was shown to be expressed in the mosquito cell, the next step would be to determine whether or not the RNA transcript was correctly processed for translation to the functional protein. The third intron (between ORF's 2-3) has already been removed from the transposase coding region in pUChsπ(Δ2-3). Correct processing of the primary transcript from this plasmid must include the removal of the remaining two introns (ORF 0 - 1 and ORF 1 - 2). The original strategy to detect any such processing was not successful

due to failure to obtain full length *in vitro* transcripts of the transposase gene. This may have been due to the fact that 300bp of the promoter, hsp-70, region was also cloned into the pSPT19 vector, inbetween the T7 promoter and the transposase coding region, so that the T7 promoter would have to read through this hsp-70 sequence before reaching the transposase coding sequences. However, transcription from an SP6 promoter at the 3' end of the coding region also did not result in full length transcripts. Following discussions with Dr. D. Rio, it was understood that it is difficult to routinely obtain full length transcripts of the transposase coding region using an SP6 promoter, due to a stop signal in this region which may be recognized by the phage promoter but not by the *Drosophila* promoter. This may also be true for the T7 phage promoter. However, if this is the case, the majority of transcripts produced would be expected to terminate at a discrete site, whereas in this work, a range of transcript sizes resulted. Full length transcripts which may occasionally arise could be isolated by selection using oligo d(T) cellulose to bind their polyA tails, providing the transposase polyadenylation site was also appropriately cloned into the SP6/T7 vector.

An alternative method of detecting RNA processing in transposase transcripts is to clone shorter regions of the coding sequences into the SP6/T7 vector, which span the intron sites. *In vitro* transcription would then give rise to transcripts including the intron sequences which could be injected into the mosquito embryo. On recovery of these transcripts, they could be hybridized to labelled, complementary transcripts as previously described in the strategy planned for use with full length transcripts. Again, if the intron to be studied has been spliced from the transcript in the mosquito embryo, hybridization of the two resulting RNA species to the complementary strand will result in a loop formation where the complementary intron sequence is represented. RNase treatment will result in splicing of the complementary strand and following denaturation of the hybrid molecules, any size differences in this labelled strand can be analysed by polyacrylamide gel electrophoresis and autoradiography. An added advantage of this method is that specific splicing events may be studied individually.

O'Brochta and Handler (1988) studied the mobility of the P element in non-drosophilid species, using the pISP plasmid assay. They detected lacZ$^+$ revertants in this assay at frequencies

ranging from one in 588 pISP molecules recovered from *D.melanogaster*, to one in 5000 pISP molecules recovered from *Zaprionus tuberculatus* (family Drosophilidae, genus Zaprionus). However no $lacZ^{+}$ revertants were recovered from insect embryos of the family Tephritidae. (The number of tranformants was corrected in each case to take into account the transformants which represent the pUChsπ(Δ2-3) plasmid, as in Table 6A)

No excision events were detected in the pISP plasmids recovered from the embryos of *Ae.aegypti* Bangkok. However, in O'Brochta and Handlers work, the lowest frequency of excision (1 in 5000 in *Z.tuberculatus*) was determined following the recovery of 28,000 pISP plasmids, and following injection of pISP into the embryos of Tephritidae, approximately 159,000 pISP plasmids were recovered from the species *Anastrepha suspensa* and approximately 117,000 from *Toxotrypana curvicauda*. Having recovered only 12,359 pISP plasmids from the embryos of *Ae.aegypti* Bangkok, it is possible that a very low frequency of excision may occur which was not detected by this screening. To compare the rate of excision in *Ae.aegypti* Bangkok to the rates obtained by O'Brochta and Handler for other non-drosophilids, the assay should therefore be repeated.

In summary, the results presented in this chapter are not conclusive in determining the role of the P encoded transposase protein in integration of pUChsneo sequences. However, each of the experiments should be repeated, incorporating the modifications described, to provide firm evidence of the nature of P element expression and function on introduction into the mosquito embryo.

CHAPTER 7

CONCLUSION

A primary aim of this research was achieved in the development of the micro-injection technique for the introduction of DNA into the developing embryos of *Ae.aegypti*, whilst maintaining their viability. Survival rates comparable to those obtained in micro-injection of *Drosophila* embryos provided sufficient material for a study of the fate of the introduced DNA. DNA from P element constructs was found to be integrated into the mosquito genome at the level of the germline so that it was detected in the genomic DNA of subsequent generations. However, its genomic location or spatial arrangement is apparently unstable. Further characterization of the behaviour of large numbers of such integration events may reveal patterns of integration in terms of integration sites and preferential integration of particular exogenous DNA sequences. Molecular studies of mobility of integrated sequences and their interaction with endogenous mosquito sequences could also be correlated with classical genetic studies using known marker loci, to investigate the activity of the integrated sequences in terms of their movement throughout individual chromosomes. Although polytene chromosomes can be isolated from *Ae.aegypti*, they do not spread well and an extensive map is not available. *In situ* hybridization to metaphase spreads of regular chromosomes may be an alternative, viable prospect which would be extremely useful in determining the locations of integrated sequences.

The instability of the integrated sequences detected in this research may be a consequence of multiple integrations of repeated exogenous sequences, or a single copy integration into a region of repetitive genomic DNA. Preferential integration into such repetitive regions may reduce the potential of P element constructs in the gene retrieval strategy of transposon tagging, since the probability of effecting a detectable mutation by their integration into the genome may decrease. In P element transposition in *Drosophila*, there may be a strong preference for euchromatic sites of integration, over heterochromatic sites (Engels, 1988). Euchromatic DNA

consists largely of single copy and middle repetitive DNA with highly repetitive sequences being concentrated in the heterochromatin. However, since the integration events described in this thesis are thought to be unlikely to have arisen as a consequence of P element transposition, their occurrence at heterochromatic or euchromatic sites may be equally likely. *In situ* hybridization of pUChsneo probes to chromosomes of transformed individuals should help to locate integrated sequences.

In the assay of *Ae.aegypti* Bangkok larvae with G418, although this was found to be a lethal treatment with particular concentrations, mortality was affected by other extraneous factors. In attempts to select individuals putatively transformed with the *neo* gene, not all survivors were found to carry this gene. On the basis of this, the G418 assay was not found to be accurate in its selection of transformed individuals, and in view of the low numbers of survivors obtained in the assays of G_2 and subsequent generation progeny, it was speculated that actual transformants may not necessarily survive treatment with G418. An advantage of a marker gene conferring a visible phenotype, as opposed to a biochemical marker such as the *neo* gene, is that partial expression may be detected. In establishing a transformation system such as that in the mosquito, it would be advantageous to detect any manifestations of integration events in these preliminary investigations, rather than just integrations resulting in normal expression of the marker gene. Phenotypic marker genes isolated from the mosquito genome are currently being sought. The genomic sequence isolated on the basis of its homology to a region of the *white* locus of *Drosophila*, should be further characterized and its function determined.

In the absence of a suitable phenotypic marker, endogenous mosquito genes acting as biochemical markers, may have advantages over the exogenous *neo* gene. For example, insecticide resistance genes isolated from the mosquito may be used as markers in transformation experiments. Putative transformants could then be assayed with the corresponding insecticide and the results compared to those obtained in standard insecticide resistance tests of non-transformed individuals. The advantages of using this particular marker include the availability of data from numerous insecticide resistance tests previously performed by other research groups so that the response of transformed individuals to treatment with the

insecticide can be compared to the known response of wild type individuals. A number of mosquito gene sequences have been isolated which appear to be associated with organophosphate insecticide resistance (Merryweather, 1988), and could ultimately serve as marker genes.

In view of the data available on insecticide resistance testing in mosquitos, exogenous genes coding for organophosphate resistance may also be useful as markers for transformation. For example, a bacterial gene coding for the enzyme organophosphorous anhydrase has been isolated which has been shown to confer some resistance to organophosphate insecticides on *Drosophila* following its introduction into the *Drosophila* genome via P element transformation (Wild, 1989). While insecticide resistance genes may not be the markers of choice in transformation experiments with a view to field releases, they may be useful in preliminary studies on the nature and behaviour of sequences introduced into the genome.

The isolation of non-P encoded proteins thought to be involved in transposition of the P element (Rio and Rubin, 1988), suggests that P element transposition may be limited to *Drosophila* species. If the transposase gene is found to be correctly expressed in mosquito cells, then transposition may be effected in these cells in the presence of these isolated non-P encoded proteins. Complete sequencing and characterization of the genes coding for *Drosophila* specific host factors could ultimately lead to the construction of a transformation 'package', whereby all factors required for transposition are encoded on plasmid vectors which may be co-introduced into the embryos of non-drosophilids, resulting in P transposition. However, *Drosophila* specific splice sites, processing mechanisms or post translational modifications could preclude this possibility.

True autonomous mobile genetic elements i.e. DNA sequences coding for all functions required for their own transposition, could be isolated from other insect species or from more distantly related organisms. The transposable genetic element '*hobo*', also isolated from *D.melanogaster*, has been isolated and its ability to mediate germline transformation demonstrated (Blackman et.al., 1989). This element can integrate into the *Drosophila* germ line with high efficiency, and

shares some genetic and structural properties, though no obvious sequence similarity, with the P element. Further characterization of this element and determination of any *Drosophila* dependent functions required for its transposition, may reveal *hobo* as a more suitable candidate for transformation of the mosquito.

The maize transposon *Ac* is capable of autonomous transposition in exogenous host plant cells of tobacco (Baker et.al., 1986, 1987). Like *hobo*, *Ac* also displays some genetic and structural similarities to the P element. Demonstration of its autonomous transposition in exogenous host cells suggests that there may be no phylogenetic restrictions on its transposition and hence may be a further candidate for use as a DNA vector in subsequent mosquito transformation experiments. Endogenous mosquito mobile elements may, however, prove to be the most effective vehicle for the introduction of foreign DNA into the mosquito genome. Further characterization of a putative mobile element isolated from *Ae.aegypti* (Warren, 1989) may lead to its future use as a DNA vector for genome manipulation.

The ability to manipulate the mosquito genome at the level of the germ line should be useful in the development of alternative strategies for the control of disease bearing insect populations. This work has established the technology for the preliminary steps involved in such manipulation and has provided an insight into the behaviour of exogenous DNA introduced into the mosquito genome. Further characterization of mosquito lines transformed with the P element construct pUChsneo, along with additional transformation experiments using alternative eukaryotic DNA vectors and marker genes, should go some way towards the realization of mosquito germ line manipulation in control programmes.

APPENDIX

Materials

General chemicals were purchased from BDH Ltd., and were of the Analar or Electran grade. Restriction enzymes, SP6 and T7 RNA polymerases, polynucleotide kinase and random priming DNA labelling kits were obtained from Boehringer Corporation Ltd..

Radiolabelled nucleotides were supplied by Amersham International plc, Amersham, U.K..

Enzyme reactions were carried out according to the suppliers specifications.

Oligonucleotides were synthesized using phosphoramidate chemistry on a Model 3810 Biosearch DNA synthesizer.

Other chemicals were supplied by:-

Sigma Chemical Co. Ltd., Poole, U.K.- Trisma base, bovine serum albumin (fraction V) and ampicillin (sodium salt);

Difco Laboratories, East Molesey, U.K.- Bacto-agar, Bactotryptone, dehydrated yeast extract and casamino acids;

Pharmacia Fine Chemicals, Milton Keynes, U.K.- Ficoll 400;

Anderman and Co. Ltd., Kingston-Upon-Thames, U.K.- nitrocellulose (Schleicher and Schuell BA85, 0.45um).

ddH20 = double distilled, deionised water

Centrifuge rotor details

SS34	Sorvall RC-5B refrigerated superspeed centrifuge
GSA	Do. GSA rotor
Eppendorf	Anderman Eppendorf centrifuge
TST 14.1	Sorvall OTD50B Ultracentrifuge, TST 14.1 rotor
T865	Do. T865 rotor

SOLUTIONS FOR NUCLEIC ACID PREPARATIONS

Single mosquito genomic DNA extractions

Solution A

NaCl	100mM
Sucrose	5%
EDTA	10mM
Tris-HCl (pH7.5)	10mM

Solution B

SDS	1.25%
Tris-HCl (pH 9.0)	0.3M
EDTA	0.1M
Sucrose	5%
DEPC (diethylpyrocarbonate)	0.8%

DEPC added immediately prior to use since it is unstable in the presence of Tris buffers.

Large scale mosquito genomic DNA extractions

TNESST

Tris-HCl (pH 7.4)	10mM
NaCl	60mM
EDTA	10mM
Spermidine	0.15mM
Spermine	0.15mM
Triton X-100	0.5%

Stored at 4°C

Isolation of RNA

All solutions were prepared and stored in glassware previously baked at 250°C for 4 hours. Plastic-ware, other items which could not be baked and solutions were treated with 0.1% DEPC for at least 12 hours and autoclaved.

Guanidinium thiocyanate solution

Sodium citrate	25mM
Guanidinium thiocyanate	5M
Sodium lauroyl sarcosinate	0.5%
β-mercaptoethanol	0.75%

pH 7.0

This solution can be stored for up to 3 months prior to the addition of β-mercaptoethanol.

CsCl (5.7M)

192g CsCl were dissolved in 0.1M EDTA to a final volume of 200ml. This solution was filtered and autoclaved.

Total embryo RNA

Solution D

Sodium citrate	25mM
Guanidinium thiocyanate	4M
Sarkosyl	0.5%
β-mercaptoethanol	0.1M

pH 7.0

Solution E

Sodium acetate	0.15M
EDTA	5mM
SDS	1%
Tris-HCl (pH 9.0)	50mM
Polyvinyl sulphate	20μg/ml

Isolation of plasmid DNA

Solution I

Glucose	50mM
Tris-HCl (pH 8.0)	25mM
EDTA	10mM
Lysozyme	2-10mg/ml

Stored at 4°C.

BACTERIOLOGICAL MEDIA

Liquid Media

L-broth

per litre:

Tryptone	10g
Yeast Extract	5g
NaCl	5g
Glucose	1g

pH 7.5

phage broth

per litre:

Tryptone	10g
Yeast Extract	5g
NaCl	5g
Maltose	2g
$MgCl_2$	10mM

pH 7.5

Media Containing Agar

L-agar

per litre:

Tryptone	10g
Yeast Extract	5g
NaCl	5g
Agar	15g

Tryptone plates

per litre:

Tryptone	12g
NaCl	5g
Agar	10g (6.5g for 'Top' tryptone agar)

ANTIBIOTICS

Ampicillin

A stock solution of 10mg/ml of the sodium salt of ampicillin in water was sterilized by filtration and stored at -20°C. Plates and liquid media were prepared as described and allowed to cool to 45°C. Sterile ampicillin was added to give a final concentration of 50 - 100μg/ml of medium.

ASEPTIC CONDITIONS

All bacterial techniques were carried out in a laminar flow cabinet. Glassware, Eppendorf tubes and tips were sterilized by autoclaving. Other plastic-ware was pre-sterilized by the manufacturers. Media and reagents were autoclaved or filtered through commercial cellulose acetate filters.

SOLUTIONS AND BUFFERS FOR ELECTROPHORESIS

Buffers were prepared as a concentrated stock solution and stored at room temperature. Stock solutions were diluted to give a 1 x working solution.

10 x E buffer

per litre:

Tris base	48g
Sodium acetate	6.4g
EDTA	7.44g

pH 7.7 (glacial acetic acid)

10 x TBE buffer

per litre:

Tris base	108g
Boric acid	55g
EDTA	9g

pH8.3

5 x loading buffer (agarose gels)

Ficoll	20%
EDTA	100mM

Mixed on a rotating wheel for 30 minutes and Orange G added to give the required colour for marker purposes.

Hybridization Solution

deionized formamide	50ml
20 x SSC	25ml
Poly (A) RNA (1mg/ml)	1ml
Herring sperm DNA (5mg/ml)	1ml
100 x Denhardt's solution	
(2% ficoll, 2% BSA,	
2% polyvinyl pyrollidine)	1ml
10% SDS	1ml
ddH_2O	21ml

Oligonucleotide Hybridization Solution

20 x SSPE	25ml
10% SDS	1ml
Herring sperm DNA (5mg/ml)	1ml
tRNA (10mg/ml)	1ml
ddH_2O	72ml

PLASMID TRANSFORMATION BUFFERS

SOB

Bacto-tryptone	2%
Bacto-yeast extract	0.5%
NaCl	10mM
KCl	2.5mM
$MgCl_2$	10mM
$MgSO_4$	10mM

SOC

As for SOB with 20mM glucose in addition.

Preparation of SOB and SOC:

Tryptone, yeast extract, NaCl and KCl were mixed in ddH_2O and autoclaved. A 2M stock comprised of 1M $MgCl_2$ plus 1M $MgSO_4$ was prepared and sterilized by filtration. A 2M stock of glucose was similarly prepared and stored at -20°C. $Mg2^+$ and glucose were added to the medium immediately prior to use.

TFB

KCl	100mM
$MnCl.4H_2O$	45mM
$CaCl_2.2H_2O$	10mM
$HACoCl_3$ *(hexamine cobalt* (III) + trichloride)	3mM
K-MES	10mM

Preparation of TFB:

A 0.5M solution of MES (2[N-morpholino]ethone sulphonic acid) was equilibrated to pH6.3 using concentrated KOH, then sterilized by filtration and stored in aliquots at -20°C. A solution of 10mM K-MES was prepared using the 0.5M MES stock and ddH_2O. Salts were added as solids, dissolved, and the solution filtered and aliquoted. Storage was at 4°C.

DnD

DTT (dithiothreitol)	1M
DMSO	90% (v/v)
Potassium acetate	10mM

Phage buffer

per 100ml:

1M Tris-HCl	(pH 7.5)1ml
1M $MgSO_4$	0.5ml
5M NaCl	4ml
gelatine	0.1g

20 x SSC

per litre:

NaCl	175g
Sodium citrate	88.2g

20 x SSPE

per litre:

NaCl	210g
NaH_2PO_4	31.2g
0.5M EDTA	40mls

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Genetic transformation of the mosquito *Aedes aegypti* by micro-injection of DNA

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ABSTRACT. We report the successful introduction of heterologous DNA sequences into embryos of the mosquito *Aedes aegypti* (L.) by micro-injection. The injected DNA carrried P transposable element sequences, derived from and known to facilitate transformation in *Drosophila melanogaster.* Two plasmids, one of which carried a dominant selectable marker, were introduced into the posterior of embryos prior to pole cell formation and subsequently taken up into the germ line of transformed individuals. Stable transfer of the selectable marker (G418 resistance) was demonstrated over two generations. The precise nature of these putative P mediated integration events is currently being investigated. However, the results presented here establish the technique of DNA transformation for the genetic manipulation of *Aedes aegypti.*

Key words. Genetic transformation, *Aedes aegypti,* micro-injection, P element, DNA, germ line transformation.

Introduction

Aedes aegypti (L.) is the major urban vector of the arboviral diseases yellow fever, dengue, and dengue haemorrhagic fever (DHF). These diseases are acute and often fatal with urban epidemics arising frequently making DHF one of the leading causes of hospitalization and death of children in many Asian countries (Rudnick, 1967). Currently, *Ae.aegypti* control involves laborious environmental sanitation activities and the use of expensive insecticides for residual treatment and larviciding.

Genetic control through the mass release of sterile males has also been attempted in certain areas. However, this has generally been unsuccessful due to the poor competitive mating ability of males following the debilitating chemical or radiological sterilization treatments. Perhaps the greatest obstacle facing vector control by conventional methods, is the development of insecticide resistance.

Aedes aegypti is a convenient species on which to undertake laboratory experiments in the study of alternative vector control strategies to complement the existing methods. Genetic manipulation of the mosquito genome, for example, may be employed to reduce population size or to prevent the transmission of the pathogen from host to host. Ideally, this manipulation will be at the level of the germ line cells so that any genetic modification may be transmitted through subsequent generations, eliminating the need for the repeated release of

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individuals as in previous attempts at genetic control methods. Here, we report the introduction of DNA sequences into the genome of *Ae.aegypti.* To facilitate transformation of the germ line tissues a eukaryotic DNA cloning vector is required which could be integrated into the host genome at a relatively high frequency. A transposable genetic element, the P element, isolated from *Drosophila melanogaster* Meigen, has been used in the successful transformation of *D.melanogaster* (Rubin & Spradling, 1982) and the distantly related species *D.hawaiiensis* Hardy (Brennan *et al.*, 1984) and *D.simulans* Sturtevant (Daniels *et al.*, 1985), suggesting that there may be no species barrier in the transposition of this element. In the light of this work, we have studied the introduction of the P element into *Ae.aegypti* for use as a potential DNA vector in subsequent control strategies.

Transformation of *Drosophila* embryos by micro-injection of the P element involves the removal of the rigid chorion and desiccation of the resulting embryo, bounded only by its transparent vitelline membrane, to allow the introduction of a DNA solution. The embryo of the aedine mosquito differs significantly from that of *Drosophila,* as does that of the anopheline mosquito (Miller *et al.*, 1987), in that it has an opaque, rigid chorion which cannot be removed but, unlike both the *Drosophila* and anopheline embryos it is extremely susceptible to desiccation during the period at which injection must take place. As a result, we have developed a new technique for the micro-injection of a DNA solution into the embryos of *Ae.aegypti.*

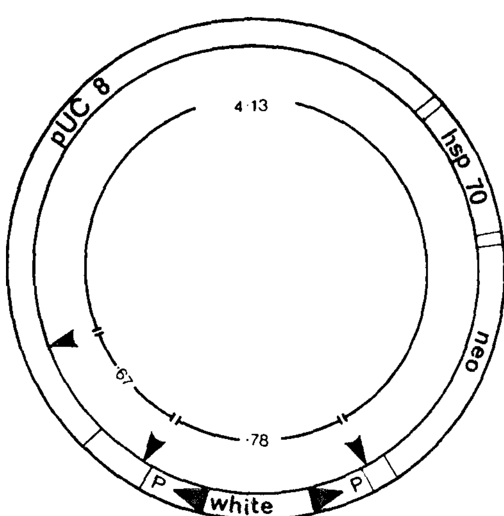

FIG. 1. The pUChsneo plasmid. This P element vector carries the bacterial neomycin gene which confers resistance to G418, under the control of a *Drosophila* heat shock promoter hsp 70. A multiple cloning site lies within the inverted terminal repeats of the P element and the P sequences are joined by 500 bp of DNA from the *white* locus of *Drosophila melanogaster.* The arrows indicate the HindIII restriction sites and fragment sizes are given in kilobases.

Materials and Methods

Plasmid DNA. The pUChsneo plasmid (Fig. 1) is a P element vector carrying the bacterial neomycin gene which confers resistance to the antibiotic G418, under the control of a *Drosophila* heat shock promoter hsp70 (Steller & Pirrotta, 1985). A multiple cloning site from pUC8 also lies within the inverted repeats of the P element to facilitate cloning of DNA into the vector. The P sequences are joined by 500 bp of DNA from the *white* locus of *Drosophila.* The arrows indicate the HindIII sites and sizes of the fragments are given in Kilobases.

A second P element construct, pUChsπ(Δ2–3) (Laski *et al.*, 1986), has been shown to act as a helper element in *D.melanogaster,* providing the transposase activity required for the effective transposition of the vector element. It has one defective inverted repeat so that it is unable to integrate into the host genome and has the intron between ORF2 and ORF3 of the intact 2.9 kb P factor spliced out to overcome the tissue specificity of transposase production encountered in *D. melanogaster.*

Micro-injection technique. Individual females from *Ae.aegypti* Bangkok strain were transferred 4 days post bloodmeal into glass tubes (3×8 cm) with a circle of moist filter paper to serve as a surface for oviposition. At oviposition, the embryo is white gradually darkening to black and doubling in size after 4 h. Dark grey-black embryos are at the optimum age for injection. The embryos were orientated on the oviposition paper with their posterior poles aligned. A strip of double-sided sticky-tape, 3×10 mm, was attached to the edge of a coverslip to immobilize the embryos which were transferred to the tape by inverting the coverslip and picking up the embryos from the oviposition paper. At this

stage, the embryos were very susceptible to desiccation and would collapse and become inviable within 1–2 min of their removal from the moist oviposition paper. Desiccation was monitored by eye under the dissecting microscope (×10 magnification) and embryos were covered with water saturated halocarbon oil (Series 700, KMZ Chemicals Ltd) after approximately 30 s. Capillary needles were loaded for injection, with the DNA solution at a total concentration of 500 μg/ml pUChsneo: pUChsπ(Δ2–3) at a ratio of 9:1 in 5 mM KCl, 0.1 mM sodium phosphate pH 6.8). The injection needles, with a tip 300 μm×4 μm (pulled using a vertical needle puller, Scientific Research Instruments) were connected to an injection system which uses two positive pressure levels (injection and holding) provided by a N_2 gas cylinder and controlled by a foot pedal (Ansorge, 1982). This reduces blockage of the needle by eliminating suction at the tip when introduced into an insufficiently desiccated embryo and when the injection pressure is reduced before withdrawal of the needle tip. The needle tip must be no more than 4 μm in diameter to prevent tearing of the rigid endochorion although a bevelled tip did not appear to be necessary. The most satisfactory needles used were those drawn from capillary tubing with a glass filament fused to the inside surface (GC120F-10, Clark Electromedical Instruments).

During injection (Fig. 2) the DNA solution must be delivered posteriorly into the region of yolk adjacent to the periplasmic space to allow its incorporation into the germ line primordia. Pole cell formation commences approximately 3 h after the eggs are laid and therefore all injections were performed within 2½ h of oviposition. Since the endochorion is opaque, it is difficult to determine the precise location of the injection site although initial orientation experiments may be carried out by bleaching the endochorion with a solution of 0.3% (w/v) sodium chlorite, 0.2% (v/v) glacial acetic acid for 30 min (Trpis, 1970) at different stages of development. However, since bleached embryos are rendered inviable all injections were performed through the opaque endochorion using the TDU500 micromanipulator (Research Instruments Ltd) at ×100 magnification. The volume of DNA solution injected was 160–800 pl, corresponding to 1–5% of the embryo volume. This injected volume was controlled by adjusting the injection pressure and time and calculated by determining the volume of a spherical droplet ejected into an oil reservoir. After injection, the coverslip carrying the embryos was placed in a petri dish lined with moist filter paper and returned to the insectary (27°C, 75–80% r.h.) for 20–30 min. The embryos were then picked from the coverslip using fine forceps, placed onto a moist filter paper and returned to the insectaries for a 4 day conditioning period to facilitate larval development. Injected embryos were stimulated to hatch in a dilute solution of hay infusion (Christophers, 1960).

FIG. 2. Micro-injection of an *Aedes aegypti* embryo showing the relative sizes of the embryo and the capillary needle.

DNA extraction, Southern blotting and hybridization. DNA extraction was based on the method of Coen *et al.* (1982). The genomic DNA from individual flies was digested with HindIII, run on a 0.9% agarose gel and blotted onto a nitrocellulose filter. The plasmid DNAs used as probes were labelled by random oligonucleotide priming (BCL) to specific activities of at least 5×10^8 dpm/μg and were hybridized to Southern blots in 50% formamide, 5×SSC, 0.1% SDS, 50 μg/ml sheared, denatured herring sperm DNA, 10μg/ml poly(A) and 1×Denhardt's solution, at 42°C for 16 h. The filters were washed in 2×SSC, 0.1% SDS at the temperatures indicated and autoradiographed at −70°C using an intensifying screen.

Results

Table 1 shows the percentage survival of injected individuals at different stages of development. These post-injection survival rates are comparable to those obtained routinely in *Drosophila melanogaster*. The resulting G_0 progeny were crossed to virgin *Aedes aegypti* Bangkok individuals at a ratio of 1:4, G_0:Bangkok non-injected individuals. The embryos of the G_1 progeny were incubated at 37°C for 30 min in a humid incubator to activate the heat shock promoter. First instar G_1 larvae were assayed with the neomycin analogue G418 to detect transformants. To determine the concentration of G418 required to kill non-transformed individuals, twenty-five first instar larvae were placed in 30 ml of dilute hay infusion in a series of 9 cm petri dishes with G418 concentrations of 0 μg/ml, 125 μg/ml, 250 μg/ml, 500 μg/ml, 1 mg/ml and 2 mg/ml. Larvae were removed from the G418 solution after 48 h and placed in flat, plastic trays 14×10×2.5 cm with fresh water and yeast. The concentration of G418 which resulted in a rapid reduction in per cent survival was determined to prevent overcrowding of the survivors. To ensure accurate selection of transformed individuals, a G418 concentration of 1 mg/ml was used in all subsequent assays.

Survivors of this G418 assay were crossed individually to virgin Bangkok individuals at the same ratio (1:4) described previously. Genomic DNA was isolated from individual putative transformants once the survival of the following generation had been assured.

Genomic DNA was prepared from twenty-four individual G_0 flies, denatured and dot blotted onto nitrocellulose using an S&S Minifold. The filter was then hybridized with the pUChsneo probe, washed in 0.1×SSC, 0.1% SDS at 65°C and autoradiographed. The probe detected pUChsneo sequences in three out of the twenty-four individuals (data not shown). Consequently, DNA from further G_0 individuals was digested with HindIII and analysed by Southern blotting using the pUChsneo plasmid as a probe. In one of the G_0 individuals, the probe detected three bands corresponding to the sizes expected for a HindIII digest of the free pUChsneo plasmid (Fig. 3a). This confirms that the pUChsneo plasmid was incorporated into the developing embryo and in this case had remained intact in the adult. Probing the Hind-

TABLE 1. Survival of injected *Aedes aegypti* embryos.

Date	Embryos injected	Hatched	Adults	% eggs to larvae	% larvae to adults	% eggs to adults
26. 5.87	63	15	↑	24	↑	↑
19. 5.87	125	44	50	35	70	16
27. 5.87	120	12	↓	10	↓	↓
29. 5.87	180	32	21	18	66	12
1. 6.87	204	51	24	25	47	12
7. 6.87	69	6	↑	9	↑	↑
15. 6.87	101	27	23	27	40	16
22. 6.87	197	27	↓	14	↓	↓
20.10.87	188	25	↑	↑	↑	↑
21.10.87	283	88	|	|	|	|
23.10.87	130	8	166	22	69	15
27.10.87	316	101	|	|	|	|
28.10.87	206	20	↓	↓	↓	↓

Total injected 2182.

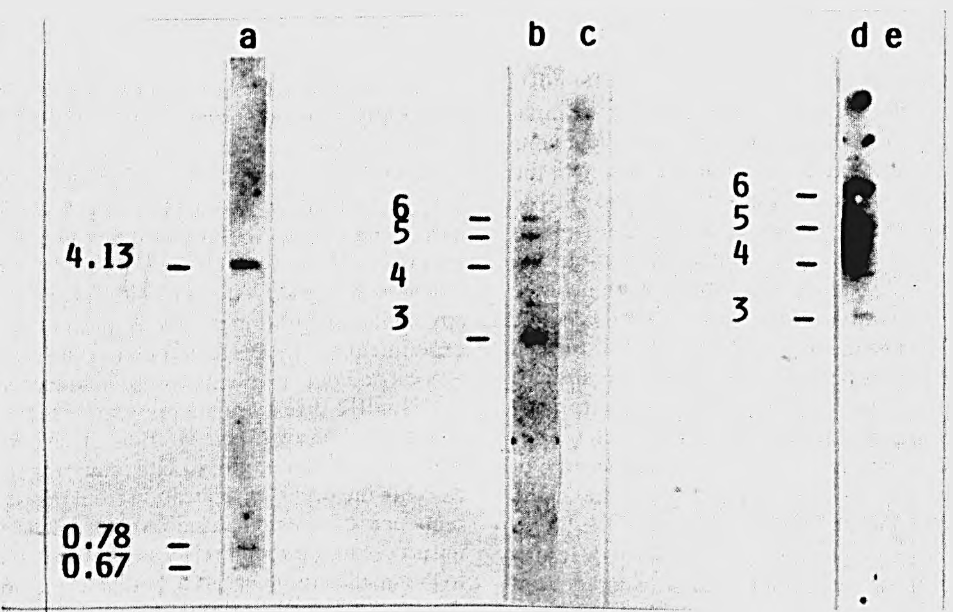

FIG. 3. HindIII digests of genomic DNA from transformed individuals. Lanes (a) and (b) are G_0 individuals, lane (d) is a G_1 or G_2 individual (which produce identical banding patterns). Lanes (a), (b) and (d) were probed with the labelled pUChsneo plasmid. Lanes (c) and (e) show lanes (b) and (d) reprobed with the labelled 1.3 kb SalI fragment of the *Drosophila white* locus carried in pCS156. Following hybridization, the filters were finally washed either at room temperature (lanes c and e), 42°C (lanes a and d) or 65°C (lane b).

III digested DNA of a further G_0 individual produced three bands unique to this individual and a fourth band of approximately 3 kb found in several other G_0 individuals and in total *Ae.aegypti* Bangkok DNA isolated from non-injected individuals (Fig. 3b). The three higher molecular weight bands were consistent with an integration event, i.e. the 4.13 kb fragment along with two fragments joined to host mosquito genomic DNA at the P element termini or within the 500 bp *white* locus sequence, giving rise to two bands with a total size greater than 0.78 kb (see Fig. 1).

HindIII digested DNA from a G_1 individual and a G_2 individual (both progeny arising from one G_0×wild type Bangkok strain cross) produced identical patterns when probed with the pUChsneo probe (Fig. 3d) suggesting a single stable integration event in one G_0 parent through two generations. This pattern is unlikely to correspond to that of a HindIII digest of the free pUChsneo plasmid since we would expect that any of the pUChsneo injected into the G_0 embryo should be degraded at this stage or would be diluted out in subsequent generations.

In the case of both the G_0 and the G_1 or G_2 integration events (Figs 3b and 3d respectively), probing with the 500 bp of the *white* locus of *Drosophila* joining the P element sequences in the pUChsneo plasmid would be expected to distinguish a P mediated transposition event from a random integration into the host genome. P transposition involves the integration of only the intact sequences flanked by the 31 bp inverted repeats of the P element (O'Hare & Rubin, 1983) and not the external *white* locus region. The appropriate filters were treated with 0.1 M NaOH at room temperature to remove the previous probe, neutralized and reprobed with a fragment of the pCS156 plasmid (Sang *et al.*, 1984) (kindly donated by David Finnegan). This 1.3 kb SalI fragment from the coding region of the *white* locus of *Drosophila* includes the 500 bp of *white* sequence present in the pUChsneo plasmid. No hybridization was detected by this probe in either case even after prolonged exposure times (Figs 3c and 3e). This suggests that the integration event was independent of the *Drosophila white* region and therefore possibly mediated by P element transposition rather

than random chromosomal integration. However, the banding patterns observed in Figs 3(b) and 3(d) are not conclusive. The HindIII digest would be expected to result in internal fragments of 4.13 and 0.67 kb plus two fragments of unknown size resulting from the fusion of the P element termini to flanking mosquito DNA. Although the larger molecular weight fragments are not inconsistent with this explanation, the small 0.67 kb band is absent and the origin of the 3 kb band (which is also found in total genomic *Ae.aegypti* Bangkok DNA from untransformed individuals) is not clear. However, the absence of the 0.67 kb band is perhaps not surprising given the small amount of DNA available for loading (<500 ng), the relative inefficiency of transfer of small fragments and the long exposure times required before even the larger molecular weight bands can be detected. It may be that these integration events resulted from homologous recombination as in the case of the *Anopheles gambiae* Giles event described by Miller *et al.* (1987). The absence of any detectable homology to the *white* sequences of *Drosophila* (Figs 3c and 3e), however, makes such an interpretation equivocal. Clearly, this would require the fortuitous and complete exclusion of the *white* region during the homologous recombination event.

Discussion

Fundamental problems in the injection of DNA sequences into the aedine embryo have now been overcome to produce survival rates comparable to those achieved in manipulations of the *Drosophila* embryo. In comparison with the anopheline mosquito (Miller *et al.*, 1987), the larval stages of *Ae.aegypti* Bangkok are much less susceptible to the effects of the G418 antibiotic. This is in keeping with the general resistant properties of the Bangkok strain to a variety of insecticidal compounds.

The putative G_0 germline integration event (Fig. 3b) was unexpected in that it is only likely to be represented in the DNA of a relatively small number of cells and so should be difficult to detect. However, a somatic integration event may have occurred which could explain this result. Whilst this is not satisfactory in terms of the genetic modification of the mosquito in successive generations, it does provide evidence that the pUChsneo:pUChsπ(Δ2–3) transposition system may be operational within the mosquito genome.

Both the G_0 and the G_1+G_2 integration events apparently occurred independently of the *white* region DNA suggesting either a precise P element mediated event or a homologous recombination event during which the *white* sequences have been excluded. Further mapping of the genomic DNA in subsequent transformed individuals may serve to elucidate the nature of any similar integration events. A plasmid rescue experiment on the DNA of the G_1 transformant was carried out, involving the circularization of the HindIII digest fragments and subsequent transformation of *E.coli* MC1061. This experiment was designed to isolate any fragments carrying the origin of replication from the pUC8 sequence of the pUChsneo vector. This was unsuccessful, although this was likely to be due to the small amount of DNA available and not to the absence of the pUC8 replication origin.

A low frequency of transformants in later generations is thought to be a fault of the selection procedure with G418. 99% of the treated control larvae die after 2–3 days, whereas progeny arising from injected individuals show only 49% mortality after 4 days rising to 96% after 8 days. A more suitable assay system would involve the use of a selectable phenotypic marker so that individuals partially expressing this marker gene may also be isolated for further study.

We have described the successful introduction of heterologous DNA sequences into the germ line of *Ae.aegypti* Bangkok embryos and obtained evidence that precise P element mediated transposition may be possible using the pUChsneo vector with the plasmid pUChsπ(Δ2–3) as helper element. Future work must include detailed analysis of the nature of these and similar integration events. A phenotypic marker assay is currently being studied which may replace the antibiotic resistance gene carried on the existing P element vector. New P element constructs and, ideally, transposable elements endogenous to the aedine mosquito may be used in the functional cloning of genes of interest and the transposon mediated mutagenesis of existing genes. Such work should ultimately lead to an understanding and control of the molecular mechanisms involved in the transmission of pathogens by their insect vectors.

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