

Genetic engineering technology for the improvement of the sterile insect technique

*Proceedings of a final Research Co-ordination Meeting
organized by the
Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture
and held in Vienna, 21 -25 November 1994*



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The originating Section of this publication in the IAEA was

Insect and Pest Control Section
International Atomic Energy Agency
Wagramerstrasse 5
P O Box 100
A-1400 Vienna, Austria

GENETIC ENGINEERING TECHNOLOGY FOR
THE IMPROVEMENT OF THE STERILE INSECT TECHNIQUE
IAEA VIENNA, 1997
IAEA-TECDOC-993
ISSN 1011-4289

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Printed by the IAEA in Austria
January 1998

FOREWORD

Since the beginning of the joint FAO/IAEA programme on the research and development of insect pest control methodology, emphasis has been placed on the basic and applied aspects of implementing the sterile insect technique (SIT). Special emphasis has always been directed at the assembly of technological progress into workable systems that can be implemented in developing countries. The general intention is to solve problems associated with insect pests that have an adverse impact on production of food and fibre.

For several insect species SIT has proven to be a powerful method for control. This includes the New World screwworm fly (*Cochliomyia hominivorax*), the Mediterranean fruit fly (*Ceratitus capitata*), the melon fly (*Bactrocera cucurbitae*), the Queensland fruit fly (*Bactrocera tryoni*) and one tsetse fly species (*Glossina austeni*).

Improvements of the SIT are possible, especially through the use of molecular techniques. The final report of the Co-ordinated Research Programme on "Genetic Engineering Technology for the Improvement of the Sterile Insect Technique" highlights the progress made towards the development of transformation systems for non-drosophilid insects and the research aimed at the identification and engineering of potential target genes or traits.

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SUMMARY

1. INTRODUCTION

The sterile insect technique is an environmentally friendly technique to eradicate or control insect pests. The primary constrain of this technology is the cost involved. It has been recognized that modifying the genetic characteristics of the insect used in SIT programmes can generate significant cost reductions. Through classical genetics medfly strains were constructed where the females can be eliminated and only the component active in the SIT, the male, is reared and released.

With the advent of molecular biology and biotechnology novel approaches for the modification of insects became available. The expected advantages, as compared to classical genetics, are:

- (a) improved set of diagnostic tools to monitor populations,
- (b) improved precision of these "gene based" techniques as compared to current "chromosome based" strategies, and
- (c) increased spectrum of possibilities as molecular approaches are not limited to genes/traits naturally occurring in the target insect species.

Based on the recommendations of a group of consultants, the IAEA initiated the Co-ordinated Research Programme on Genetic Engineering Technology for the Improvement of the Sterile Insect Technique. The objective of this programme was to conduct research in two complementary areas:

- (a) to develop a transformation system for economically important insects:

A technique is needed that allows the introduction and stable integration of genes/gene constructs into the germline of the target organism. As the routine *Drosophila* system, based on the transposable element P, does not work in non-drosophilid species, alternative transposable elements have to be identified. In addition to the identification of new elements, appropriate markers to monitor transformation have also to be found.

- (b) to investigate genes/promoters that could be utilized to generate better strains for the SIT:

Depending on the trait that will be the target for molecular manipulations (e.g. sex determination), the appropriate genes have to be identified and, eventually, cloned. In addition, appropriate control elements (promoters) have to be found to allow the required specificity of gene expression (e.g. inducible, sex specific, tissue specific, etc.).

This report describes the outcomes of the final Research Co-ordination meeting of this Co-ordinated Research Programme. Previous meetings were held in 1989 and 1992. The two most important achievements of this research programme are:

- (a) The isolation of genes and transposable elements from a variety of insect species.
- (b) The demonstration that insect transposable elements can transpose in heterologous species. As a consequence of these experiments, preliminary results indicate that genetic transformation of a commercially important tephritid species may have been achieved.
- (c) Several different promoters have been isolated.

These achievements have been published in the relevant scientific literature and constitute significant progress toward the establishment of genetic transformation technology in pest insect species of agricultural importance.

This report highlights the developments made in the field during the past two years and describes how many of the recommendations made by this research co-ordination group in the previous meeting in 1992 have been fulfilled. This report concludes by listing a number of recommendations for the short- and long-term directions of research aimed at further establishing genetic engineering technology in economically important insect pests.

2. CONCLUSIONS AND RECOMMENDATIONS

2.1. TRANSFORMATION

2.1.1. Progress to date

Genetic transformation will benefit the SIT by enabling the relatively rapid construction of strains which can be used for SIT. In addition, genetic transformation will enable, through gene tagging, the isolation of new mutations which could be used to improve and expand the SIT.

Significant progress towards the establishment of a genetic transformation technology in non-drosophilid insects has been made since this research co-ordination group met last in 1992. This progress can be best described in terms of the recommendations made by this group in 1992. Specifically:

2.1.1.1. "That characterized transposable elements, such as *mariner*, *hobo*, *hermit*, *Activator*, *Minos* and *Juan* be the subject of research aimed at determining whether they can be used as transformation vectors in non-drosophilid insects."

- The transposable elements *Minos*, *hobo* and *Hermes* have been shown to be mobile in non-host species. The *Minos* element, isolated from *Drosophila hydei*, can transform *Drosophila melanogaster* and is capable of transposase-dependent excision when introduced into *D. melanogaster*. This indicates that *Minos* may function as a transformation vector in other insect species. The *Hermes* element of *Musca domestica* is a member of the hAT family of eukaryotic transposable elements which includes the *hobo* element of *D. melanogaster*, the *Ac* element of *Zea mays* and the *Tam3* element of *Antirrhinum majus*. *Hobo* has been used as a transformation vector in *D. melanogaster* while both *Ac* and *Tam3* are capable of mobility in non-host species.
- *Hermes* has recently been shown to be a functional element in that it can transpose not only its host species, *M. domestica*, but can also transform the heterologous species, *D. melanogaster*. Moreover, it appears to do so at a high frequency indicating that *Hermes* may be an efficient transformation vector to use in other insect species. *Hobo* has recently been shown to be capable of accurate plasmid to plasmid transposition in a number of non-host species such as *M. domestica*, the Queensland fruit fly, *Bactrocera tryoni*, and the Old World cotton bollworm, *Helicoverpa armigera*, indicating that *hobo* may also be able to be used as a transformation vector in these species. Experiments aimed at achieving this in *B. tryoni* have been attempted using resistance to the antibiotic G418 as a selectable marker. Preliminary results indicate that integration of the *hobo* element into the *B. tryoni* genome has occurred.

2.1.2.2. "That the search for transposable elements endogenous to each pest species be intensified. Two approaches which should be encouraged are:

- (a) Identification and characterization of DNA sequences homologous to existing transposable elements that are already used successfully as transformation vectors in eukaryotes."**

A number of transposable elements endogenous to many species of insects have been isolated and characterized over the past two years. For example, retrotransposon-type elements have been isolated from the important mosquito pests, *Anopheles gambiae* and *Aedes aegypti* while the isolation of *Hermes* from *M. domestica* has already been described. Another hAT element, *homer*, from the tephritid, *B. tryoni* has been isolated and is partially characterized. The characterization of these, and other, transposable elements, has increased our ability to isolate other member of these transposable element families through the design of specific PCR primers. This has been particularly true for the Tc1 super-family of transposable elements of which the insect-based mariner family is a member. Mariner elements have been isolated and characterized from a large number of insect species, including *Anopheles gambiae*.

- (b) "Identification of dysgenic traits in particular pest species and the subsequent characterization of the molecular basis for this dysgenesis. By comparison with *Drosophila*, this approach may lead to the Identification of genes into which an endogenous mobile transposable element has inserted."**

The occurrence of hybrid dysgenesis in *C. capitata* has now been well documented and, by analogy with the P-M system of hybrid dysgenesis in *D. melanogaster*, it is quite likely that the molecular basis for this dysgenesis is the occurrence of actively mobile transposable elements in some strains, but not others, of *C. capitata*. By definition, such elements, because of their mobility, could be harnessed as genetic transformation vectors in *C. capitata* and other pest insect species. It would be predicted that the analysis of mutant lines arising from these dysgenic crosses should lead to the rapid isolation of these transposable elements.

2.1.2.3. "That electroporation, biolistics and pole cell transformation (by electroporation or lipofection) and transplantation be explored as viable alternatives to embryo micro-injection as a means by which foreign DNA can be introduced into insect nuclei."

Microinjection of blastoderm insect embryos remains the most reliable technique to introduce foreign DNA into some insect species. However, this technique is laborious and, as explained below, may be inapplicable to many other insect species, such as the tsetse fly. The recent reclassification of the *gypsy* transposable element as a virus capable of infectious transfer in *D. melanogaster* may open up the possibility of using *gypsy*, and related retroviruses as transformation vectors. These could be delivered by topical application or ingestion.

2.1.2.4. "That new visible and selectable marker systems be explored as an alternative means of detecting transformation events."

- Homologues of the *white* gene of *D. melanogaster* have been isolated and sequenced from *C. capitata* and *An. gambiae*. Their use as marker genes that will permit the identification of transgenic individuals in these two species. Use of these markers depends on the

existence of the appropriate stable mutations in the target insect species. However, the availability of eye pigmentation mutants in other insect species (e.g. *M. domestica*, *An. gambiae*, *B. tryoni*, *C. capitata*, *Lucilia cuprina*) together with the availability of the appropriate *D. melanogaster* genes will facilitate the extension of transformation technology into these species.

- Recently, a gene isolated from the jellyfish, *Aequora victoria*, has been shown to be usable as dominant marker in *D. melanogaster* and in the nematode, *Caenorhabditis elegans*. This gene, encoding the autonomous green fluorescent protein (gfp), is likely to be of use in non-drosophilid transgenesis. It can be envisaged that this gene will be capable of being used with little modification, as a marker in a wide range of dipteran and lepidopteran pest species.

In summary, the past two years have seen the development of techniques that have enabled the identification and isolation of transposable elements from pest species, the development of methods that permit the mobility properties of transposable elements in host or heterologous species to be rapidly determined, and the characterization of a marker gene which may be of general use in insect transformation. Thus, the tools available to researchers in the field of non-drosophilid transformation has rapidly expanded and further significant advances in this field can be eagerly anticipated.

2.1.2. Recommendations for future research

2.1.2.1. Short-term research

The transposable elements *Minos* of *D. hydei*, *hobo* of *D. melanogaster* and *Hermes* of *M. domestica*, are mobile in some non-host insect species. The mobility of these elements in *C. capitata* is unknown. The following recommendations have been made:

1. Evaluation of the mobility of these elements in *C. capitata* within the next 12 months.
2. Completion of the characterization of the *C. capitata white* gene and its development into a genetic marker for this species.
3. Characterization of the molecular basis of hybrid dysgenesis in *C. capitata*.
4. Extension of the current technologies and tools (mobility assays, transposable elements) into insect pests which are subject to SIT.

2.1.2.2. Long-term research

The short-term recommendations described above are clearly highly focused. However, in order to ensure that the technology may have future application to a broad range of pest species, it is appropriate that a series of longer term goals are also identified. For example, the current research on using transposable elements as gene vectors in specific insect species is encouraging. In the longer term, however, we believe that a single "universal" gene vector based on a single transposable element is an unrealistic expectation because:

- transposable elements have limited host ranges.
- transposable elements may be unstable in heterologous species due to interactions with host transposable elements.

- transposable elements may be incompatible with the DNA delivery system required in certain species.

Over the next five year period the following areas demand research:

(a) Gene transfer vectors

- (i) Transposable elements are available from a number of other systems, such as *mariner*, retroposons and retrotransposons etc, and the potential of these elements as vectors should be explored. In addition, novel elements may be identified in insect systems using a range of approaches, including the use of direct PCR analysis, the detailed analysis of the basis of hybrid dysgenesis in pest species, as well as the investigation of specific gene systems (such as the ribosomal genes) for the presence of insertional elements. The identification, characterization and assessment of mobility of such elements specifically addresses the issues of mobility and stability indicated above.
- (ii) A number of viral-based vectors systems may have potential for the genetic manipulation of pest species. Such systems, currently under investigation in a range of insect species, include *gypsy* and *densovirus*s. The development of these systems as vectors not only addresses the issue of developing new transformation systems, but also may provide very attractive alternative methods for delivering gene constructs into insects where the introduction of DNA by direct microinjection is not feasible (see below).

(b) Physical delivery

Microinjection is currently the best means to deliver DNA into insect germ cells. However, microinjection is inappropriate for many insect pest species, such as tsetse flies and sandflies, which are larviparous or have small egg sizes. Alternative approaches for delivering DNA to germ cells should be developed. It should also be stressed that the problem of delivery of DNA to specific target tissues and organs is a common one in the field of genetic manipulation. We therefore recommend that every opportunity be explored to utilize approaches developed for gene therapy and plant genetic manipulation in the insect pest systems. There may also be merit in exploring how transposable elements move between species in the natural situation, as these mechanisms may be harnessed to allow the introduction of specific genes and constructs into the genomes of target insect species.

(c) *Marker Genes*

As the possibility for genetic manipulation of some insect pest species becomes more likely, it is clear that the need for suitable marker systems for the efficient selection of transformed individuals becomes a major priority. It is also important to note that it is desirable that a number of selectable marker systems should be available, they should be non-sacrificial, and it may be necessary to eliminate the selected gene at some stage during construction of the SIT strain. As indicated above, the white gene for medfly should be available shortly and the strategy of using dominant selectable markers will certainly be applicable in a limited range of pest insects. However, the limited genetic information for most insect species dictates that markers be developed which can be used without the need for any established genetics in the target pest. For example, the use of intracellular lethal gene products placed under cell-specific promoters may generate easily scorable transgenic individuals. Similarly, the use of histochemical markers, such as GUS, and autonomous markers, such as green fluorescent protein, may be generic marker systems applicable to a broad range of pest species. Marker systems may not just have application for the generation of transgenic

insects, but also be suitable as tags for the release of insects in SIT (see Section 2). Other dominant selectable marker systems that should be explored include those that select for resistance to alcohol, hygromycin and heavy metals.

(d) *Genetics*

The combination of molecular and classical genetics will enable to address important problems related to improving the SIT. This includes:

- Development of diagnostic genetic markers for the target populations, to be used before, during and after release of sterile insects. In certain species, this should include identification of cryptic species.
- Development of genetic tools, such as chromosomal rearrangements and balancer chromosomes, that will facilitate the construction of sexing strains based on transgenic technology.

Long-term recommendations

This Research Co-ordination meeting recommends the:

1. Continuation of the search for mobile systems which enable the efficient and stable integration of DNA into pest insect genomes.
2. Utilization of technology available in other fields of biotechnology in which gene delivery systems are required.
3. Development of fully characterized genetic markers of general use . The search for these markers is central for the implementation of transgenic technology in insect pests, since the development of an efficient gene transformation technology is contingent on both mobile elements and markers.
4. Isolation of chromosome rearrangements (e.g. balancers) through classical genetics.

2.2. TARGET SYSTEMS FOR TRANSFORMATION: APPLICATION TO SIT

During the past five years progress has been made in the analysis of a number of gene systems that are useful improvement of the SIT. However, most of the goals of the following section are contingent on the availability of a transformation system. In that regard, the results reported in the previous section indicate that such a system will be available for some pest species in the near future, and that practical applications to SIT will follow.

2.2.1. Progress on previous recommendations

In the previous RCM report the following recommendations were made:

2.2.1.1. "That genes from pest insects be cloned and analyzed that are:

(a) involved in sex determination."

The sex-lethal homologs from *C. capitata* and *M. domestica* have been partially characterized. Although further characterization is needed, it appears that the function of this gene is very different in these pest species as compared to *Drosophila*. *Sex lethal* is the primary sex determination switch in *D. melanogaster* but so far no sex specific difference in expression of this gene in the two non-drosophilid species mentioned above has been detected. This suggests that the mechanism of sex determination in Diptera is much more divergent over evolutionary times than previously believed. This critically important result demonstrates the need for the characterization of sex determination systems in pest species. As a consequence, the isolation of other sex determination genes in pest insect species is underway.

(b) involved in insect/pathogen interaction either using heterologous probes or through direct approaches.

Many genes encoding the proteins of the insect immune system have been cloned and sequenced. The mode of action of these proteins are known. Several groups are involved in isolating genes involved in resistance to specific human or animal pathogens.

2.2.1.2. "That sex-specific, developmentally regulated or inducible promoters from target species be isolated and analyzed."

There are examples of genes cloned from pest insects that are sex and developmental stage specific, or inducible. With the availability of a transformation system, the promoters of these genes can now be analyzed. Yolk protein, chorion and vitelline membrane genes, produced only in the female, have been isolated and sequenced from many species of insects, including pest species that are actual or potential targets of SIT, such as *Adh* genes from medfly, segmentation genes from *Tribolium*, *M. domestica* and locusts, and gut-specific genes from blood feeding insects. Most genes are regulated to some extent in a developmental or tissue specific manner, and these are potential sources of promoters. Finally, inducible promoters, such as heat shock, metallothionein and P450 are available from *Drosophila*. There is evidence that at least heat shock and metallothionein promoters can function in other species. Inducible genes available from other insects are the heat shock genes from mosquito and medfly and the metallothionein gene from medfly.

2.2.1.3. "That sex determination mechanisms in pest insects be studied for exploring possibilities of constructing sexing strains."

The male determining element on the Y chromosome of medfly has been mapped. The function of the Y chromosome in *Anopheles quadrimaculatus* has been studied. Clearly the elucidation of sex determination mechanisms in each pest species is now of critical importance in view of the results of the *M. domestica* and *C. capitata* sex lethal gene discussed above.

2.2.1.4. "That mechanisms leading to an effective sex-distortion system be identified and analyzed."

To our knowledge, little research has been undertaken on this subject since the previous Research Co-ordination Meeting.

2.2.1.5. "That alternative approaches be explored for long-term improvement of SIT by molecular techniques."

The availability of newly cloned and characterized genes, not only from insects but also from heterologous systems, has resulted in many suggestions for new approaches to improvement of SIT. Several of these are discussed in the following section.

2.2.2. Specific areas for future research

We have identified a number of areas in which genetic engineering of pest insects will improve the SIT.

2.2.2.1. Monitoring of released insects:

The first practical application of transgenic insects to SIT is likely to be improved monitoring. For example, 30% of the costs of current medfly SIT programmes are due to the problems involved in monitoring released insects and the remaining wild population. An inexpensive and accurate method of differentiating these two types of insects would significantly reduce these costs. Currently, identification is made by tagging released insects with fluorescent dye which is debilitating to the insects and requires additional labor and handling. As an alternative, incorporation of the *gfp* gene into the germline of the currently used SIT stocks might eliminate the need for this step. Evaluation of the use of such genes for insect identification is strongly recommended.

2.2.2.2. Genetic sexing:

- (i) Sex determination mechanisms from pest insects. It is critical to investigate the mechanisms of sex determination from all pest insects selected for SIT. The aim is to develop a system which will enable the inducible production of only males during mass-rearing in the SIT. Manipulation of sex is central to improvement of SIT using genetic tools. As mentioned above, the mechanism of sex determination is very divergent amongst insects, and caution should be used in making assumptions regarding homology to *Drosophila* or any other system.
- (ii) Synthetic genes for sexing. A number of sex-specific promoters are currently available, but they represent relatively late-acting genes. Research directed at the isolation of promoters that act earlier should be undertaken. There are a number of conditional promoters that could be combined with sex-specific promoters to create a conditionally expressed, sex-specific promoter. Many lethal genes are available that could be attached to such promoters to kill one sex only. Alternatively, sex-specific promoters could be fused to lethal genes for the same effect.
- (iii) Synthetic genes for genetic sterilization. Irradiation and chemical-based methods used to sterilize insects also cause somatic damage, resulting in lower viability and competitiveness. In a manner similar to genetic sexing, it should be possible to create a strain that could be rendered sterile using an innocuous inducer.
- (iv) Increased efficiency. The current genetic sexing strains all have the feature that they normally produce fully fertile insects of both sexes. Using genetic engineering, it might be possible to combine research described in (ii) and (iii) above to create a strain that must be induced to produce fertile males.

2.2.2.3. Vectorial capacity:

Mosquitoes, tsetse, and other blood-feeding insects are usually important not because of the direct physical damage that they cause, but rather because they transmit pathogens. This is an active interest in many laboratories, and many systems are under active investigation or consideration. These include insect genes that provide natural resistance to pathogens, genes involved in the insect immune system, mammalian antibody genes, pathogen antigen genes, and blood-feeding behavior. A strain that is unable to transmit pathogens would be advantageous in an SIT program.

2.2.2.4. Symbionts:

It has been established that symbionts can also cause reproductive incompatibility in a wide variety of species. Particular symbionts play an important role in host-pathogen interactions. In tsetse fly they are required for the establishment of trypanosome infections in the midgut of the fly. For the SIT control of this species the released males have to be blood-fed before release to ensure that they do not contribute significantly to any increase in trypanosome transmission. If symbionts could be genetically engineered to interfere with trypanosome development, then the SIT would be more efficient. There is also considerable interest in trying to transfer symbionts between different species. Work in this area should be encouraged.

2.2.2.5. Reduction of damage by pest insects:

Much of the objection to large scale SIT in species such as mosquitoes and medfly has been the associated damage or nuisance produced by even sterile insects. Mosquitoes that do not feed on blood and medflies that do not sting fruit would eliminate these problems. A classical genetic approach to creating a non-blood-feeding mosquito is currently underway and it is possible that isolation of genes involved in oogenesis might make this project easier (most mosquitoes normally require a blood meal before laying eggs).

2.2.2.6. Stockpiling:

It would be beneficial to be able to store insects for release. In some species, this is possible (for example *Aedes* mosquitoes). Diapause genes or cryopreservative genes might make it possible to maintain insects of other species at low temperatures for extended periods of time.

2.2.2.7. Competitiveness of SIT strains:

Mass-reared flies are usually less competitive for mates and more susceptible to predation than wild flies. Genes encoding mating peptides, genes involved in production and release of pheromones, and genes involved in behavioral traits may have eventual application in SIT programmes.

2.2.3. Recommendations for future research

This Research Co-ordination Meeting recommends the:

1. Introduction, by transformation, and evaluation of a marker detectable in the field (such as GFP or GUS) into one or more strains used for SIT. This would be the first demonstration of the use of genetic engineering to provide practical benefits to an operational SIT program.

2. Isolation of inducible, sex, stage and tissue specific promoters.
3. Construction and testing of promoter cassettes which are simultaneously sex-specific and inducible.
4. Construction and evaluation of chimeric genes suitable for genetic sexing.
5. Isolation of genes and promoters involved in sex determination systems in pest insects for use in constructing sexing strains.
6. Exploration of alternative approaches for the long-term improvement of the SIT by molecular techniques.

2.3. CONCLUSIONS

The development of tools and strategies for the construction of transgenic pest insect strain is advancing rapidly. It is envisaged that within the next years functional transformation systems will be available. These have to be tested for their applicability in large scale rearing in SIT programmes. In parallel, research is needed to identify genes/traits that will be the target of genetic engineering.



MOBILITY OF *HOB*O TRANSPOSABLE ELEMENTS IN NON-DROSOPHILID INSECTS

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Abstract

We will describe the development and implementation of assays which permit the mobility of *hobo* elements injected into developing insect embryos to be detected and examined. These assays have enabled us to classify *hobo* elements as members of a transposable element family which includes the *Ac* element of maize and the *Tam3* element of snapdragon - two plant transposable elements that have wide host ranges. We will present data that show that *hobo* also has a wide host range in that it can excise and transpose in a number of non-drosophilid insect species. These results have led us to use *hobo* as a gene vector in the tephritid, *Bactrocera tryoni*, and we will discuss the progress of these ongoing experiments.

1. Introduction

The inability to genetically transform insects of medical and agricultural importance has prevented the application of the full repertoire of molecular biological and modern genetic techniques to these species. For example, reverse genetics, gene tagging and enhancer trapping are three powerful techniques which are, at present, restricted for use in only one insect species, *Drosophila melanogaster*. *P* transposable element mediated genetic transformation was developed for this species over 12 years ago [1]. As a consequence, our understanding of many of the basic developmental and biochemical mechanisms of *D. melanogaster* has increased dramatically. In addition, similarities between these processes in *D. melanogaster* and vertebrates have also been revealed.

The ability to genetically transform other insect species will not only have a similar impact on our understanding of basic biological approaches across a very diverse class of arthropods but will also enable us to design and implement effective pest control strategies based on the use of, for example, autocidal genes. This will serve to reduce our dependency on chemical insecticides and thus will impact not just on pest control but also on the quality of the environment in regions where this control is implemented.

A number of attempts have been made to develop genetic transformation technologies in non-drosophilid insects. Most of these have involved the use of the *P* transposable element which has proved to be successful in *D. melanogaster* and some closely related drosophilid species [2,3]. A few have been successful in that transgenic insects have been obtained, however subsequent analysis has revealed that in no cases were transposable element sequences involved in the integration of the foreign DNA [4]. Rather, integration appeared to occur via a low frequency non-homologous recombination mechanism [4,5,6]. In the majority of cases no evidence for transformation of any nature was detected for the insect species examined.

We have chosen an alternate approach to the development of transposable element-based transgenic systems in non-drosophilid insects. This approach, which utilizes assays to detect the excision and transposition of transposable elements, is based on research previously performed in *D. melanogaster* and plants [7, 8]. It was first utilized for non-drosophilids by O'Brochta and Handler [9] in their analysis of *P* transposable element mobility in non-drosophilid insects. We have used this approach to examine

the mobility of *hobo* elements in *D. melanogaster* and non-drosophilid insects. We have found that *hobo* based mobility assays are reliable indicators of the *hobo* element's potential to mediate genetic transformation in insects. In addition, these assays have enabled us to identify the presence of endogenous *hobo*-like elements in some non-drosophilid species.

2. Excision assays

Hobo excision assays enable the removal of the *hobo* element from an indicator plasmid to be detected. The DNA sequence of the empty excision site can be obtained thereby permitting the nature of the excision event to be determined. A diagram of the *hobo* excision assay is shown in Figure 1. The indicator plasmid contains a *hobo* element with an internal deletion which renders it non-autonomous. A suitable marker gene, such as β -galactosidase or *supF* is then inserted into this deleted *hobo* element at the site at which the deletion was created. The indicator plasmid is co-injected into blastoderm insect embryos together with a helper plasmid containing the *hobo* transposase-encoding region placed under the control of the *hsp70* promoter of *D. melanogaster*. Upon appropriate heat shock conditions, this promoter enables the production of high levels of *hobo* transposase. Injected embryos are allowed to develop and, approximately 20 hours post-injection, indicator plasmid DNA is rescued from developed embryos or hatched larvae. This plasmid DNA is then transformed into an appropriate strain of *E. coli* which permits the loss of the marker gene to be detected. Plasmids are then recovered from colonies lacking this marker and analysed, by restriction enzyme mapping and DNA sequencing, to determine the structure of the excision event.

When these assays are performed in a strain of *D. melanogaster* lacking *hobo* elements, the production of deletions was found to be dependent both on the presence of *hobo* transposase on the helper plasmid and on the presence of *hobo* sequences on the indicator plasmid [10]. Moreover, the DNA sequence remaining at the empty excision site following the excision of the *hobo* element was very similar to empty excision sites remaining after the excision of the *Ac* and *Tam3* transposable elements from maize and snapdragon respectively [10]. These empty sites did not contain any transposable element DNA, but rather contained additional DNA which was an inverted repeat of DNA flanking the transposable element. This similarity, together with the similarities in structure and sequences of these elements, led us to support the proposition that *hobo*, *Ac* and *Tam3* belong to a single family of transposable elements - the *hAT* element family [11]. Excision assays performed in *D. melanogaster* using a *hobo* element located in the chromosomes led to the recovery of empty excision sites that had identical structure to those recovered from the plasmid-based assays [12]. This indicates that the plasmid-based *hobo* excision assay accurately reflects the mechanism of *hobo* excision from chromosomal DNA.

We further examined the pattern of *hobo* excision in *D. melanogaster*. We were specifically interested to determine if target site duplications were required for subsequent excision of the *hobo* element and whether the flanking DNA located either side of the element made equal contributions to the templated addition of the additional DNA remaining at the empty excision site. When 8bp target site duplications were present a common class of excision product was obtained (Figure 2). A second indicator plasmid identical to the first except that the *hobo* element was not flanked by an 8bp target site duplication was constructed and used in assays in which helper plasmid was co-injected. The frequency of excision products obtained from these experiments decreased relative to the experiments using the original indicator plasmid [12]. In addition, no common class of excision product was obtained however all products contained additional DNA which was a templated inversion of flanking genomic DNA. Significantly, flanking DNA on both sides of the *hobo* element made approximately equal contributions to this additional DNA present at the empty excision site [12]. Since, in many cases, some of the flanking DNA was deleted during the excision of the *hobo* element, we believe that the templated addition of DNA during excision must occur prior to the creation of these deletions. We suggest that this indicates that 8bp target site duplications are not necessary for the subsequent excision of *hobo* elements and that the mechanism of *hobo* excision involves the linking of *hobo* terminal sequences thereby

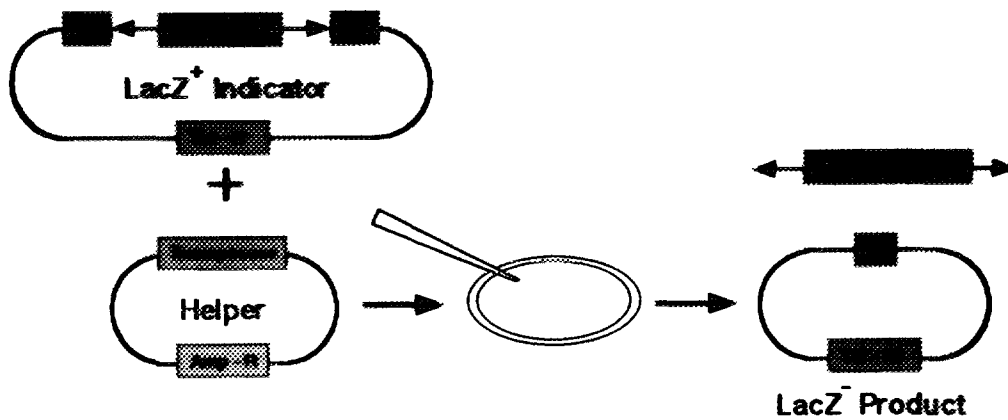


FIG. 1. *Hobo excision assay*.

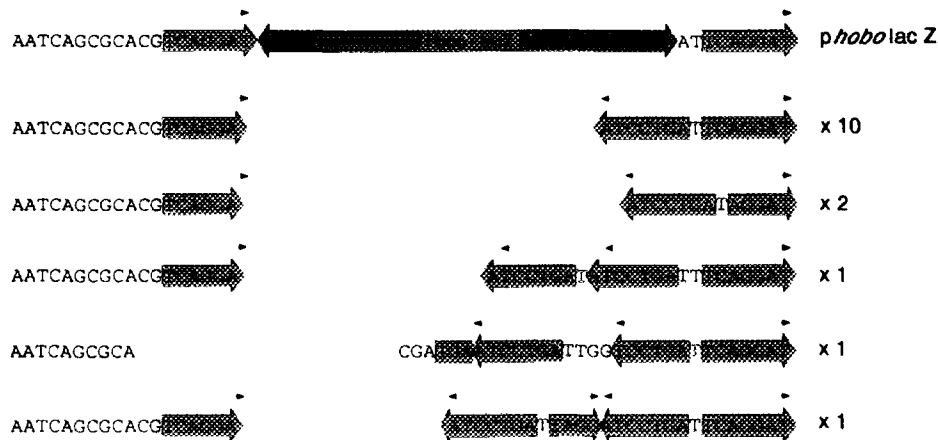


FIG. 2. *Excision products recovered from D. melanogaster*. The solid bar represents the *hobo* element while the arrows show the 8bp target site duplications flanking the *hobo* element. Additional DNA sequences present at the empty excision sites are shown. 5 different types of excision product were recovered however the first type was the most common form recovered.

enabling the interaction of *hobo* flanking sequences. This is similar to the mechanism proposed for the addition of P DNA to the coding joints generated during V(D)J recombination in the developing vertebrate immune system [13].

We also constructed an indicator plasmid in which the *hobo* element was flanked by 40bp of directly duplicated DNA. Chromosomal and plasmid-based excision assays revealed that the *hobo* element flanked by this DNA excises at a high frequency compared with a *hobo* element flanked by the normal 8bp target site duplication. All excision products were characterized by an absence of *hobo* element DNA and an absence of additional DNA at the empty excision site [12]. We propose that the presence of the long direct repeats increases the efficiency of the DNA repair process which acts upon the double stranded gap left by the excision of the *hobo* element.

We performed these assays in a number of non-drosophilid species including the housefly, *Musca domestica*, the Queensland fruitfly, *Bactrocera tryoni* and the Old World cotton bollworm, *Helicoverpa armigera*. [10, 14]. A common feature of these experiments was that *hobo* helper transposase was not required in order to recover deletions in the indicator plasmid. Addition of *hobo* helper transposase had little or no effect on the type or frequency of deletions recovered and no precise deletions were recovered from any of these species. Some of the deletion breakpoints were located in or near the inverted terminal repeats of the *hobo* elements, however there was no clear pattern of deletion other than a dependency on the presence of *hobo* sequences on the indicator plasmid. Figure 3 shows the excision products obtained from *M. domestica*. Despite the absence of precise excisions, these experiments showed that the *hobo* element was capable of mobility in these insect species. By comparison, similar assays performed with the *P* element in non-drosophilid species, did not produce excision products which were dependent on the presence of *P* element sequences on the indicator plasmid [9].

We hypothesized that the occurrence of deletions in non-drosophilid insect embryos injected without transposase helper plasmid was caused by the presence of *hobo*-like factors that were capable of recognizing *hobo* sequences in these insects but were incapable of excising these sequences correctly. The genes encoding these factors would most likely be contained on *hobo*-like elements endogenous to these species. Subsequently we have been able to isolate the *Hermes*, *hermit* and *homer* transposable elements from *M. domestica*, *Lucilia cuprina* and *B. tryoni* respectively that will be described in subsequent manuscripts [15,16, 17].

3. Transposition assays

The demonstration that *hobo* was capable of mobility in non-drosophilids as measured by excision assays led us to examine whether this element was also capable of transposition in these species. To explore this, we developed a plasmid-based transposition assay [18]. This is shown in Figure 4. Three plasmids are co-injected into blastoderm insect embryos. One of these plasmids is a helper plasmid similar to that used for excision assays. A second plasmid contains a *hobo* element containing a gene encoding resistance to the antibiotic, kanamycin. The third plasmid contains a target sequence, the disruption of which can be detected by an appropriate genetic test. We have used the *sucraseRB* gene of *B. subtilis* as a target sequence. This encodes the enzyme levansucrose which is toxic when expressed in *E. coli*

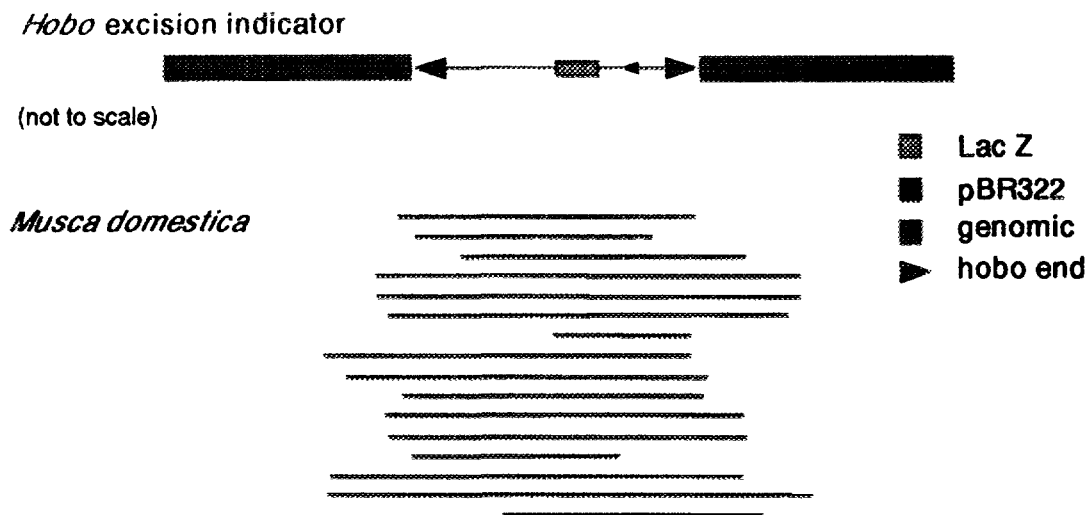


FIG. 3. Excision products recovered from *M. domestica*. The solid bar represents *hobo* element DNA and flanking *D. melanogaster* genomic DNA. Arrows indicate the location of the *hobo* terminal sequences. The empty bar represents pBR322 vector sequence. The lines under the bar represent the DNA deleted in each excision event characterized.

grown on media containing sucrose as a carbon source. Thus inactivation of this gene can be detected by growth on sucrose containing media. Plasmid DNA is rescued from developed embryos approximately 20 hours following injection and then transformed into the appropriate *E. coli* strain which is then grown on media containing sucrose and antibiotics which select for both the target plasmid and the *hobo* element. Plasmid DNA is prepared and examined for the presence of the *hobo* element in the *sucraseRB* gene.

When performed in *D. melanogaster*, transpositions of *hobo* occur at a frequency of approximately 1 per 50,000 target plasmids screened [18]. Transposition of *hobo* is accurate, the sequence transposed is delimited by the inverted terminal repeats of *hobo* and an 8bp target site duplication is created in the target gene. Thus there appears to be no apparent difference between the plasmid to plasmid transposition of *hobo*, the chromosomal transposition of *hobo*, or the plasmid to chromosome transposition of *hobo* that occurs during *D. melanogaster* transgenesis. This is further supported by the similarities in consensus target sequence obtained from chromosomal insertions of *hobo* [19] compared with the 15 insertions of *hobo* in the *sucraseRB* target sequence.

We performed transposition assays in three non-drosophilid species, *M. domestica*, *B. tryoni* and *H. armigera*, in which we had evidence, from excision assays, that *hobo* was capable of mobility. *Hobo* was found to be capable of accurate transposition in all three species [18, 14]. As for *hobo* transposition in *D. melanogaster*, the sequence transposed was delimited by the inverted terminal repeats of the *hobo* element and an 8bp duplication was made at the target site. There was also a similarity between the consensus target sequence obtained and the consensus target sequence obtained from *hobo* insertions into *D. melanogaster* chromosomes [19]. The frequency of transposition was, however, reduced relative to that observed in *D. melanogaster*, in *M. domestica* the frequency was approximately 1 per 200,000 target plasmids screened, in *B. tryoni* it was approximately 1 per 540,000 and in *H. armigera*, it was approximately 1 per 3 million target plasmids screened [18, 14].

Comparison of the target sites recovered from these transpositions revealed that *hobo* preferentially inserted into a particular location within the *sucraseRB* gene. Eleven out of the 40 transpositions recovered occurred at nucleotide position 1210 in this target gene. The 8bp target site duplication created at this site has little similarity to the consensus target sequence obtained from *hobo* transposition in *D. melanogaster*. We have commenced our examination of the length of target sequence required for this insertional specificity by removing a 39bp fragment centered on the 1210 site and cloning it into pUC19. This plasmid was then used as the target plasmid in transposition assays performed in *D. melanogaster*. Over 50% of the transpositions obtained were located at the 1210 site indicating that the nucleotide sequence at this location was a preferred site for the integration of the *hobo* element [18].

The ability of *hobo* to undergo accurate plasmid to plasmid transposition in these three non-drosophilid species suggested that the *hobo* element could be used to mediate genetic transformation of these species by a plasmid to chromosome transposition. *Hobo* has already been shown to mediate this in its host species, *D. melanogaster* [20]. We were therefore interested to determine if it could mediate genetic transformation of these non-drosophilid species.

4. Transformation

We investigated whether the *hobo* element could achieve genetic transformation of the Queensland fruitfly, *B. tryoni*. *B. tryoni* is a member of the family tephritidae and is estimated to cost the Australian horticulture industry approximately AUD\$300 million per year through lost production and sales. We determined that *B. tryoni* is sensitive to the antibiotic G418 when reared on Carolina instant media [21] and therefore decided to use the neomycin phosphotransferase gene encoding resistance to this antibiotic

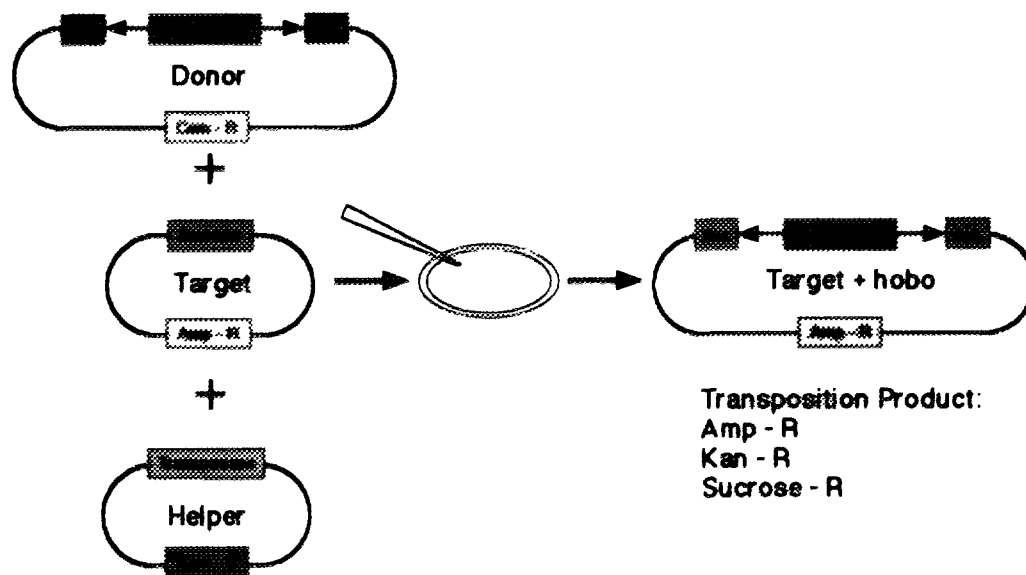


FIG. 4. *Hobo* transposition assay

as a marker for *hobo* integration. We inserted this gene, placed under the control of the *D. melanogaster hsp70* gene, into the *hobo* element. This was co-injected with a helper plasmid into blastoderm *B. tryoni* embryos. G1 embryos were placed on media containing G418, thereafter, from each line, embryos were placed on media containing or not containing G418. Lines exhibiting resistance to G418 were examined for the presence of the *hobo* element by Southern blot analysis. A total of 5 lines were obtained which contained individuals displaying resistance to G418 and which contained *hobo* sequences [21]. We are currently determining the precise breakpoints of integration of the *hobo* element in these lines using inverse PCR.

5. Conclusions and Future Work

Our results indicate that *hobo* element excision and transposition assays are accurate indicators of this transposable elements ability to mediate genetic transformation of those species in which it is mobile. We believe that the *hobo* element, and other *hAT* elements, will be useful vectors for insect transgenesis. The ability of *hobo* to accurately transpose in these species suggests that either this element has no requirement for host-encoded factors which participate in the transposition reaction or that, if host factors are required, they are conserved across the species we have so far examined. The extended host range of *hobo* is consistent with other *hAT* elements such as *Ac* and *Tam* which are capable of mobility in a number of plant species [22]. These mobility assays can be applied to other transposable elements such as *Hermes* and *mariner* [15,23] as well as other *hAT* elements that we have isolated. We will be examining the mobility properties of each of these transposable elements not only in their host species, but in other insects as well.

It is also clear from our results that *hAT* elements are capable of cross-mobilizing one another. This may present problems when a *hAT* element is introduced into a species already containing an endogenous *hAT* element. We will be examining whether the *homer* element of *B. tryoni* is capable of mobilizing the *hobo* element present in our transgenic lines. If so, it may be necessary to construct *hAT* element vectors which can be rendered suicidal following their initial integration into the recipient genome. It is also clear that a renewed emphasis should be placed on the development of marker genes which will enable the efficient detection of transgenic individuals.

ACKNOWLEDGEMENTS

This research was supported, in part, by NIH grant GM48102 .

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HOBO-LIKE TRANSPOSABLE ELEMENTS AS NON-DROSOPHILID GENE VECTORS

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Abstract

Using genetic and physical methods we discovered short-inverted repeat type transposable elements in non-drosophilid insects including, *Bactrocera tryoni*, *Musca domestica*, *Musca vetustissima* and *Lucilia cuprina*. These elements are related to *hobo*, *Ac* and *Tam3*. The *Hermes* element from *M. domestica* is 2749 bp in length and has terminal inverted repeats and a transposase coding region very similar to those in *hobo*. *Hermes* is functional in *M. domestica* and can act as a gene vector in this species. When *Hermes* is introduced into *D. melanogaster* it is hyperactive, relative to existing vector systems used in this species. *Hermes* will be useful as a gene vector.

1. Introduction

The transposable elements *hobo*, *Ac* and *Tam3* are structurally similar, encode proteins with similar amino acid sequences and have similar mechanisms of movement [1-4]. These similarities suggest that these elements are members of a family of elements that we call the *hAT* (*hobo*, *Ac*, *Tam3*) family. *hAT* elements are used as gene vectors and/or gene tagging agents in the species from which they were isolated and are capable of transposing when introduced into heterologous species. The ability of *hAT* elements to function in species other than their hosts distinguishes them from most other eukaryotic transposable elements that have been analyzed [5]. As part of our efforts to develop gene vector technology for insects of economic and medical significance to man we discovered and are characterizing *hAT* elements from *Musca domestica*, *Musca vetustissima*, *Lucilia cuprina* and *Bactrocera tryoni*. We show that some of these elements are functional and capable of serving as gene vectors in their hosts and in diverged species.

2. Material and Methods

hAT element detection *hAT* element excision and transposition require transposase. We have used this requirement to assess non drosophilid insect embryos for the presence of *hAT*-like transposases that can cause the excision of *hobo* elements from plasmids [4]. This method has been described [4].

hAT element isolation Based on the limited sequence similarities between *hobo*, *Ac* and *Tam3* we designed oligonucleotide primers that were used in polymerase chain reactions with non-drosophilid insect genomic DNA as template. Primer design and amplification conditions have been described [4]. Inverse PCR was used to isolate the remaining sequences of the element [6].

hAT element analysis *hAT* element transposition was tested in vivo using plasmid-based element mobility assays as described [5]. Germline transformation of *Drosophila melanogaster* with

h11 vectors relied on established methods [7, 8]. In experiments reported here *Hermes* vectors containing the *D. melanogaster* mini-white gene were constructed [9]. In addition, a *Hermes* helper plasmid was constructed containing the *Hermes* transposase coding region under the regulatory control of *D. melanogaster* hsp70 promoter. This plasmid provided a source of transposase. The *D. melanogaster* strain *w¹¹¹⁸* was used as a host [10].

3. Results

3.1. *hobo* transposase-like activity in *Musca domestica* embryos

When plasmids containing non-autonomous *hobo* elements were introduced into *M. domestica* embryonic cells by injection of preblastoderm embryos *hobo* elements excised frequently. *hobo* excision was completely dependent upon the presence of the *hobo* terminal inverted repeats. Excision frequently resulted in deletion of sequences flanking the *hobo* element. These "footprints" arising from *hobo* excision were qualitatively different from those observed in *D. melanogaster* embryos expressing *hobo* transposase. Expression of *hobo* transposase in *Musca domestica* embryos resulted in reduced excision frequencies but did not alter the "footprints" of the excision products recovered.

3.2. *hobo* transposase-like coding regions in *Musca domestica*, *Musca vetustissima*, *Lucilia cuprina* and *Bactrocera tryoni*.

We designed degenerate oligonucleotides similar to regions previously identified as being conserved among *hobo*, *Ac* and *Tam3* and used them as primers in a PCR with *M. domestica* genomic DNA as template [4]. These primers amplified the predicted 454-bp *hobo* fragment from a *hobo*-containing Oregon-R strain of *D. melanogaster*, and a similar sized fragment was amplified from the genome of a strain of *M. domestica*. The fragment was cloned, sequenced and shares 61% amino acid identity with *hobo* transposase. Similar results were obtained using *B. tryoni* and *M. vetustissima* genomic DNA.

3.3. The *Hermes* transposable element from *M. domestica*

Sequence and structure. We used an inverse PCR-based strategy to isolate sequences flanking the 454 bp transposase-like fragment isolated from *M. domestica*. Using this method we isolated overlapping segments of several *Hermes* elements from *M. domestica*. Alignment of the overlapping regions yielded a full-length consensus *Hermes* sequence of 2749 bp. The data were generated by compiling the sequences of several independent recombinants of each inverse PCR generated product. In this way sequence variation introduced during amplification by Taq polymerase was distinguished from naturally occurring sequence variation between elements. *Hermes* elements are quite homogeneous in sequence. Very low levels of nucleotide polymorphism were found between the different *Hermes* elements sequenced. No large DNA insertions or deletions were observed.

Variation between strains. We used oligonucleotide primers specific to subterminal *Hermes* sequences in a PCR reaction to investigate sequence length heterogeneity of *Hermes* elements among *M. domestica* strains. These oligonucleotides were used to amplify internal *Hermes* sequences from genomic DNA extracted from single flies of various strains. Elements without deletions will yield a 2.4 kb amplification product. All strains examined contained a 2.4 kb band, indicating that all contain at least one full-length or near full-length element. Most strains contained between 1 and 5 different-sized elements. The pattern of size variation we observed is similar to that observed for other active transposable element systems, including *P*, *hobo*, *Tam3* and *Ac*.

Hermes transposase. *Hermes* contains a single long open reading frame beginning at nucleotide 450 and ending at 2285. Conceptual translation of the ORF yields a protein sequence comprising 612 amino acids that displays 55% identity and 71% similarity to the *hobo*-transposase. The ORF appears to encode the *Hermes* transposase protein. Comparisons of the *Hermes* sequence with those of *hobo*, *Ac*, *Tam3* and the *Ac*-like element from *P. glaucum* clearly show that *Hermes* transposase protein is most similar to that of *hobo*. We find that all five transposases are alignable over their entire length.

Hermes terminal and subterminal sequences. Comparison of the left and right terminal sequences of *Hermes* reveals that they are composed of 17 bp imperfect inverted repeats. The left terminal inverted repeat of *Hermes* differs from that of *hobo* by two bases, while the right terminus of *Hermes* differs from the corresponding region of *hobo* by only a single nucleotide. Comparison of the terminal inverted repeats of other members of the *hAT* element family revealed that all share a conserved A and G at positions 2 and 5, respectively, in their left inverted terminal repeats and a complementary C and T in their right terminal sequences. This A2G5 pattern is not universal to all short inverted repeat-type elements.

3.4. *Hermes* mobility in *M. domestica*

We used the transposition assay developed by us and described elsewhere [5]. Our only modification of this assay was to use a 'donor plasmid' that contained a *Hermes* element instead of *hobo* sequences and a 'helper' plasmid consisting of the *Hermes* transposase coding region under the regulatory control of the *D. melanogaster hsp70* promoter. Embryo injections, plasmid recovery and plasmid screening were done as described [5].

After screening 10⁶ target plasmids (pUCSacRB [5]) we recovered two interplasmid transposition events. Transposition resulted in the movement of only sequences delimited by the inverted repeats of *Hermes* and resulted in an 8 bp duplication of the insertion site. These features are characteristic of transpositional recombination mediated by *hAT* elements.

3.5. *Hermes* mobility in *D. melanogaster*

Three independent experiments resulted in the production of transgenic *D. melanogaster* with integrated *Hermes* elements. An average of 32% of fertile G₀ adults developing from injected embryos produced transgenic progeny. Comparable frequencies are seen using *P* elements [11]. 88% of the G₀ adults producing transgenic progeny had multiple insertions of *Hermes* in the germline. This was indicated by the presence of multiple eye phenotypes ranging from light orange to dark red. We confirmed the presence of multiple insertions by genetic mapping. 57% of the G₀ progeny with integration of *Hermes* in the germline produced clusters of transgenic progeny caused by premeiotic insertion of *Hermes*. We defined a cluster as 10% or more of the progeny. In some flies almost the entire germline was transformed resulting in over 90% of the progeny with an integrated *Hermes* element.

We confirmed the presence of *Hermes* sequences in G₁ progeny with pigmented eyes using PCR. *Hermes*-specific oligonucleotide primers were used with genomic DNA isolated from G₁ adults. *Hermes* sequences were detected in all progeny with pigmented eyes but never detected in non-transformed white-eyed siblings. Donor-plasmid sequences flanking *Hermes* were never detected in progeny with pigmented eyes, confirming that *Hermes* integrated into the *Drosophila* genome by transpositional recombination.

4. Discussion

A genetic test for detecting *hobo* excision showed that *hobo* excision is a transposase-dependent reaction. We also showed that *hobo* excision occurred in *M. domestica* embryos in the absence of *hobo*-encoded transposase suggesting this species contains proteins with transposase-like activity. However, differences in *hobo* excision footprints seen in *M. domestica* and *D. melanogaster* indicated the protein responsible for excising *hobo* in *M. domestica* was not identical to *hobo* transposase [4]. Using PCR and degenerate oligonucleotide primers we tested this hypothesis by screening *M. domestica* genomic DNA for sequences similar to the coding region of *hobo* transposase. We isolated middle repetitive sequences with high sequence similarity to *hobo* transposase. These sequences displayed copy-number and insertion-site variation between two *M. domestica* strains suggesting they were transposable elements (*Hermes*, [4]).

Isolation and identification of a complete *Hermes* element revealed 17 bp imperfect terminal repeats nearly identical to the inverted repeats of *hobo*. The internal sequences of *Hermes* contain a single long open reading frame with remarkable similarity to the amino acid sequence of *hobo* transposase [6]. These data indicate that *Hermes* is a short inverted repeat-type transposable element belonging to the *hobo*, *Ac* and *Tam3* (*hAT*) element family and suggests *Hermes* is the source of *hobo* transposase-like activity detected in *M. domestica* embryos [4].

Comparison of the terminal inverted repeats of *Hermes* with other members of the *hAT* family, including the *Bg* and *Tag1* elements, revealed a previously undocumented sequence similarity. These elements, although having inverted repeats of various lengths and sequence compositions, all have an A at position 2 and a G at position 5 of their left termini, and complementary bases at the corresponding positions in their right termini. This observation suggests these nucleotides play a central role in the biochemistry of recombination in this family of elements. Elements with the A2G5 motif share the property of generating 8 bp insertion site duplications upon insertion and supernumerary nucleotides forming short palindromes at the site of rejoining following excision [6].

Identification and characterization of *Hermes* in *M. domestica* indicates that *hobo*-like elements are not restricted in their distribution within insects as thought previously. Although we have not yet undertaken a large-scale search for related elements in other species, we have identified additional members of this family from other non-drosophilid insects including *Musca vetustissima* (Muscidae), *Lucilia cuprina* (Calliphoridae) and *Bactrocera tryoni* (Tephritidae). Insect *hAT* element sequences appear more similar to each other than to the *hAT* elements of plants. These data are consistent with the hypothesis that this family of elements is of ancient origin.

Hermes is a functional transposable element, capable of transposing in *M. domestica*. Using interplasmid transposition assays we showed that *Hermes* can transpose from a donor plasmid to a target plasmid and insertion resulted in 8 bp duplications of the target site. Although *Hermes* transposase was supplied by a helper plasmid we do not know at this time if this was required. Our previous efforts testing *hobo* mobility in *Musca* revealed an endogenous transposase activity [4]. That this activity originated from *Hermes* and was responsible for promoting transposition of *Hermes* in *M. domestica* remains to be tested directly.

Hermes can transpose when introduced into cells of divergent species such as *D. melanogaster*. Not only can *Hermes* transpose but it can be used as a germline transposition vector in this species. *Hermes* is the first transposable element shown to be capable of acting as a germline transformation vector in an insect outside the family of insects from which it was isolated. In this case *Hermes* is an efficient gene vector in an insect species that last shared a common ancestor with *M. domestica* 150 million years ago. The mobility properties of *Hermes* in *D. melanogaster* are similar to those of *Ac* and *Tam3* in heterologous plant species. However, unlike *Ac* and *Tam3* in heterologous plant species, *Hermes* appears hyperactive in heterologous hosts. The large clusters of transgenic progeny from single

G₀ adults (in some cases over 90% of the progeny were transgenic) arose because transposition occurred very soon after injection into preblastoderm embryos. Approximately 20-50 pole cells are present prior to gastrulation of *D. melanogaster*. These arose from the division of 2-4 pole cells that budded from the pole plasm. Large clusters of transgenic progeny from a single G₀ requires transposition to occur before or shortly after budding of pole cells. Early transposition is uncharacteristic of *D. melanogaster* transposable elements currently used as gene vectors in this species, including *P*, *hobo* and *mariner*. In addition to early movement of *Hermes* upon injection into *D. melanogaster* *Hermes* has high rates of transposition. Many of the G₀ individuals producing transformed progeny had multiple *Hermes* insertions at different chromosomal locations. In one case we recovered at least 7 independent integration events from a single G₀ adult. Multiple insertions were seen in many G₀ individuals. Multiple independent transgenic progeny arising from individual G₀ are detected infrequently using *P* elements in *D. melanogaster*.

Our results allow us to make a number of conclusions. First, using *hAT* element excision as a bioassay for *hAT* transposases is a reliable means for detecting the presence of related, functional transposable elements in non-drosophilid insects. Second, *hAT* elements are present in a number of non-drosophilid insect species and appear to be a family of elements of ancient origin. Third, the *Hermes* element from *M. domestica* is a functional *hAT* element from this species. Fourth, *Hermes* is capable of acting as a germline transformation vector in a species of insect 150 million years diverged from *M. domestica*. Fifth, *Hermes* is hyperactive in *D. melanogaster*, relative to the activities of *P*, *hobo* and *mariner* elements. Finally, *Hermes* is likely to be functional in tephritid fruitflies.

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DETECTION OF CRYPTIC SPECIES

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Abstract

Morphologically similar cryptic species are common in insects. In *Anopheles* mosquitoes, most morphologically described species are complexes of cryptic species. Cryptic species are of great practical importance for two reasons: first, one or more species of the complex might not be a pest and control efforts directed at the complex as a whole would therefore be partly wasted; and second, genetic (and perhaps biological) control strategies directed against one species of the complex would not affect other species of the complex. At least one SIT effort has failed because the released sterile insects were of a different species and therefore did not mate with the wild insects being targeted.

We use a multidisciplinary approach for detection of cryptic species complexes, focusing first on identifying variability in wild populations using RFLPs of mitochondrial and ribosomal RNA genes (mtDNA and rDNA); followed by confirmation using a variety of other techniques.

For rapid identification of wild individuals of field collections, we use a DNA dot blot assay. DNA probes can be isolated by differential screening, however we are currently focusing on the sequencing of the rDNA extragenic spacers. These regions are repeated several hundred times per genome in mosquitoes and evolve rapidly. Molecular drive tends to keep the individual genes homogeneous within a species.

1. INTRODUCTION

Surprisingly, the problem of cryptic species has not received adequate attention in pest control. Two recent examples of important pests that are cryptic species are the silverleaf whitefly (originally thought to be the sweet potato whitefly), which has caused enormous economic damage in the US in the last few years, and was not described as a separate species until last year [1]; the fall armyworm, which was recently described as two species [2]. In each of these two cases, an enormous amount of research was conducted on the wrong species in an effort to control the pest.

For the past few years, our research program has focused on cryptic species of anopheline mosquitoes. There are four related problems: 1) detection of a species complex in a single morphologically described species, 2) determination of the phylogenetic relationships among these species, 3) elucidation of the ecological and vector biology of the different species, and 4) development of a rapid screening method for mosquito control personnel. In this paper we discuss detection of cryptic species and the use of DNA probes for ecology, vector biology, and routine surveillance.

2. DETECTION OF CRYPTIC SPECIES

The most critical and difficult problem is the detection of a cryptic species complex. In the simplest case, where two species are sympatric, it is usually sufficient to show that the population is

subdivided into two groups that are out of Hardy-Weinberg equilibrium. However, when populations are allopatric or collections are limited, definite proof is often hard to obtain. Hybridization crosses are probably the most powerful method, however many species can not be bred in the laboratory. Luckily, from a practical point of view, it is largely irrelevant whether widely separated allopatric populations are identical or closely related sibling species. In either case, it is necessary to examine the ecology and pest status of each population separately.

Note that it is impossible to prove that a species is monotypic- all we can do is demonstrate that within the limits of our collections and techniques there are no significant differences. This does not rule out the possibility of a new collection or technique showing that a species complex does exist. In a multidisciplinary approach, the conclusive evidence rests with any technique that shows differentiation among groups. Showing that a collection is genetically uniform with one technique, for example polytene chromosome cytology, does not in any way diminish the impact of discovering that it is separable using another technique, for example allozyme electrophoresis.

Our approach has been multidisciplinary (see figure 1), resting on the assumption that we are most likely to detect differences between closely related species by using several different techniques. We routinely screen collections of wild mosquitoes for mtDNA and rDNA RFLPs, allozymes, and polytene chromosomes. All of these techniques are relatively rapid and detect different types of genetic variation.

2.1. Mitochondrial DNA RFLPs

MtDNA RFLPs are easy to interpret, because the mtDNA is circular and a constant length. The fragment sizes should always add to the same value (about 15.5 kb in mosquitoes). Too large a size is due to contamination or partial digestion; too small a size is due to comigrating bands or fragments migrating off of the gel. Differences between mitochondrial patterns are almost always due to gain or loss of restriction sites, which are simple to use in constructing maps or creating phylogenies.

Since mtDNA is maternally inherited and hemizygous, Hardy-Weinberg equilibrium is not applicable. There is no theoretical reason why different individuals of a single species can not have two or more very different mtDNAs, or that individuals of two different species can not have identical patterns. In practice, usually individuals within a species have similar mtDNAs, and different species have different mtDNAs [3]. However, exceptions such as introgression between species and lineage sorting within species have been observed.

MtDNA restriction site data is of dubious usefulness for constructing phylogenies. Unless a large number of restriction enzymes are used, the number of informative characters will be too small to be significant. [4], working with viral isolates with known relationships, used approximately 60 restriction sites to generate phylogenies with various methods. About 10% of the branches in all of their derived phylogenies were incorrect. We find that we seldom get more than 30 significant characters using eight restriction enzymes, so the branches in phylogenies generated using our data are likely to be incorrect 20% of the time. Part of the problem seems to be that the same sites tend to appear in different lineages, because the sequence of the mtDNA is highly restricted due to coding and base composition constraints.

2.2. Ribosomal DNA RFLPs

In our experience, rDNA is the most useful tool for identifying sibling species. Because it is chromosomally inherited and is present in tandem arrays, it is subjected to molecular drive [5] which tends to homogenize the individual repeats. Generally, the rDNA patterns of individuals within a species are essentially identical- there is usually as much variation within an individual as between individuals [6,7]. However, there are usually striking differences between species, either in restriction sites or length [7,8].

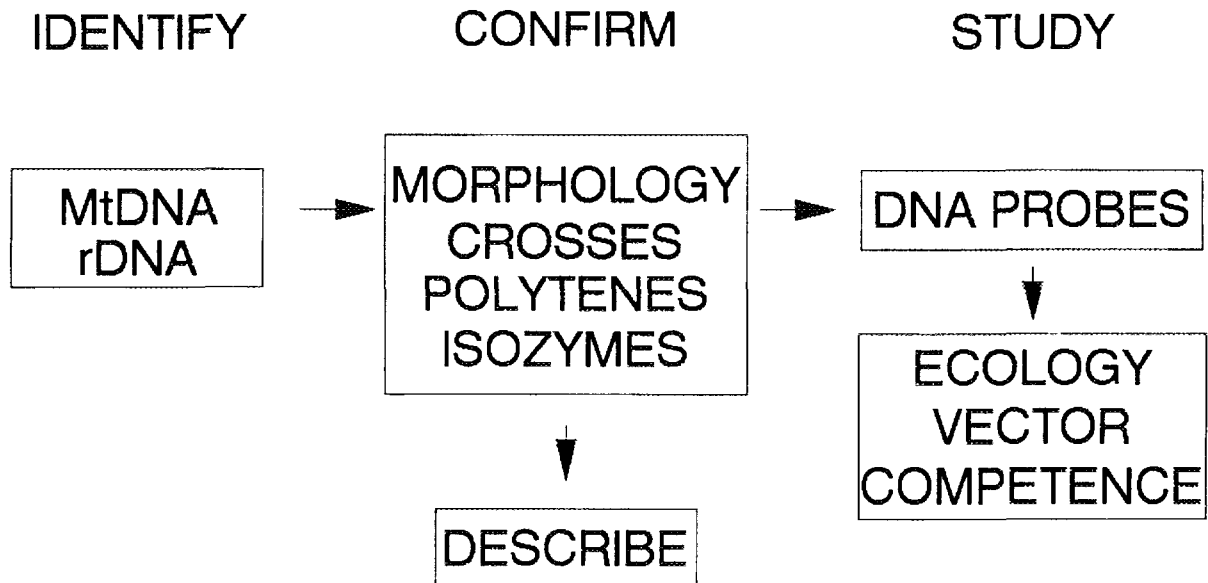


FIG. 1. Characterizing new cryptic species.

The significance of differences in the rDNA between individuals is magnified by the copy number. Mosquitoes and other insects usually have several hundred copies of the rDNA per genome, so any noticeable differences involve dozens of loci. A fixed difference between two individuals represents fixation at hundreds of loci. While these loci are not independent (because of gene conversion or unequal crossing over), neither are they equivalent to a single locus. The probability of getting such fixed differences between individuals in a freely interbreeding population is negligible.

rDNA patterns are much harder to interpret than mtDNA patterns. There are frequently differences in length among the different members of the repeat family even within an individual- this gives rise to bands that are blurred and impossible to measure accurately. It is also possible to gain or lose sites between different repeat units, giving rise to minor bands. Since the nontranscribed spacer diverges rapidly, a generic mosquito rDNA probe will only hybridize to the coding sequences, and fragments that contain only spacer will not be detected. Because much of the variation in rDNA patterns is insertion/deletion length variation and many of the bands are not detected, rDNA is essentially useless for phylogenetics.

3. DNA PROBES

There are three types of data that epidemiologists generally want to know about the individual mosquitoes in a collection: the species, the type of blood meal taken, and whether they are infected with a particular pathogen. Generally these are determined using different techniques- morphology for species identification; antibodies for blood meal identification; and ELISA, cell culture, and others for pathogens. This makes epidemiology expensive and difficult to do, since it requires equipment and expertise for a variety of techniques. We are trying to develop an integrated DNA probe based method for collecting all of this information.

3.1. Mosquito identification

The easiest way to identify a species is to find a single qualitative character that separates it from all other organisms. After determining the existence of a sibling species, we develop DNA probes for the rapid identification of species in samples of natural populations such as would be collected during distribution or epidemiological surveys. We currently have clones of repetitive DNA sequences that are useful for identifying species of the *A. quadrimaculatus* complex [9], species of the *A. crucians* complex,

A. perplexans, *A. punctipennis*, *A. atropos*, and a number of South American species [10]. We have shown that these can be used to identify thousands of individual mosquitoes by means of a convenient squash technique and standard DNA detection methodology [11].

Table 1: Cost estimates for identifying 1,000 individual mosquitoes using three different procedures used in our laboratory. Labor includes not only technical assistance, but also professional time needed for data analysis.

	Equipment	Supplies	Labor
Probes	\$500	\$50	\$75
PCR	\$5,000	\$2,000	\$1,000
Electrophoresis	\$1,000	\$200	\$500

We have used a differential screening strategy to isolate the species-specific clones that we have isolated [9]. However, we have found that many of these clones originate from the non-transcribed spacer of the rDNA locus. Therefore, we are beginning to sequence this region from all of the mosquito species that we have available, in order to create a database of potential species-specific sequences. When a new species is identified, it will be possible to determine whether existing probes will hybridize to it, and it will be simple to predict putative specific probes that will detect it. This should greatly simplify the task of identifying and validating these probes.

3.2. Blood meal identification

We have used a human Alu repeat to detect human blood in field collected mosquitoes. This probe is sensitive enough to detect human blood meals 48 hours after feeding, when the meal is almost totally digested. Using this approach, we have studied collections of blood fed *A. quadrimaculatus* from Manatee Springs, a park in North Florida.

We have previously shown that three species of *A. quadrimaculatus* occur at this location: species A, B, and C1. An excellent probe already exists for species A [9], and we isolated two other probes, one which hybridizes to both species A and B, and one that hybridizes to species C1, C2, and D. Since species C2 and D do not occur at this site, these three probes are sufficient.

Four identical sets of filters were screened with the Alu probe and the three *A. quadrimaculatus* probes. By superimposing the filters, it was easy to determine the species of each individual and which individuals had taken human blood. The higher prevalence of human blood in species A females indicates that this species is much more likely to feed on humans than sympatric species B or C1 (figure 2, table 2). When we pooled the data for the two campgrounds, the human blood feeding rates were significantly higher for species A than for species B or C1. The numbers of mosquitoes collected at the B pond site were too few for statistical analysis, but the low number of human blood meals in species B and C1 is consistent with the two campgrounds.

3.3. Disease detection in individual wild mosquitoes

In collaboration with researchers in Venezuela, we are using our species-specific probes in conjunction with the US Army's ELISA kit for detection of malaria parasites in mosquitoes to study the infection rates of species of the subgenus *Nyssorhynchus* in a hyperendemic region of South America. DNA sequences are available for most important arboviruses, and these can be used to predict sequences for use as probes or PCR primers. Kits for the detection of arboviruses are under development at the Centers for Disease Control and Colorado State University laboratories in Fort Collins. We plan to collaborate with these groups on the development and application of these kits in screening wild populations.

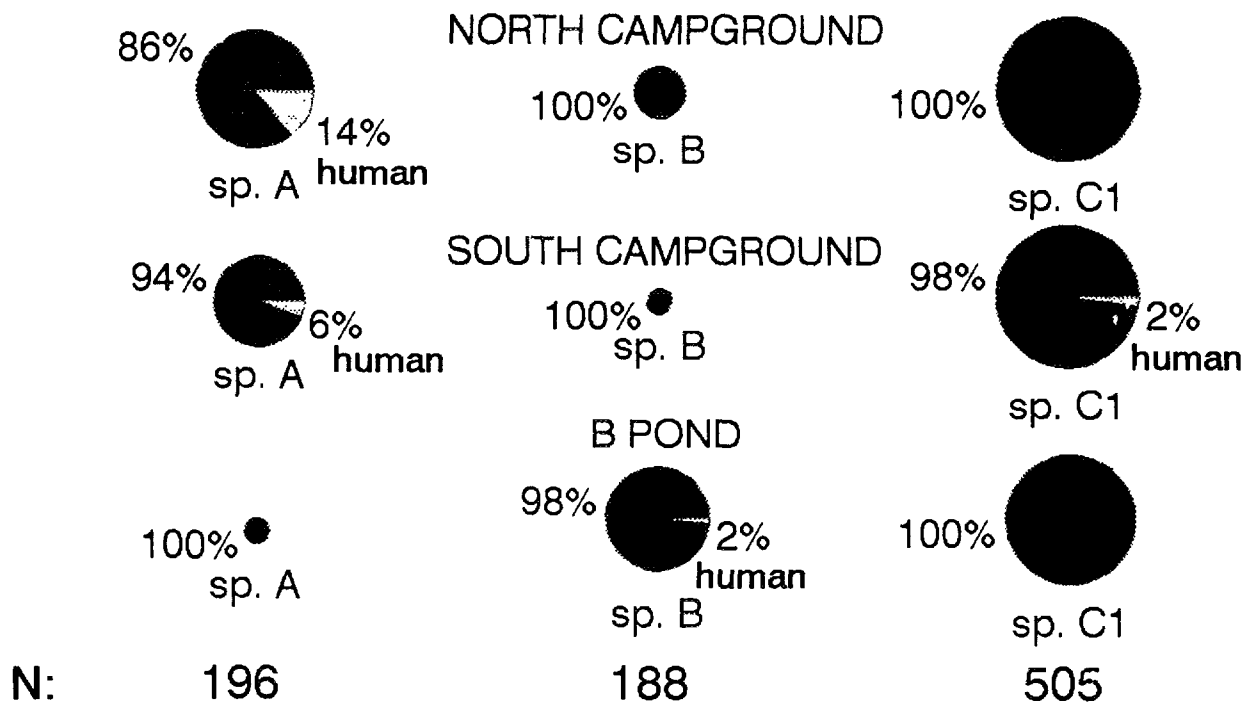


FIG.2. Human and nonhuman blood in *An. Quadrimaculatus* (from Manatee Springs, Florida).

Table 2: Numbers of blood fed mosquitoes of three species of *A. quadrimaculatus* complex from three locations at Manatee Springs State Park (blooded columns) and numbers that contain human blood (human columns).

	species A		species B		species C1	
	blooded	human	blooded	human	blooded	human
N. campground	106	15	36	0	167	0
S. campground	71	4	23	0	179	4
"B" pond	19	0	129	2	159	0
TOTALS	196	19	188	2	505	4

4. CONCLUSION

A common situation encountered when working with insect pests is the occurrence of several different species that look identical, at least to humans. This can be a problem because control efforts are partly wasted on innocuous species or because specific biocontrol agents (such as diseases or predators) attack the wrong species. Therefore, it is important to know whether a particular pest is a single species or a collection of several indistinguishable species. This paper discusses a multidisciplinary approach to identifying these species complexes, and explains how that approach has been applied to the study of several important mosquitoes.

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THE USE OF GENETIC TRANSFORMATION IN THE STUDY OF OVARIAN-SPECIFIC GENE EXPRESSION

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Abstract

We are using genetic and molecular approaches to understand the mechanisms controlling the establishment of the cellular specificity of expression during oogenesis. Female-sterile mutations have been isolated and the molecular analysis is revealing interesting cell-cell interaction systems that work not only during oogenesis but also at other developmental stages. We will review in this paper our most recent studies on genes involved in ovarian development.

1 INTRODUCTION

Different cell types of germ-line and somatic origin are involved in oogenesis which in *D melanogaster* has been divided into 14 morphological stages [1,2] and can easily be approached at genetic, cellular and molecular levels. To study the role of maternal genes involved in oogenesis and early embryogenesis the mutants to be selected have a large variety of phenotypes sharing a common characteristic: females are unable to generate offspring either because they are unable to lay eggs (female sterility) or because the embryos die (maternal effect). Notwithstanding the large collection of mutants isolated [3], it has been difficult to assign a functional role to the various genes. In addition, classical genetic approaches are unable to detect all the genes necessary for the production of a functional egg. It has been demonstrated that when a system of cell-cell communication has been well established, the genes involved will be used repeatedly during development [4]. Consequently, mutations in such genes will be zygotic lethal, and it will be impossible to assess the female sterility or the maternal effect produced by these genes. A very elegant method has been developed that allows the identification *in situ* of control elements of genes regulated during different developmental stages [5]. The method is called "P-element-mediated enhancer detection". The P transposon detects neighbouring genomic transcriptional regulatory sequences by means of a β -galactosidase reporter gene, which, in the majority of cases, responds to nearby transcriptional regulatory sequences in the *D melanogaster* genome.

Other methodologies, such as *in situ* hybridization [6] or DAPI staining [7] of ovaries, isolated egg chambers and early embryos can provide significant insight into the role played by genes expressed at different stages of ovarian development.

Our interest in genes involved in oogenesis started with the study of mutations isolated by Sandler in region 32 of the standard salivary gland chromosome map [8]. The mutations are named *hold up* (*hup*), *wavoid-like* (*wdl*), *daughterless-obo-like* (*dal*), and *abnormal oocyte* (*abo*) and are all recessive, hypomorphic and female semi-sterile. The semi-lethal phenotype shown by these mutations [8] and the demonstration of their zygotic action [9] seem to indicate that the corresponding genes are expressed not only during oogenesis but also in other tissues and developmental stages and that null alleles will be lethal for the flies.

Our present study on the control of gene expression during oogenesis avails of all the powerful recent methodologies (P-mediated mutagenesis, P local transposition and enhancer trapping) to isolate new mutations conferring female sterility to the flies in 32 and other regions of the second chromosome.

On the other hand, the molecular work carried out in the last years has led to the identification in region 32 of two genes with specific ovarian patterns of expression the Vitelline Membrane Protein gene 32E [10,11] and the gene coding for a receptor form of Guanylate Cyclase [12] We have started a detailed analysis of their structure and expression by P-mediated genetic transformation that in *Drosophila* is very well established [13]

We review in this paper the more recent data on the expression and regulation of these two genes, show the phenotypes of female sterile mutants recently isolated in our laboratory and illustrate the cellular specificity of expression of other genes identified in genetic screen by P-mediated enhancer detection or P-local transposition

2 RESULTS AND DISCUSSION

Starting with a plasmid clone containing a segment from region 32D, we screened a library constructed in the EMBL4 lambda phage vector with embryonic DNA from *D melanogaster Oregon R* stock and walked about 200 kb in this region, from band 32D to band 32E-F We used the recombinant phages by the following approaches 1) analysis of the transcription pattern of the DNA region isolated in our recombinant phages by screening of stage-specific cDNA libraries and 2) investigation of the molecular organization of region 32 to identify possible restriction enzyme site polymorphism correlated with the presence of one of the mutations under study

1) We hybridized restriction fragments of the recombinant phages isolated during the chromosome walk in that region with labelled cDNA prepared from poly-A RNAs extracted from different developmental stages of *Drosophila* (embryos, larvae, pupae, adult females, and adult males) We identified in this way genes selectively expressed in particular developmental stages We started our characterization with a fragment strongly hybridizing with a transcript present in females and not in 18 hr embryos as happen with a maternal transcript and discovered the gene coding for a Vitelline Membrane Proteins (VMP) We called the gene VMP32E from his chromosomal location [10]

2) By using as probes the DNA fragments from the phages isolated in the chromosomal walking, we performed restriction enzyme site polymorphism analyses in wild type and homozygous mutant flies We identified, in stocks carrying the *abo* mutation, a copia-like *blood* transposon in region 32E We demonstrated that the *abo* phenotypic expression is correlated with the presence of the *blood* transposon [14] The membrane form of Guanylate Cyclase (GC) *Drosophila* homolog gene was cloned as the first gene downstream (4.2 Kb) from the *blood* transposon insertion site [12]

2.1. The 32E Vitelline Membrane Protein Gene

The VMP32E gene was characterized in detail A peculiar feature of the VMP gene family was discovered a conserved hydrophobic domain, constituted of about 30 amino acids, is present in all the genes so far isolated [10,15,16] In the late stages of oogenesis, the follicle cells surrounding the growing oocyte are engaged in the elaboration of the egg-shells The eggshell proteins are produced in a characteristic temporal and spatial pattern that reflects the transcriptional regulation of the respective genes During stages 9-10 Vitelline Membrane Proteins are maximally synthesized, later, in stages 11-14 chorion proteins are produced Synthesis of the proteinaceous eggshell in *D melanogaster* is a system particularly well suited for cis-regulatory signals controlling gene expression in eukaryotes [17,18]

We are analyzing the VMP32 gene expression and regulation *in vivo* by P-mediated transformation We have demonstrated the existence of a compartmentalization within the follicle cells In transformation experiments performed with a VMP- β -gal gene fusion we demonstrated the existence of a cellular specificity of VMP32E gene expression [11] Expression is localized initially in a very small ventral group of columnar follicle cells at stage 10A egg chambers and progressively extends to the dorsal region as a large stripe surrounding the oocyte in stage 10B We do not observe expression in the polar terminal domains, which remain silent (Fig. 1) This is a peculiar feature of the VMP32F gene

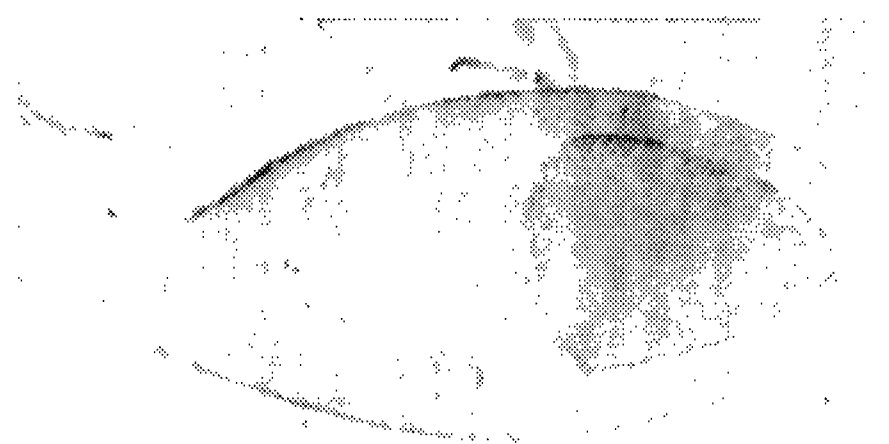
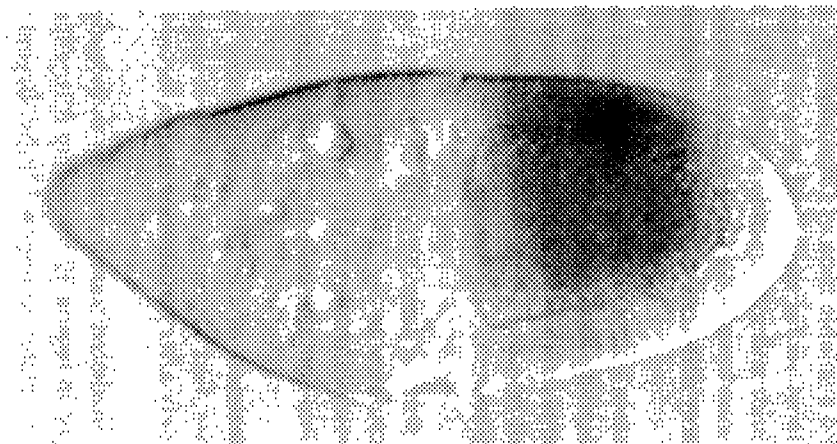
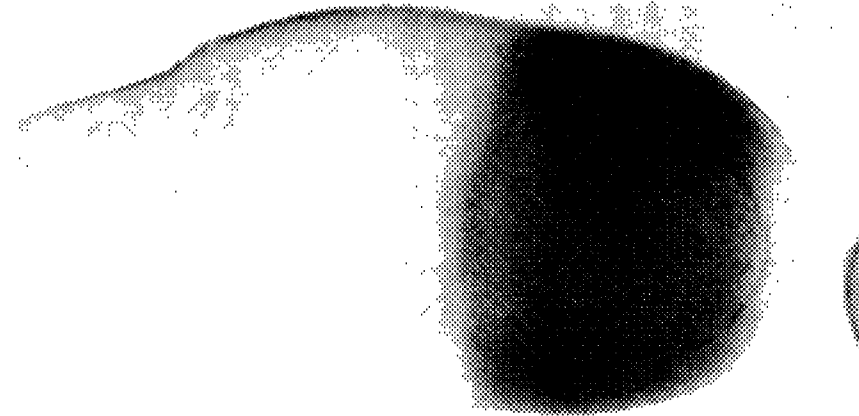


FIG. 1. β -galactosidase activity in ovaries of females transformed with the VMP32E- β -gal fused gene. The gene fusion carries the 5' upstream region plus the first 232 bp of the VMP32E coding region fused in frame with the b-gal gene. After P-mediated transformation, the ovaries of the transformed females were dissected by hand and examined by histological staining for b-galactosidase activity.

while the other genes so far tested, VM26A1 and VM26A2, are expressed all round the follicular cells, including the most posterior ones [15,19]. The peculiar temporal and spatial VMP32E gene expression may reflect a particular function of this VMP component and could be made possible by those genetic mechanisms co-ordinating the development of asymmetry in both the oocyte and the enveloping follicular epithelium [20].

We have dissected the promoter region of the VMP32E gene and we have found that DNA sequences extending from -465 to -39 bp upstream from the transcription initiation site are sufficient to direct the specific expression of the VMP32E gene. Deletion analysis in transformant lines revealed the presence of at least two cis-acting regulatory elements (-465/-249, -135/-39). Both these regulatory regions are necessary to determine the specificity of the VMP gene expression in ovaries [11]. We have recently demonstrated, in collaboration with F. Kafatos, that in the functionally defined -465/-249 fragment, where substantial similarities are found to the hormone response elements of chorion s-15 gene, the transcription factor CF1/Usp [21,22] which bind to the cis-regulatory sequences of the s15 chorion gene also binds the VMP32E regulatory region (Gargiulo et al., unpublished). This provides the first indication that similar regulatory strategies may be shared by chorion and VMP genes.

2.2. The Guanylate Cyclase receptor gene

Cyclic GMP levels change in response to a great variety of agents, including hormones and neurotransmitters. Several soluble and membrane forms of Guanylate Cyclase have been studied and described in detail. However, only recently, by taking advantage of the existence of conserved domains of the proteins, the corresponding genes have been identified in several species, including man [23,24]. It has been discovered that Guanylate Cyclases in sea urchins are strongly implicated as cell-surface receptors on spermatozoa for chemotactic peptides causing behavioral changes in spermatozoa that include both kinetic and directional effects on motility. In mammals, several forms of GC genes have been described and they are expressed in aortic smooth muscle, brain, kidney, adrenal gland, adipose tissue, ileum, human placenta, pituitary gland, olfactory tissues, and retina.

Only recently also a soluble form of *Drosophila* GC gene has been isolated in 63A1-2 [25]. All the GC membrane proteins so far described have a single hydrophobic sequence that divides the molecule into extra cellular and cytoplasmic regions. The intracellular catalytic region contains two different domains: a tyrosine kinase-like and a cyclase domain (Reviewed by Garbers [23]). The various regions of the protein show different degrees of conservation among the species.

Although the protein kinase-like region contains 25 out of the 33 amino acids highly conserved in protein kinases, this activity has not been demonstrated for GCs. The molecular organization of the *Drosophila* receptor gene is complex: it is made up of many small exons, each one coding for less than 50 aa, separated by small introns. All the functional domains, including the small transmembrane and the catalytic regions, are separated by introns.

The *Drosophila* protein shows varying degrees of conservation with other Guanylate Cyclases and also with adenylate cyclases. In Fig. 2 is reported the putative translational product of the *Drosophila* receptor GC with the *Drosophila* soluble GC, as obtained by the pileup GCG Wisconsin program. The soluble form is smaller and contains only the cyclase catalytic domains underlined in the figure [25].

The level of expression of the GC gene is very low. The transcript is detected by *in situ* hybridization [6] in ovaries, first in germaria and later at stage 10 (Fig. 3). A signal is also present in very early embryos, immediately after fertilization and in larval imaginal discs ([12] and unpublished).

We are using P-mediated transformation to investigate if the GC gene can rescue any of the mutations already mapped in region 32, including *l(2)gd* that is a tumor suppressor gene affecting the imaginal disc growth and isolated in region 32E by Bryant and Schubiger [26].

```

VIDGG LPLAI EDVVK NPILL PGKKL AFKPV DIGHK MSAYR VKPLR AMTQM 50
REAGV TAFIG PDESC TTEAL LASAW NTPML SFKCS DPIVS NKSTF HITAR 100
TLAPA SKVSK SVISL LNAFH WNKFS IVVSS KPIWG SDVAR AIQEL AEARN 150
FTTSH FKYIS DYIPT TKILS QIDKI IEETY ATTRI YVFIG EHIAM VDFVR 200
GLQNR RLLES GDYTV VSVDD EYDYS NRRVN IMERN YLDPY IRKEK SKSLD 250
KISFR SVIKI SMYYP QNPHI RVPIY GLHLY DSVMI YVRAI TEVLR LGGDI 300
YDGNL VMSHI FNRSY HSIQG FDVYI DSNED AEGNY TVITL QNDVG SGASI 350
GSLAK MSMQP VGFFA YDKNS VIPEF RYIKN DRPIQ WLNGR PPLAE PLOGF 400
HGELC PRKKL DWRYL VSGPL CALVV VVAIA LLIKH YRYEQ TLAGL LWKVD 450
MKDVT VINLG EYNNP TNKNI FQICR QSILV VGEPN KRSFT NIALF RGNIV 500
.....
MACPFFRRAD SLTRQPSVIA EPGGHWALED EELSDDALTL THLQMAIQLL

..... AMKKIHKKS VDI.TRSIRK ELKLMREVRH
TAPSNEDLNT AVTSLVAKYR QNWPNIHKLK LDPQTFKSCA NYDYLADI.Q

ENIINFIGAS TDHGSVII.. .FTTYCAR.. .....GSLE
ELLLKMDEAS ASEILVLLGE ELITCCCTGI IERAFCRLGT DLQEFGLSLD

DVLANEDLHL DHMFISLVS DILKGMIIYLH DSEIISHGNL RSSNCL....
GVYDVLKLQE EDVTDGTGVC AGEGELIFTS ERPVIAWLLL GSLKALTRML

.....IDSR WVCQISDFGL HELKLGQEEP .....
YKVDVNIKIE PVERCPAVSL PLLAGQGQLP DHADGSSTSV SKTIPETVQR

....NKSELE LK..... .RALCMAPEL ..... .LRDAYRPGR
SNSSNASDLQ MNSSSFCKMF PWHFIMNEQL ELVQLGRGFS KLYKPYMADF

GSQKGDVY.. .....SFGI LLYEMIGRKG PWGDTAYSKE
GCQATTYFDF KRPKGLTMKF RDIVRRTYTP FLIGLNNPPG AVDFPAIGLE

EIIQFVKCPE MLQHGVFRPA LTHTHLDIPD YIRKCLCQCW DEDPEVRPDI
IKGQMVHCPE SNSLLFIGSP FLD...GLDG LTCNGL...F ISDIPLHDAT

RLVRMHLKEL QAGLKPNIFD NMLSIMEKYA YNLEGLVQEA NQFVVRREEE
RFVILVGEQA RAQ.....D GLRRRMDK.. ..IKNSIEEA NSAVTKERKK

TDMLLYQMLP RPVAELLKRG DPVEAECFDC VTILFSDIVG FTELCTTSTP
NVSLHLHLIFP AEIAEKLWLG SSIDAKTYPD VTILFSDIVG FTSICSRATP

FMVVEMLNDW YTCCDSIIWN YDVYKVVTIG DAYMVVSGLP LQNGSRHAGE
FMVISMLEGL YKDFDEFCDP FDVYKVETIG DAYCVASGL...HRASIYDAH

IASLALHLLT TVGNLKIRHK RTETVQLRIG VHSGPCAAGV VGOKMPRYCL
RULDGLK MID ACSKHITHD. .GEIKMRIG LHTGTVLAGV VGRKMPRYCL

FGDTVNTASR MESTGDSMRI HISEATYQLL QVIGSYVC.I ERGLTSIKGK
FGHSVTIANK FESGSEALKI NVSPITKDWL TKHEGFEFEL GPRDPSFLPK

.....GDMR TYWLTKRQQP ELTPGSD... .....
EFPNPGGTET CYFLESFRNP ALDSELPLVE HINVSMTIS EGAMPRSPA

....
PIPP

```

FIG. 2. Amino acid sequence comparison between *Drosophila* receptor and soluble form guanylate cyclases, as shown by alignment produced by the pileup program in the GCG software package. The proposed catalytic domain and the region which is likely to be involved in recognition of the base by GCs are underlined. The sequence of the soluble form is from Yoshikawa et al., 1993.

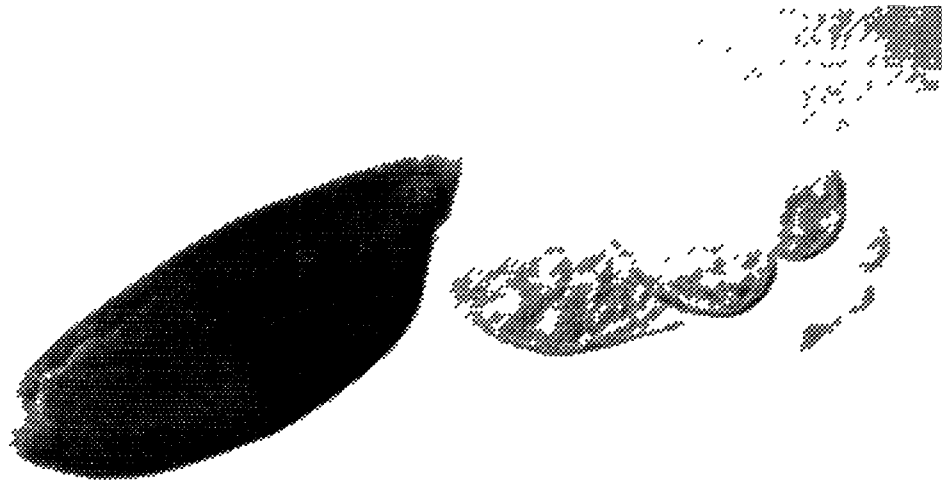


FIG 3 Whole-mount in situ hybridization on wild type egg chambers with digoxigenin-labelled GC cDNA probe performed according to Tautz and Pfeifle (1989)

For the moment, nothing can be said about the potential ligands of the *Drosophila* receptor protein except that they must be present in various tissues and developmental stages. Sea urchin egg peptides interact with the GC and cause behavioral changes in spermatozoa. Natriuretic peptides apparently elicit their physiological effects of natriuresis, diuresis, and vasorelaxation through the interaction with the receptor GCs and finally it has been shown that a heat-stable enterotoxin responsible for acute diarrhoea binds to an intestinal membrane form of GC [24]. The identification of possible ligands of the *Drosophila* receptor protein will be highly dependent on the identification of the tissues and cell populations expressing this gene and of mutant phenotypes due to the disruption of the GC gene function approaches which are presently followed in our laboratory.

2.3. Other mutants and lines recently isolated

We performed various sets of experiments either by using insertional mutagenesis with the "mutator" *Pry11* or *PlacZ* and the "jump starter" *P(D2-3-ry)* combination [27] or by mobilizing *Pry+* elements already inserted in region 32 [28].

With these approaches we have isolated new female-sterile mutations on the second chromosome: enhancer trapping lines showing β -gal expression in various ovarian cell subpopulations, two new *hup* alleles and homozygous lethal deletions in 32 region. In Fig. 4 are shown egg chambers stained with DAPI and isolated from females homozygous for the mutations *teg*, *abo* and *pla*. In these mutations the control of germ-line cell proliferation is affected.

In Fig. 4A, the *tegamino* (*teg*) mutation is shown. In this new mutant, obtained in a P element mediated enhancer detection set of experiments performed in our laboratory, sterility is complete. The oocyte position within the egg chamber is no longer fixed. Nurse cells are quite irregular in size, shape and number.

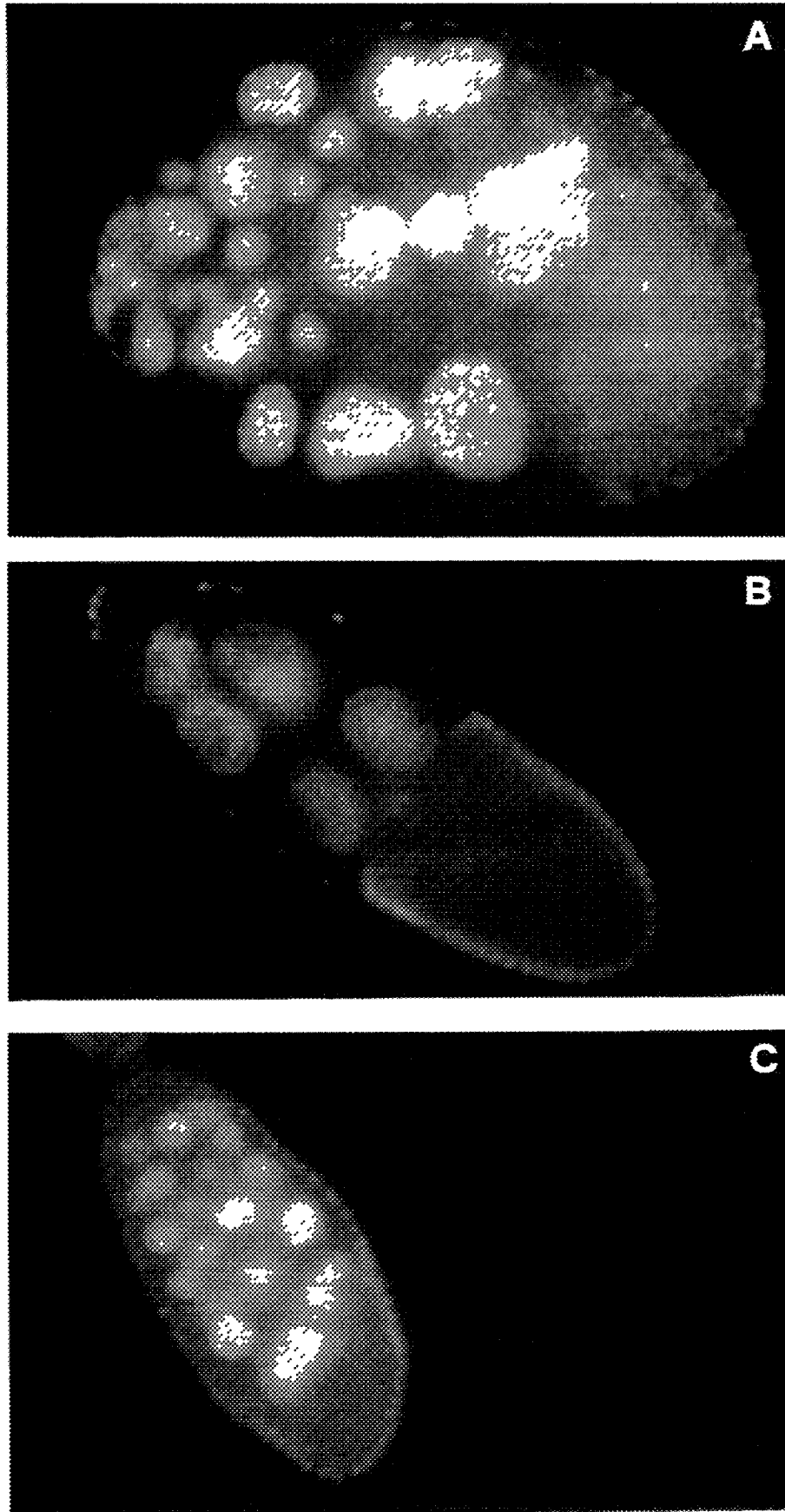


FIG 4 DAPI staining of egg chambers isolated from females homozygous for the following mutations A= *teg*, B= *abo*, C= *pla* For details see text

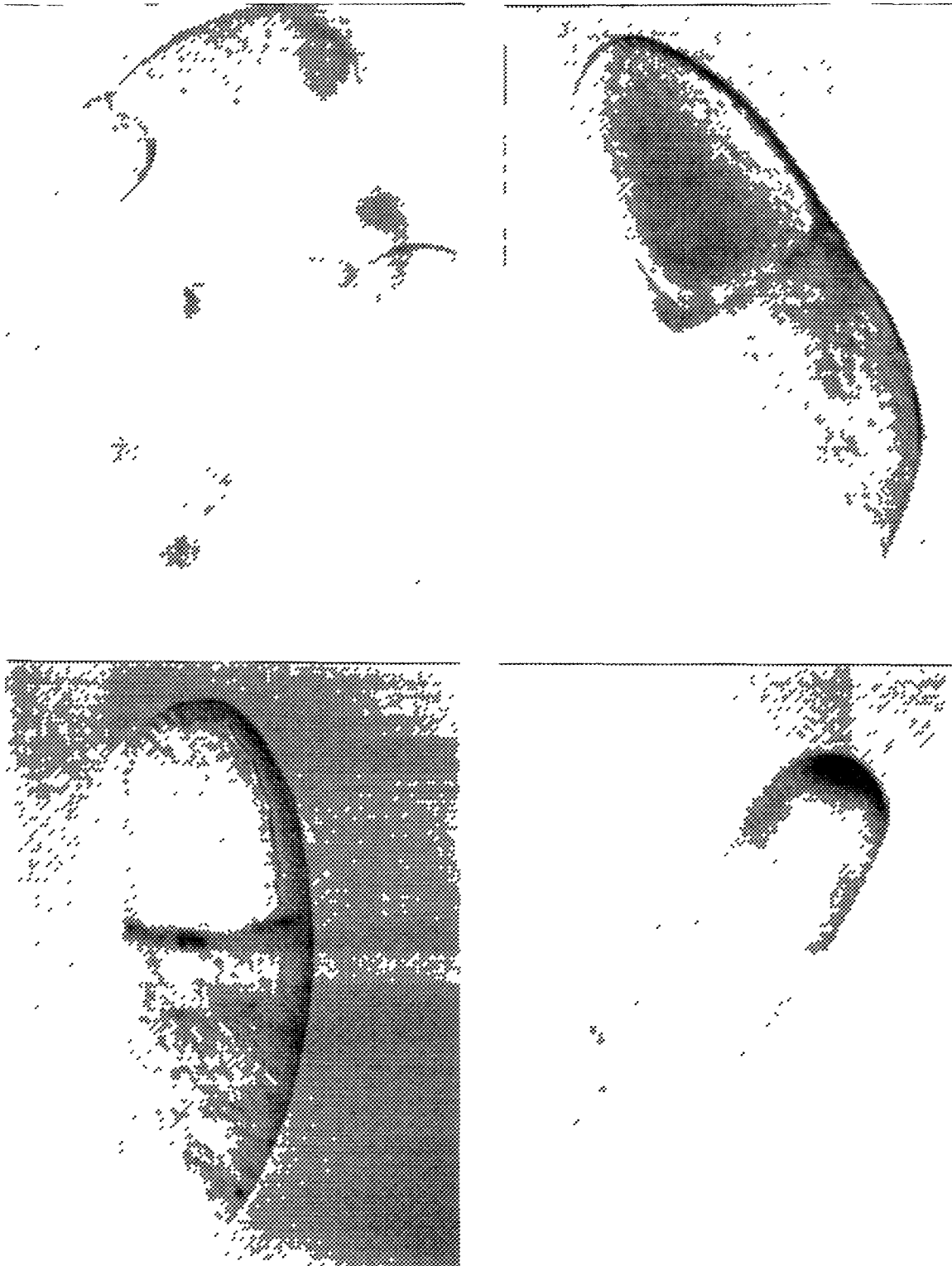


FIG 5 β -galactosidase activity in egg chambers of different enhancer trap lines isolated in our laboratories For details see text

In ovaries of *abo* homozygous females (Fig. 4B) we observed a reduced number of nurse cells in about 30% of egg-chambers, suggesting that the *abo* mutation affects germ-line cell divisions. Females homozygous for the *palla* (*pla*) mutation (Fig. 4C) do lay eggs, indicating that in most of the egg chambers many aspects of oocyte development occur relatively normally. Follicle cell nuclei show an uneven distribution around the oocyte and the eggs are shorter and flatter and show reduced dorsal appendages. A stronger phenotype is shown by 10% of the ovarioles, where fusion of adjacent egg chambers or tumorous follicles are observed (Fig. 4C). For some of these mutations we have already isolated lethal, wild type and weaker or stronger alleles due to imprecise P excision. We have mapped the genes by *in situ* hybridization and have isolated genomic DNA fragments. The β -gal expression of enhancer trap lines recently isolated in our laboratory are reported in figure 5.

Experiments are in progress to better characterize some of these lines and to clone some of the detected genes. In addition, we are using the β -gal expression of the different lines as ovarian specific markers to gain more insight on the positional cues existing in oogenesis.

ACKNOWLEDGEMENTS

We thank Mr. Enzo De Falco and A. Bellopede for technical assistance. This work was supported by National Research Council of Italy Special Project "Genetic Engineering" and is part of the FAO/IAEA Agreement No. 5116/CF.

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ANALYSIS OF THE *CERATITIS CAPITATA* Y CHROMOSOME USING *IN SITU* HYBRIDIZATION TO MITOTIC CHROMOSOMES

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Abstract

In *Ceratitis capitata* the Y chromosome is responsible for sex-determination. We used fluorescence *in situ* hybridization (FISH) for cytogenetic analysis of mitotic chromosomes. FISH with the wild-type strain EgyptII and two repetitive DNA probes enabled us to differentiate between the short and the long arm of the Y chromosome and gives a much better resolution than C-banding of mitotic chromosomes. We identified the Y-chromosomal breakpoints in Y-autosome translocations using FISH. Even more complex rearrangements i.e. deletions and insertions in some translocation strains were detected by this method. A strategy for mapping the primary sex determination factor in *Ceratitis capitata* by FISH is presented.

1. INTRODUCTION

Genetic Sexing is achieved by linking the wild-type allele of a selectable marker, via a reciprocal translocation, to the Y chromosome. Large scale separation of homozygous females and heterozygous males opens the possibility to release only males in SIT programs. Due to the structure of current sexing strains some disadvantages arise: a) occurrence of unbalanced gametes leads to semi-sterility and b) recombination in heterozygous males, although a rare event, leads to breakdown of the genetic sexing system. It is envisaged that sexing systems developed with molecular approaches will not have these problems. Isolation and germline transformation of a gene suitable for genetic sexing might overcome problems that arise with the use of translocation strains for genetic sexing. Genes involved in sex determination of *Ceratitis capitata* are good candidates for such an approach. This report will summarize a strategy to map the primary sex determination signal in *Ceratitis capitata* in order to facilitate cloning of this factor.

2. MATERIAL AND METHODS

Wild-type strain Egypt II was used to characterize the Y-chromosomal hybridization pattern of two DNA probes. The translocation strains T(Y;3)1-30 [1], T(Y;5)1-61 [2] and T(Y;5)30C [3] were used for mapping the Y-chromosomal breakpoints. As the Y chromosome does not polytenize, mitotic chromosome spreads were analyzed. Brain tissue of 3rd instar larvae was incubated for 15 min in 1% Na-citrate, prefixed in methanol:acetic acid (3:1) for 5 min, dissected in a little drop of 60% acetic acid on a slide and fixed on a hot plate. After 2 min in 80% ethanol, the slides were air-dried and frozen at -20°C over night up to one year.

Two probes were used to analyze the Y chromosomes: pY114 is a cloned DNA fragment from *Ceratitis capitata* that contains Y-specific, repetitive DNA [4]. 2H8 is a clone containing ribosomal DNA (rDNA) from *Drosophila hydei* [5]. DNA probes were labelled with DIG-11-UTP according to the instructions of the supplier (Boehringer).

For *in situ* hybridization, slides were baked for 2 h at 80°C and dehydrated in 80% and 100% ethanol. Chromosome spreads were denatured in 25 mM NaOH for 60 sec., washed for 10 sec. in 0.4 x

SSC, 0.1% Tween, and then dehydrated through a series of 70% (precooled at -20°C), 80% and 100% ethanol for 2 min each. The hybridization solution consisted of 20 ng DIG-labelled DNA probe, 50% formamide (deionized) and 35% master mix (1 ml 20 x SSC, 1 ml dextran sulfate, 1 ml aqua bidest. and 0.5 ml salmon sperm DNA (10 mg/ml, sheared to 200 - 500 bp). 10 µl hybridization mixture was denatured at 80°C for 8 min, cooled on ice and then applied to the slide, covered with a cover-slip and sealed with rubber cement. Hybridization took place at 37°C in a humid chamber for 12 - 18 h. After two washes in 0.4 x SSC, 0.1% Tween at room temperature, anti-DIG-antibodies (fluorescein-conjugated) were applied according to the instructions of the supplier (Boehringer). Antifade (0.233 g DABCO in 0.9 ml glycerol, 0.1 ml 0.2 M Tris pH 8) containing 0.2 µg/µl propidium iodide was placed on the slide. Pictures from epifluorescence microscopy were taken with Kodak Ecta GoldII (400 ASA) films.

3. RESULTS AND DISCUSSION

3.1. FISH with wild-type strain EgyptII

Fluorescence *in situ* hybridization using mitotic chromosome spreads of the wild-type strain Egypt II, probed with clone pY114, showed hybridization signals exclusively on the Y chromosome (Fig.1). The long arm of the Y chromosome was labelled except for the region next to the centromere and a gap of approx. 10% of the whole Y chromosome length, near the tip. The centromere and the short arm were not labelled.

In contrast, 2H8 labelled the short arm, the centromere region and approx. 10% of the long arm of the Y chromosome (Fig.1). Besides that, a region at the tip of the short arm of the X chromosome was labelled. This hybridization pattern is in good agreement with published results [6]. In addition, the centromere region of chromosome 4 showed a faint hybridization signal. On the long arm of the Y chromosome the hybridization signals of both probes overlap partially.

In situ hybridization with 2H8 and pY114 proved to increase the resolution compared with C-banding techniques of mitotic chromosomes. Even small parts of the Y chromosome, representing roughly 5 - 10% of the whole Y, can be detected. In addition, probes 2H8 and pY114 allow to distinguish between the long and the short arm of the chromosome. Therefore, *in situ* hybridization was used to identify breakpoints of several Y-autosome translocations.

3.2. Detection of translocation breakpoints

The Y-chromosomal breakpoint of the Y-5 translocation in strain T(Y;5)1-61 was mapped to the middle of the long arm (Fig.2). The Y⁵ consists of the short arm, the centromere and a part of the long arm, equivalent to approx. 30 % of the whole Y chromosome, while the 5^Y harbors the rest of the long arm of the Y. Inspecting the chromosomes of four male individuals, two were found to carry only the Y⁵, but not the 5^Y chromosome. This karyotype is caused by adjacent-1 segregation and its frequency fits well to the expected 50% unbalanced gametes in this translocation strain.

In strain T(Y;3)1-30 the Y-chromosomal breakpoint was mapped in the neighborhood of the centromere at the long arm (Fig.2). Probe 2H8 labels the Y⁵, but not the 5^Y chromosome, indicating that the breakpoint is located approx. 10% of the total length of the Y chromosome distal of the centromere on the long arm. One out of the six individuals that were analyzed proved to have an adjacent-1 karyotype.

The hybridization pattern of T(Y;5)30C showed, besides the Y-5 translocation, a more complex configuration (Fig.2). A considerable part of the long arm of the Y chromosome is deleted in T(Y;5)30C,

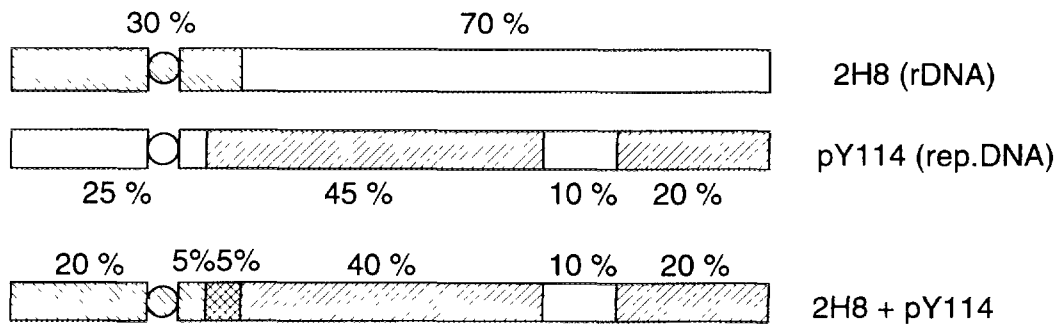


FIG. 1. *Y* chromosome of the wild-type strain EGYPT II.

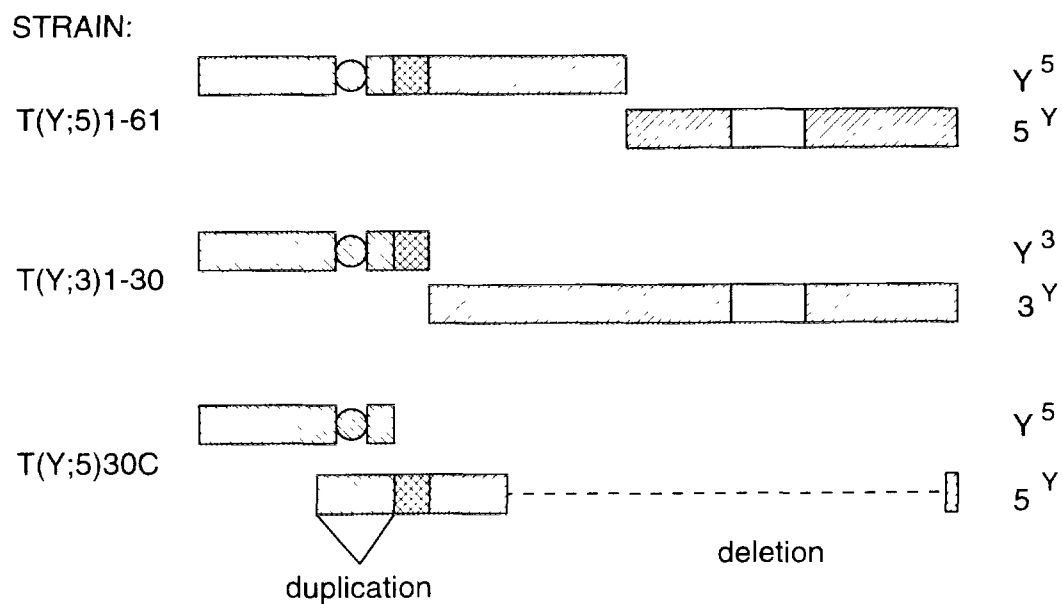


FIG. 2. *Y* chromosome in translocation strains.

the exact position of the deletion cannot be determined. The Y-chromosomal breakpoint mapped close to the centromere on the long arm of the Y. The rDNA signal on the 5^Y chromosome appeared to be twice as long as the one found in the wild-type karyotype. This is caused either by a duplication of this region or by an insertion of an rDNA containing segment of unknown origin. The pY114 signal appeared to be shorter than in the wild-type situation, the region around the "gap" is missing, thus a deletion of half of the long Y chromosome arm has occurred in this strain. Hybridization signals of pY114 and 2H8 overlap, as found in the wild-type karyotype, suggesting that this region of the long arm is not rearranged. As the signal of pY114 labels the tip of the 5^Y chromosome, the telomere of this arm should be of Y chromosome origin. Three out of eight individuals analyzed, showed an adjacent-1 karyotype with the Y⁵ and two chromosomes 5.

3.3. A mapping strategy for the sex-determination factor

Mapping of the breakpoints and of deletions in Y-autosome translocation strains opens the possibility to identify those parts of the Y chromosome that are relevant for sex determination. In Y-autosome translocation, the Y chromosome is split into two parts. Adjacent-1 individuals have a part of the translocated autosome in triplicate. Furthermore, the Y-chromosomal segment without centromere is absent. In strains T(Y;5)1-61, T(Y;3)1-30 and T(Y;5)30C 3rd instar larvae with adjacent-1 karyotype were found. In some strains such adjacent individuals develop into adults and they can be distinguished by their phenotype using the appropriate combination of genetic markers. In 1-61 ♂ x *or* ♀, all females are homozygous *or*, while all males are heterozygous and, therefore, wild-type. Adjacent-1 reach the adult stage and have a X,Y⁵,5,5 karyotype. The wild-type allele of *or* is located outside of the triplicated chromosome segment [2] and, consequently, adjacent-1 individuals are *or/or*. In this translocation strain adjacent-1 flies are male and can, therefore, be distinguished from the normal, balanced, males. The Y⁵ chromosome of strain 1-61 is sufficient for the development of male individuals.

In case of strain T(Y;3)1-30, the Y³ carries the *dp*⁺ allele while the free chromosomes 3 carries the mutant allele. This strain generates adjacent-1 females with wild-type phenotype. Therefore, the primary sex determining factor cannot be located on the short arm or on the centromere region of the long arm of the Y chromosome.

In strain 30C, half of the long arm is deleted. As this strain produces viable and fertile males, factors relevant for male sex determination cannot be located in the deleted part of the Y chromosome. Due to the complex rearrangement of the Y chromosome, at least one deletion, one insertion or duplication and one translocation breakpoint, we cannot map the deleted part in detail. *In situ* hybridization with other Y-specific DNA probes might overcome this problem.

Fig.3 summarizes the mapping of the primary sex determining factor on the Y chromosome by analyzing adjacent-1 offspring. Absence of the long arm of the Y chromosome leads to the development of female individuals, presence of this part to the development of male individuals. The primary sex determination factor in *Ceratitis capitata* is therefore a maleness factor.

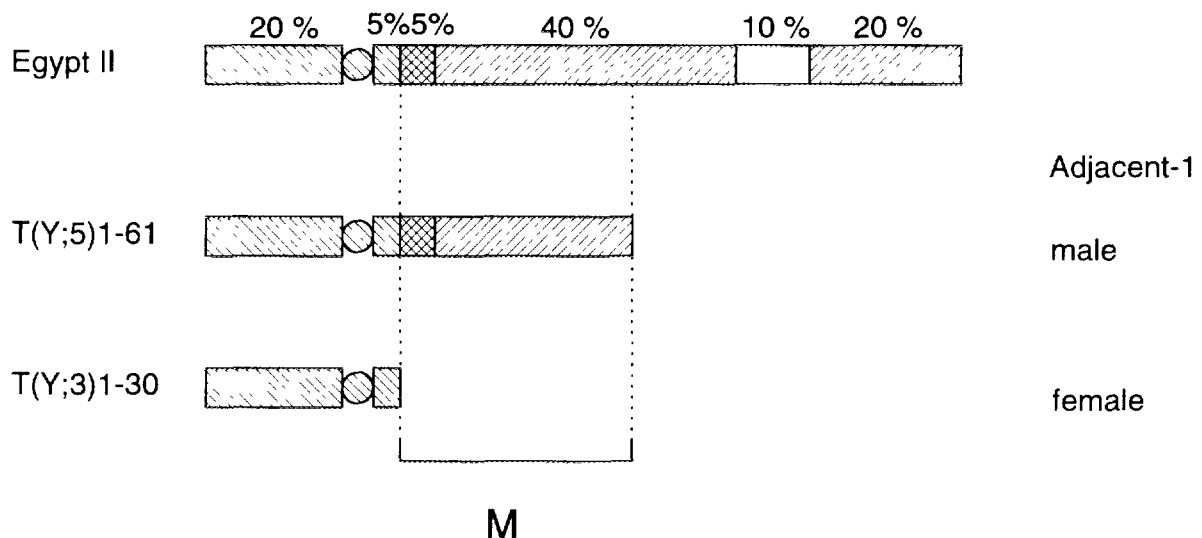


FIG. 3. Deletion mapping of Y factors.

These result is in good agreement with data obtained in studies of sex chromosome aneuploids [7], who found XXX individuals to be females and XXY individuals to be males. The number of X chromosomes is not of importance, while the presence of Y chromosome determines the male sex. A maleness factor responsible for sex determination was found in other Dipterean species like *Megaselia scalaris*, *Musca domestica* and *Culex tritaeniorrhynchus*. A different system of the sex determination is present in *Drosophila melanogaster*, where the X:autosome ratio is responsible for sex determination, while the Y chromosome has no impact.

In theory, a single maleness factor could be sufficient for sex determination. This factor would trigger a cascade of genes that determine somatic and germline development of either female or male characteristics. However, at present we cannot be certain whether one factor or several factors, responsible for sex determination, are located in the mapped part of the Y chromosome of *Ceratitis capitata*.

ACKNOWLEDGEMENTS

We thank Drs. D. Haymer and R. Renkawitz-Pohl providing us with the DNA probes, E. Schorn, O. Ibantschitz and M. Taher for excellent technical assistance in breeding the strains.

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TOWARDS THE GENETIC MANIPULATION OF MOSQUITO DISEASE VECTORS

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Abstract

Our research is aimed at developing the technologies necessary to undertake the genetic manipulation of insect vector genomes. In the longer term, we wish to explore the potential that this technology may have for developing novel strategies for the control of vector-borne diseases. The focus of our current research has been to: i) identify and characterise endogenous transposable elements in the genomes of mosquito vectors - research has focussed on identifying both Class I and Class II elements and determining their structure and distribution within mosquito genomes; ii) develop and use transfection systems for mosquito cells in culture as a test bed for transformation vectors and promoters - transfection techniques, vector constructs and different promoters driving reporter genes have been utilised to optimise the transformation of both *Aedes aegypti* and *Anopheles gambiae* cells in culture; iii) identify putative promoter sequences which are induced in the female mosquito midgut when it takes a blood meal - the *Anopheles gambiae* trypsin gene locus has been cloned and sequenced and the intergenic regions assessed for their ability to induce reporter gene expression in mosquito gut cells. The progress we have made in each of these areas will be described and discussed in the context of our longer term aim which is to introduce genes coding for antiparasitic agents into mosquito genomes in such a way that they are expressed in the mosquito midgut and disrupt transmission of the malaria parasite.

1. INTRODUCTION

Molecular biology has been used in a variety of ways to extend our understanding of insect vectors and their ability to transmit disease to human populations. It is hoped that in the longer term novel vector control strategies might be developed through the application of these techniques, possibly by creating transgenic insect vectors, either to suppress the vector population or to reduce their ability to transmit disease-causing organisms [1].

It also is important to consider what we are trying to achieve by applying genetic manipulation techniques to insect vector populations. This technology can be used as an analytical tool to provide a greater understanding of insect vector biology and ecology and the interactions between vectors, the disease-causing organisms they transmit and the human host. The expectation is that this knowledge will allow the development of new approaches to the control and management of these diseases by an increased ability to interrupt or disrupt disease transmission. This might be achieved through the identification of new targets for attack, the development of novel methods for insect vector population suppression, the ability to alter insect vector behaviour and hence disrupt transmission, and finally to alter the ability or efficiency of insects to transmit pathogenic organisms [2].

In any consideration of transgenic technology and how it may be applied to medically important insects, the following factors need to be considered: i) the practical requirements for creating transgenic insects; ii) how best to apply the technology and thus what gene systems from insects or other organisms

need to be defined in order to undertake the desired manipulations. Our recent research has focused on developing methods for introducing DNA constructs into cells and embryos, the identification of endogenous transposable elements which could form the core of transformation vectors, and the characterisation of promoters which are induced in the mosquito gut when it takes a blood meal. In the longer term we wish to introduce genes coding for anti-parasitic agents into mosquitoes in such a way that parasite transmission is blocked.

2 THE REQUIREMENTS FOR GENETIC MANIPULATION

Despite the dramatic advances made recently with respect to genome manipulation in *Drosophila melanogaster* there is an urgent need for a much greater understanding of the molecular biology of mosquito disease carriers. This must include an analysis of the complexity and organisation of their genomes and an understanding of the distribution of coding and repetitive sequences. These details form an integral part of the design and interpretation of cloning and hybridisation experiments. In addition, the exploitation of transgenic technology requires methods for the introduction of DNA both into living mosquitoes and into cultured cells. Ideally, this will involve a transformation vector which is capable of directing efficient and stable integration into the chromosomes of the recipient. The introduced DNA has not only to be expressed but should also carry a selectable marker for the identification of transformed individuals or cells. Ultimately, there will be a need to study alternative promoter and enhancer sequences so that the spatial and temporal expression of the introduced DNA may be controlled. Finally, none of this will be of any relevance unless appropriate genetic target systems can be identified, cloned and characterised at the molecular level.

2.1. Methods for introducing DNA into mosquito embryos

In relation to embryo microinjection, the early development of all insects is relatively similar in general terms to that of *Drosophila*. There may, however, be significant differences in developmental rate and embryo physiology. For example, mosquito embryos differ from those of *Drosophila* by having opaque, rigid chorions which cannot be removed without loss of viability. In addition, the mosquito embryo is extremely susceptible to desiccation during the period at which injection must take place.

Here, we describe the system which we have developed in our own laboratory for transformation of the mosquito *Aedes aegypti* [3]. However, similar techniques have been used elsewhere both for *Anopheles* and for other *Aedes* species, and all are based in general on the methods developed for *Drosophila melanogaster*. As indicated above, the rigid, opaque endochorion of the mosquito embryo cannot be removed, and the embryos are extremely sensitive to desiccation. However, glass capillaries with tips of 100-300µm x 4-10µm can be used to puncture the rigid endochorion without tearing it and deliver the DNA solution without damage to the embryo. The slightly viscous DNA solution can not be expelled manually from such a fine needle and is therefore injected by means of a two phase nitrogen supply. The lower pressure prevents backflow and the higher pressure delivers 160-800pl of DNA solution (corresponding to 1-5% of the embryo volume) into the posterior pole of the embryo at the syncytial blastoderm stage, before cell partitioning occurs. This is where the pole cells, which are the germ line primordia, develop. Injection of DNA close to the site of pole cell formation is not critical to germ line incorporation, but the timing is clearly important if the DNA is to be taken up by the developing germ line cells. All of our injections are normally completed within 2 hours of oviposition. After injection, the embryos are covered with a water-saturated halocarbon oil, which permits the normal uptake of water until they are returned to standard insectary conditions. In this way DNA has been introduced into mosquito embryos [3,4,5], with survival rates comparable to those obtained with *Drosophila* [6]. Similar methodologies have also been employed to introduce DNA into the embryos of silk moth [7], medfly [8], the sheep blowfly and the housefly [9].

2.2. DNA vector systems used in non-drosophilid transformation

The experimental design for the creation of transgenic insects, including mosquitoes, is based on that developed for *Drosophila melanogaster*. G_0 individuals which survive micro-injection with the P element vector/helper DNA, pUCHsneo/pUCHs $\pi(\Delta 2-3)$, are mated *inter-se* and allowed to produce progeny. These G_1 individuals are the first which might be expected to express antibiotic resistance throughout all tissues and larvae are therefore subjected to selection with the neomycin derivative, G418. The molecular nature of any transformation events is determined by DNA analysis using radioactively labelled transformation vector DNA to probe Southern blots of genomic DNA extracted from the putative transformants and their progeny [3,4]. Intact vector P elements have been detected in 5-10% of adults that have developed from injected embryos (G_0), confirming that the introduced DNA is not immediately broken down by the mosquito. Furthermore, we have detected the chromosomal integration of vector DNA in several G_0 individuals. This probably reflects direct incorporation into a proportion of the somatic cell nuclei since it is only in the following generation (G_1) that we might expect a germ line integration event to have been transmitted to every nucleus. More promisingly, vector DNA has been identified in the chromosomes of the G_1 and G_2 progeny of injected embryos, suggesting that integration has occurred in the germ line of the mosquito and that this DNA shows normal Mendelian inheritance. Some of these events, however, appear to be unstable from one generation to the next and this phenomenon, together with the molecular basis of the transformation events, awaits further investigation.

As indicated above, chromosomal integration of the introduced P element DNA has been observed in both *Anopheles* and *Aedes* mosquitoes and the integration events appear, in some cases, to be heritable and clearly involve the germ line of the transgenic mosquitoes [3,4,5]. Although these events did not result from normal P element transposition, some functional role of the P sequences can not be excluded. This is particularly true since similar experiments in *Lucilia cuprina* [9] and *Ceratitis capitata* [8] have failed to produce any integration of vector sequences. Research in other laboratories is now being directed towards the identification of these accessory *Drosophila* proteins and the cloning of the genes involved may facilitate high efficiency P transposition in non-drosophilids. It is clear, however, from this work and from other experiments involving the transfection of the same DNA into cultured mosquito cells [10] that the P element system in its present form is not suitable for routine use in the mosquito. Thus, whilst the means are currently available for introducing DNA into both mosquito embryonic germ lines and cultured cells, a major stumbling block is the lack of an appropriate, high efficiency, DNA transformation vector system for manipulating the mosquito genome.

In addition to the use of the P element derived DNA vector systems, experiments to assess the mobility of a number of other transposable elements in non-drosophilid systems have been attempted. These experiments have included the use of fully processed cDNA copies of both the *Ac* and *Spm* elements from *Zea mays* [11] which have been shown to transpose actively in a number of evolutionarily disparate organisms and may prove to act autonomously in mosquitoes [12]. In addition, experiments involving the introduction of *hobo*, *gypsy* and *mariner* into *Anopheles gambiae* embryos are currently underway.

2.3. The search for mobile genetic elements in mosquito genomes

It is clear that the germ line integration events so far observed in mosquitoes do not involve normal P element transposition. The absence of this controlled mobility poses certain limitations, for example with respect to transposon tagging for functional cloning. Research elsewhere is concentrating on the precise mechanism of P transposition and attempts are being made to modify the P element system for more general use [13]. Such research may yet lead to the "universal vectors" originally envisaged. At the same time, there remains the possibility that P elements may never function as efficient transposition mediated transformation vectors in non-drosophilids. We and others are therefore actively searching for endogenous transposable elements which may yet prove to be the most suitable transformation vectors.

The isolation of endogenous transposable genetic elements may ultimately prove central to the development of efficient transformation and transposon tagging systems in mosquitoes. A number of approaches have been taken to identify such mobile elements and one of these was to analyse specific gene systems, such as the ribosomal DNA of mosquitoes, in an attempt to isolate variants of these genes which may have arisen from the insertion of transposons. No such insertions have, as yet, been identified in *Aedes aegypti* DNA [14] but insertion events have been detected in the rDNA of *Anopheles gambiae* and these elements are being fully defined [15]. The elements appear to resemble a particular class of mobile element known as non-viral retrotransposons. It is unlikely, however, that these elements will prove useful as transformation vectors because of the ill defined nature of their mode of transposition. More recently, the polymerase chain reaction has been employed to identify sequences related to the *mariner* transposable element from *Drosophila mauritiana* in the genomes of a wide range of insect vector species [16]. It appears that *mariner* is of ancient origin and is widely distributed within insects. Active elements have been identified in *Drosophila* which can transpose into the genome of different *Drosophila* species. One can readily postulate the existence of active elements in other species, which can likewise cross species-specific boundaries. Currently, five principal subfamilies of *mariner* have been identified, although more may follow. As such, there may be restrictions on the transposition activity of these elements, but it seems that *mariner* has a far wider scope as a transformation vector than does the P element.

A major difficulty arises in the identification of active elements in species of insect, including *An. gambiae*, that carry a large number of copies of *mariner*. Another consequence of this may be that any *mariner*-based vector may be redundant in such an insect due to the presence of host/element repressor systems. However, a *mariner*-based vector should have great potential for the genetic manipulation of species with no, or very few, copies of the element present. This possibility seems unlikely, at least in the case of *An. gambiae*, where all strains so far studied possess large numbers of *mariner*-like elements.

Mariner could yet prove to be a potent tool for studying insect systems. Many in the field of transgenic mosquitoes are interested in the use of transposable elements to incorporate specific genes, such as those for refractoriness to *Plasmodium falciparum*, into wild populations of *An. gambiae*. As Kidwell and Ribeiro [18] point out, once a transposable element has become fixed in a population it is unlikely that a second opportunity would be available for using the same transposable element as a DNA vector in an autonomous system. In their review, they further explore the problems associated with the use of loaded transposons and the mechanisms that could be used to drive specific gene constructs through populations. These problems relate not only to *mariner*, but probably to any other potential transposable element-based transformation system. While there are high hopes and great expectations for *mariner* as a future transformation vector for the genetic manipulation of insect vector species, it would seem prudent to continue our search for other endogenous transposable elements in an attempt to develop a number of transformation systems and DNA vectors for the insect species of interest.

We originally adopted an alternative strategy to identify directly a specific class of mobile elements, known as retrotransposons, in the mosquito DNA. The approach relies on utilising the characteristic biochemical and structural properties of these elements. This work has led to the successful isolation of several retrotransposon-like elements from the *Aedes aegypti* genome [1,19]. We have also used PCR as a particularly rapid methodology to identify endogenous Class I, retrotransposon-like elements and Class II transposable elements in mosquito DNA [20]. Using PCR technology, together with oligonucleotide primers corresponding to highly conserved amino acid motifs in the *copia/Ty1* group LTR retrotransposon reverse transcriptases, we have amplified DNA sequences from an *Ae. aegypti* total genomic template. The major PCR product is a DNA fragment of approximately 250bp. One would expect to observe a product of this size following amplification of autonomous elements from the *copia/Ty1* group using these same primers. Following DNA sequencing, the deduced amino acid sequences were aligned with the amino acid sequences of reverse transcriptases of well characterised LTR retrotransposons including *copia*, *Ty912*, *Tal-3* and *Int-1*, and the *Ae. aegypti* sequence clearly exhibits considerable homology to these counterparts.

The cloned PCR product was used to identify and isolate homologous genomic clones from a representative EMBL4 total genomic library. Interestingly, when the entire sequence of one of these genomic clones was determined, the clone contained two incomplete non-LTR retrotransposons in opposite orientation in the DNA. One element contains an open reading frame showing considerable homology to *copia*-like elements (including protease, integrase and reverse transcriptase motifs), and the other homology to the second open reading frame of LINE-like elements. Neither element seems to be complete, and neither contains LTRs. We also have evidence that sequences homologous to the LINE-like ORF are actively transcribed in *Ae. aegypti* cells in culture, whereas the *copia*-like sequences are not. We are currently analysing other representatives of both these elements in an attempt to find more complete elements which may have retained their ability to mediate their own transposition in mosquitoes.

Class II transposable elements are characterised by their mode of transposition through a DNA intermediate and short inverted terminal repeat sequences flanking a transposase encoding sequence required for transposition. Class II elements have proved to be powerful tools for gene cloning through transposon tagging, as markers for genome elucidation and as DNA transformation vectors. Several elements have fulfilled these roles, the most notable being the P-element of *Drosophila melanogaster* [21]. However, as with the P element these transposons appear to be seemingly ineffective outside closely related species and endogenous elements may be required to carry out similar functions in a species or genus of interest. The *Tc1* element was first recognised genetically in the nematode *Caenorhabditis elegans* [22]. It is 1610bp long with 54bp perfect terminal repeats [23]. The *Tc1* transposase has been recently elucidated by Vos *et al* [24]. *Tc1* has proved invaluable for transposon tagging, leading to the isolation of genes and genome elucidation of *C. elegans* [25]. Given the broad spectrum of animals in which *Tc1*-like elements have been discovered it seems highly probable that members of this family of transposons will be identified in many more diverse organisms. We have now identified *Tc1*-like sequences in the *An. gambiae* genome using PCR and oligonucleotide primers corresponding to conserved amino acid motifs within the transposase encoding region of *Tc1*-like elements. The *An. gambiae* *Tc1*-like PCR clone, TRANG, exhibited homology to the transposase region of several *Tc1*-like elements from a variety of organisms and shares 78% amino acid sequence identity with the corresponding region of the active *Tc1* element from the nematode *C. elegans*. Southern blot analysis of genomic DNA from several strains of *An. gambiae* probed with TRANG indicates a very low copy number of its counterparts within this mosquito genome. Variations in the position of TRANG in the genome further suggests that it is, or was, capable of transposition. Genomic clones containing this sequence have been obtained and sequence analysis is nearing completion.

2.4. The search for stage and tissue specific promoters for use in mosquito transformation vectors

At some stage it will be desirable to express defined genes in mosquitoes and other insects in a tissue or stage specific fashion. For this to be envisaged, stage and tissue specific promoters have to be defined. None are, as yet, available but attempts to characterise the DNA sequences responsible for expressing certain genes, particularly in mosquito systems, are underway by identifying genes which are expressed in a tissue specific fashion and then defining the upstream, putative tissue-specific promoter sequence. One example of this approach, has been the identification of an *Ae. aegypti* sequence which is only expressed in the female salivary gland [26]. The expectation is, therefore, that this will allow the definition of a salivary gland-specific promoter sequence which may eventually allow the controlled expression of an introduced gene sequence in this tissue. More recently, trypsin genes have been cloned and characterised from *Ae. aegypti* [27], the Blackfly, *Simulium vittatum* [28] and *An. gambiae* [29]. In each case, the expression of one or more of these trypsin genes has been shown to be induced in the insect midgut by a blood meal. It is therefore likely that gut-specific, blood meal inducible promoters will shortly be available for each of these insects. Such promoters are clearly of interest as they will allow the expression of antiparasitic agents in the insect gut when it takes a blood meal, that is, when the insect first comes in contact with the organisms which it can transmit to the human population.

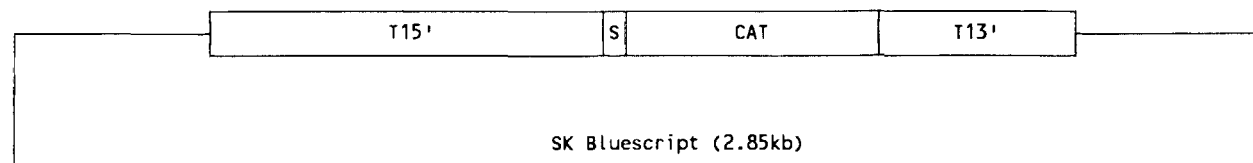
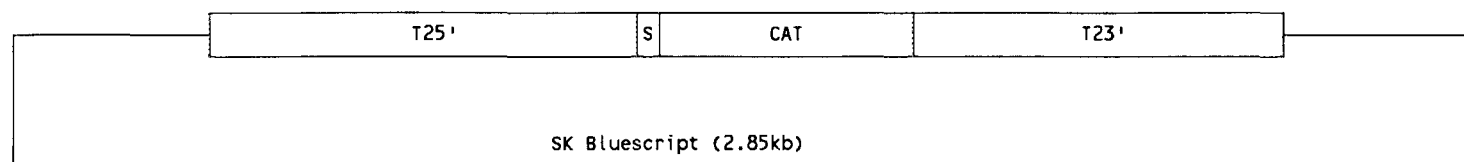
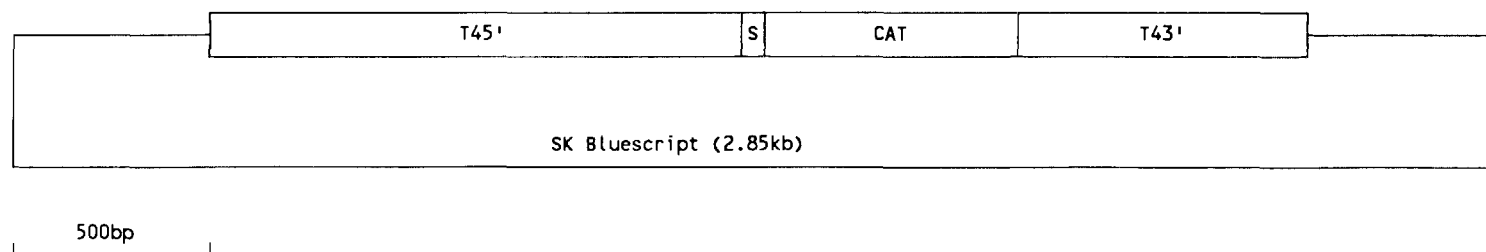
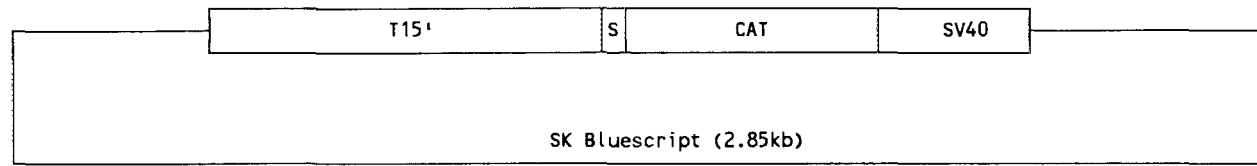
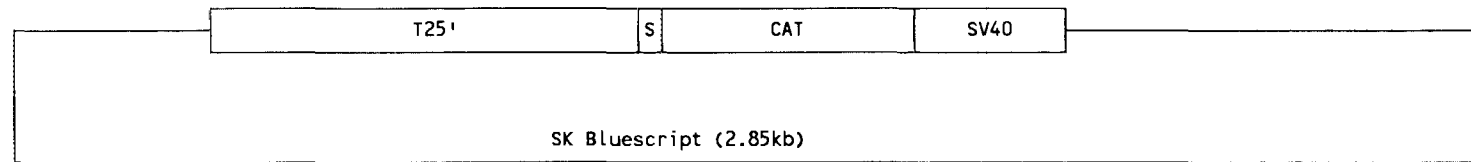
T1C3' (5.18kb)**T2C3'** (5.67kb)**T4C3'** (5.71kb)

FIG. 1. Maps of plasmid constructs incorporating *Anopheles gambiae* trypsin gene 5' regions, signal sequence (S), the CAT reporter gene and SV40 terminator. Numbers refer to the trypsin genes of *An. gambiae* as described by Muller et al [29].

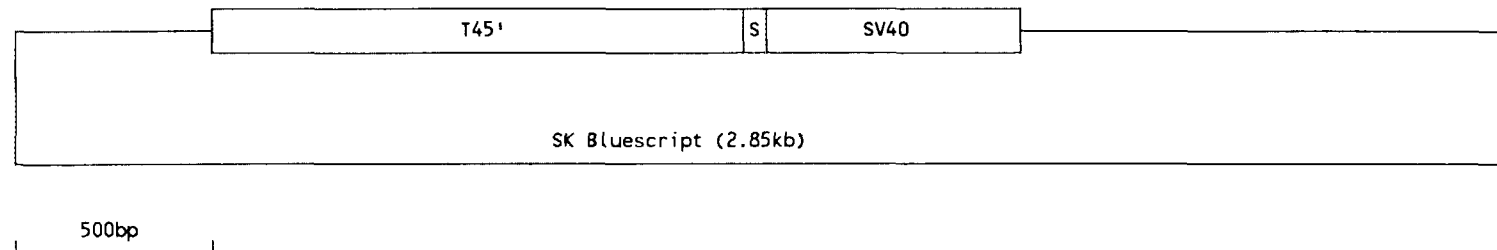
T1C



T2C



T4C



*FIG. 2. Maps of plasmid constructs incorporating **Anopheles gambiae** trypsin gene 5' and 3' regions, signal sequence (S), and the CAT reporter gene.*

However, it will be necessary to develop methods for establishing the functionality of putative promoter sequences. To this end, we and others have begun to develop the methodologies for transfecting mosquito cells in culture.

The simple, controlled environment of cultured cells allows one to follow the expression of cloned genes, and so delineate promoter and enhancer sequences. Genetically manipulated cell cultures are also able to over-produce specific proteins which facilitates their isolation and purification. Cultured mosquito cells have been used to examine different transfection techniques and vectors and to help establish a suitable system for germline transformation of *Ae. aegypti* [30]. Initially, experiments involved introduction of the P element vector and helper constructs into several cell lines by a variety of techniques devised to generate transient cell membrane pores, including calcium phosphate precipitation [31], dextran sulphate [32], polybrene [33], electroporation [34] and lipofection [35]. Much of this work has concentrated on the immortal Mos20 fibroblast cell line which was derived from minced, trypsinised, neonate larvae of the *Aedes aegypti* London strain in 1969. Polybrene and electroporation mediated transfection have proved to be most successful for these cells, producing approximately 30 and 4000 transformants per 10^6 cells, respectively. Subsequently, constructs incorporating the chloramphenicol acetyl transferase (CAT) reporter gene system have been utilised to optimise expression of the CAT gene under the control of the *Drosophila* heat shock promoter, hsp70, in the Mos20 mosquito cultured cells [36].

Subsequently, in collaboration with A. Crisanti and M. Muller (Rome) and R. Sinden (Imperial, London), we have used these transfection techniques to introduce reporter gene constructs into *An.gambiae* gut cells in culture as a means to begin defining functional blood meal inducible promoters derived from the *An.gambiae* trypsin and chymotrypsin-like genes. Genomic clones have been isolated encompassing both these loci [29], and constructs derived utilising the 5' and 3' regions from trypsins 1, 2 and 4, and chymotrypsins 1 and 2. In one set of constructs, the CAT reporter gene together with the trypsin-derived signal sequence has been placed downstream from the 5' region of each gene (Fig. 1); in a second set of constructs the same signal/reporter has been placed between the 5' and 3' region from each gene (Fig. 2). The Act/CAT/SV40 construct has been employed as a positive control, and each of these constructs has been transfected into cultured primary gut cells derived from blood-fed and non-blood-fed female mosquitoes. 2.2kb of the 5' region of trypsin 2 was sufficient to induce CAT expression in transfected gut cells from blood fed females, but not from non-blood fed females. In the case of Trypsin 1, both the 5' region (2kb) and 3' region (1kb) were necessary to induce CAT expression in transfected gut cells from blood fed females. Deletion constructs of these regions are being assessed to determine the minimum region of DNA necessary to induce expression in similar cells. Preliminary gel shift experiments are underway to define whether there is any evidence of factors binding to these regions. Similar experiments are being carried out with the chymotrypsin genes from *An.gambiae*.

3. THE POTENTIAL APPLICATION OF TRANSGENIC TECHNIQUES IN MOSQUITO VECTORS

Once the systems necessary to create transgenic insects have been developed, how may this technology be applied? Three possible uses of transgenic technology in insects include: i) its use as an analytical tool to increase our understanding of insect biology (including ecology, behaviour, population genetics, evolution, insect/pathogen interactions, etc); ii) to introduce anti-parasite genes into mosquitoes so as to eliminate parasites, or disrupt their onward transmission to vertebrate hosts (this requires the 'useful' genes to be driven through insect population with high efficiency); iii) the technology could be used to improve the SIT so as to suppress insect populations more effectively. Our recent studies have focused on the second of these applications.

3.1. Potential target genes for manipulation

There are a number of obvious targets for manipulation including the genes involved in the insect immune system, developmental control genes and insecticide resistance genes. Genes influencing all of these factors have now been characterised at the molecular level for a number of different insects and it is now feasible to consider manipulating them in the germ line of these insects. In addition, a number of genes are of particular interest because they are directly implicated in the ability of insects to transmit disease causing organisms. Examples include the filarial susceptibility (f^m) and *Plasmodium* susceptibility (*pls*) loci of the mosquito, *Aedes aegypti*. The f^m locus is genetically well defined and there is good data on its linkage relationships. Refractoriness to infection is due to a partially sex-linked, dominant gene [37]. There is marked variation in the susceptibility of this mosquito to different filarial worms, although all of the alleles concerned map at about the same place on the sex chromosome. Also of particular interest is a strain of *Anopheles gambiae* which has been selected for refractoriness to the malaria parasite and characterised genetically [38]. Attempts are currently underway to clone these genes but it is difficult to undertake such a cloning exercise in the absence of any knowledge of the gene product. Considerable progress has now been made towards the molecular characterisation of this gene, with the development of a linkage map for the X chromosome of *Anopheles gambiae* [39,40]. Once fully refined, this map should allow the genes influencing refractoriness to be cloned and fully characterised. Clearly, the use of transgenic technology through transposon tagging will assist in the characterisation of refractory genes and their products.

An important genotypic characteristic not met by the majority of genes encoding refractoriness is that any such gene introduced into the insect would have to be capable of altering the phenotype through the expression of a single gene copy. Unfortunately, at present, there is no gene or gene product defined at the molecular level which is known to directly affect phenotype in relation to pathogen development in, or transmission by, any insect. However, in the mosquito system a number of molecules are known to affect the transmission of malaria by anophelines. Foremost among these are the so called transmission blocking vaccines, which can achieve a total transmission blockade [41]. These vaccines attack antigens present on the gametes and ookinetes of the malaria parasite and antibodies which recognise these antigens are able to block the development of the parasite in the mosquito midgut. A very exciting possibility therefore is to introduce the genes coding for such antibodies into the mosquito genome thus directly conferring the transmission blocking phenotype to the insect. In this case, a transgenic mosquito would be created incorporating an antibody gene expressed in the insect midgut in response to a blood meal and which therefore blocks transmission of malaria. In its simplest form, this approach would involve introducing what is, essentially, a monoclonal antibody into the mosquito. This would not be ideal for a number of reasons, and some way of introducing polyclonal transmission blocking antibody coding genes would be required. However, for the present, the introduction of a single transmission blocking antibody gene under the control of a gut specific, blood meal inducible promoter is being attempted as a model to assess the system.

This type of approach is an attractive one for a number of reasons. It eliminates the need for the detailed molecular analysis of refractory mechanisms in mosquitoes and it would be a 'dominant' gene system (ie one gene copy only would be needed in each cell of the mosquito). The antigen target on the stage of the malaria parasite present in the mosquito is highly conserved, suggesting that the parasite may be less able to avoid this type of transmission control mechanism. Finally, the use of transgenic insects incorporating an antibody gene could be applied to any vector transmitted pathogen (parasite or viral) where a target antigen can be identified as being inhibited by the expressed molecule.

To date, mouse antibody genes have been cloned and introduced into mosquito cells in culture and mouse Fab molecules have been expressed and detected using immunohistochemical staining techniques. The transmission blocking antibody genes coding for Pbs21 have now been cloned and are being expressed in mosquito cells to ensure that they produce functional antibody. As indicated above, we have available DNA sequences which will induce gene expression in blood fed mosquito gut cells. It may therefore be possible in the near future to link the transmission blocking antibody gene to these

promoter sequences and create a mosquito expressing the transmission blocking antibody genes in such a way as to block or disrupt the transmission of malaria. If successful, transgenic mosquitoes expressing antimalarial antibodies may represent a potential strategy for controlling malaria and may establish a precedent for a wide range of new anti-disease strategies.

4. TRANSGENIC INSECTS: THE FUTURE

The questions posed by considering the release of transgenic insects emphasise the need to assess the biological consequences of such a release. It is, however, difficult to gauge the possible hazards of such a release in the absence of experimental evidence and these ethical and safety considerations need to be faced at an early stage. In order to undertake an informed appraisal where the possible net benefits may be balanced against the potential hazards, considerable effort will have to be devoted to utilising caged populations and the controlled release of molecularly tagged individuals together with mathematical modelling of these populations. One particular problem of releasing transgenic insects into natural populations is the concern that the transgenes may 'escape', ie move through both the target insect population as well as other organisms with which they have contact. Such organisms may include symbionts, or closely related species with which the target population may (rarely) interbreed. This clearly poses a problem, particularly where a major aim will be to drive beneficial genes through target populations, obviating the necessity to release large numbers of transgenics. Regulatory bodies may require assurances that gene constructs will never be able to spill over into closely related species. Whilst the probability of such events may be extremely rare, concern about the possibility may well stop the application of transgenic insect technology. In the case of direct release of transgenics to a defined target population in a limited locality such a problem could be overcome by pre-sterilising the insects. This would be similar to the current SIT, and would necessitate repeated, large scale release of the relevant transgenic insects. These possibilities are for the future, and await the development of reliable and efficient DNA vectors systems to construct the desired transgenic insects.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support provided by the Wolfson Foundation, Wellcome Trust, Medical Research Council, Liverpool University and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. JMC is a Wellcome Trust Senior Research Fellow in Basic Biomedical Sciences.

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Abstract

In the animal kingdom, many species with euchromatic heteromorphic sex chromosomes have developed mechanisms for the equalization of gene products in the homo- and the heterogametic sex. This mechanism, called dosage compensation, is achieved in *Drosophila* by the doubling of the transcriptional activity of X-linked genes in the male, in comparison to the female. Any failure in achieving dosage compensation causes lethality: haplo-X male individuals that fail to hyperactivate their single X-chromosome as well as diplo-X female individuals that hyperactivate their X-chromosomes die. Five genes that are involved in the regulation of this process have been identified. In males, a group of four genes, the so called male-specific lethals (*mle*, *msl-1*, *msl-2*, *msl-3*), must be active in order for hypertranscription to occur, whereas in females the *Sxl* gene, the master key gene of sex determination, has to be active to prevent the *msl* genes from becoming active. However, XX individuals with mutations in *Sxl* cannot be rescued by mutations in the *msl* genes indicating that at least one member of this group is yet to be uncovered. Furthermore, the *msl* gene that is regulated in a sex-specific manner has not yet been identified. Given that *msl-1* is transcribed in both sexes but its protein product is nearly absent in females, we have investigated whether this gene is the target of sex-specific regulation. We have also carried out extensive genetic screens for the purpose of identifying additional members of the *msl* group. These investigations are necessary prerequisites to the development of genetic sexing techniques based on the constitutive expression of *msl* genes in females causing female-specific lethality.

1. INTRODUCTION

As a prerequisite for the development of genetic sexing techniques, genes have to be identified that are expressed in one sex and not in the other. In the case of the dipterans, *Drosophila melanogaster* seems to be the model system of choice for this purpose, with the ultimate goal of characterizing homologous genes in the other species of interest.

One process known in *Drosophila*, which when disturbed causes sex-specific lethality, is the mechanism of dosage compensation. Measured at the level of X-linked transcripts or enzyme activities, males and females are equal in spite of the difference in number of X-chromosomes present in their karyotypes. This equalization takes place by a twofold enhancement of transcription of the single X-chromosome in males. The process is initiated in the preblastoderm stage and is maintained throughout development and in adult stages. Five genes were identified as playing an important role in the regulation of the process of dosage compensation (for reviews see [1,2,3]).

In females, the *Sex-lethal* gene (*Sxl*) has been shown to be responsible for the repression of X-chromosome hypertranscription. Any failure to initiate the production of SXL protein causes female-specific lethality in embryonic stages while failure to maintain this production is lethal throughout development. Lethality is caused by the hypertranscription of X-linked genes [4].

In males, four genes (*mle*, *msl-1*, *msl-2* and *msl-3*), generally called the male-specific lethals (*msl*), are necessary for the proper hypertranscription of the X chromosome. The lack of

hypertranscription in mutant animals results in male-specific lethality [5,6,7] throughout larval development [5,8,9,10,11]. Three of the *msl* genes have been characterized at the molecular level. The *mle* gene encodes the Drosophila analogue of human RNA helicase A [12,13], the *msl-1* gene encodes a novel protein with an acidic N terminus that is characteristic of proteins involved in transcription and chromatin modeling [14] and the protein encoded by *male-specific lethal-3* (*msl-3*) is novel, as well [3]. Antisera made against these three gene products (MSL-1, MSL-3 and MLE) detect the presence of their respective antigens at numerous sites along the X-chromosome in males; MSL-1 and MSL-3 are associated with only a few autosomal sites in males and MLE is associated with some autosomal sites in males and females. Loss-of-function mutations in any of the four *msl* genes prevent the binding of the three identified gene products in mutant males. These results indicate that the preferential association of each of the *msl* gene products with the X-chromosome in males depends on the presence of the wild-type products of the other three *msl* genes [15,16,17,18]. These observations lead to the important conclusion that the *msl* gene products must interact as a precondition to X-chromosome binding in males.

Surprisingly, no sex-specific differences could be detected in the transcripts of any of the three characterized genes; furthermore, their proteins seem to be present in both males and females [3,12,14,15,17]. However, in the case of MSL-1, the concentration in females is much lower than in males [17]. Whether this difference is caused by sex-specific regulation at the translational level or whether it is the result of decreased stability in females due to the failure of MSL-1 to bind to the X chromosomes, is the first question asked in this paper.

Given the evidence that *Sxl* represses *msl* gene functions either directly or indirectly, and given that loss of *Sxl* function in females results in lethality because of hypertranscription of their two X chromosomes, one could expect that XX animals mutant for *Sxl* could be rescued by mutations in any of the *msl* genes. However, this is not the case [4,19]. Additionally, it has been shown that the transcription rate of the X-linked gene *runt* (*run*), transcribed at the blastoderm stage, is not affected by *msl* mutations, whereas it is affected by *Sxl* mutations defective in the initiation process [20,21]. Moreover, the failure to achieve hypertranscription due to the constitutive expression of *Sxl* in haplo-X animals causes lethality in embryogenesis, while mutations in *mle*, *msl-1*, *msl-2* or *msl-3* (or any combination of these) result in a much later lethal period [5,8,9,10,11]. These observations indicate that there must exist at least one undiscovered gene responsible for dosage compensation in males. Here, we describe two extensive screens performed to identify genes located on the X-chromosome that are involved in the process of dosage compensation.

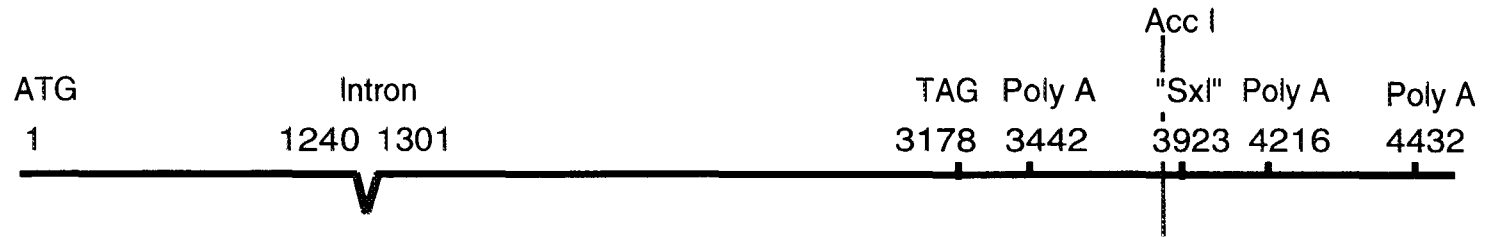
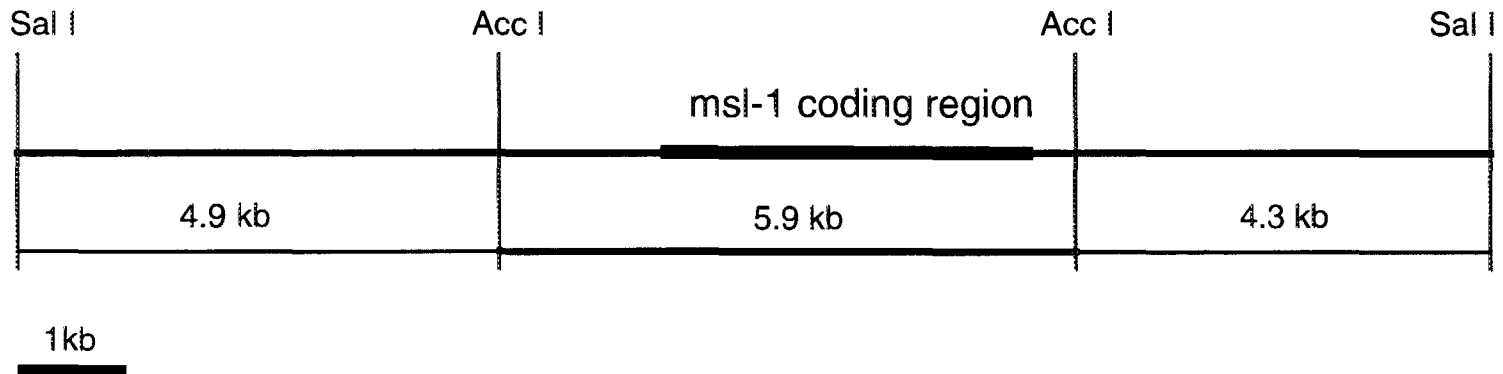
2. MATERIALS AND METHODS

2.1. Fly stocks and culture conditions

Unless noted otherwise, all crosses were done at room temperature that ranged from 22°C to 25°C on standard food (corn meal, sugar, yeast, agar, and molt inhibitor). For genetic symbols see [22].

2.2. Construction of a truncated *msl-1* gene and germline transformation with *msl-1*ΔE3'

A 15 kbp Sal I genomic DNA fragment containing the *msl-1* gene [14] was digested with Acc I. A 5.9 kbp fragment containing approximately 2 kbp of upstream sequences and lacking the putative SXL binding site and the two most distal polyadenylation signal sites at the 3' end was selected (see Figure 1 A, B and C). The ends of this fragment were filled in and it was inserted by blunt end ligation into the EcoRV site of pBluescript, recovered as a Kpn I - Not I fragment and subcloned into the transformation vector pCaSpeR4 [23] marked with the *w⁺* minigene. This construction was co-injected with the helper plasmid pUChs1Δ2-3 into host embryos of genotype *w¹¹¹⁸* using standard techniques [24]. Transformants were recovered according to their colored eye phenotype. The construction of

A**B****C**

<i>tra</i>	T C T T T T G T T G T T T T T T T C - - T A G T G T C A T A
	* * * * * * * * * * * * * * * * *
<i>msl-1</i>	A T T T T T A A A T A T T T T T T T T C T T T A G C C C T T A T
	* * * * * * * * * * * * * * * * * * * * *
<i>Sxl</i>	G T G T A G A C A T A T T T T T T T T C - A C A G C C C A G A A

Figure 1: - Structure of the *msl-1* gene (A). Situation of the *Acc I* sites in the 15kbp *Sal I* fragment, which provides fully rescue when introduced in *msl-1* mutant males (B). Sequence homologies between the SXL binding sites of the *tra* and *Sxl* pre-mRNA compared with the trailer sequence of *msl-1* (C).

homozygous transformant lines, the identification of the chromosome containing the insertion and the introduction of the transgene into *msh-1*^{Y222} mutant lines were performed using standard genetic procedures.

2.3. EMS mutagenesis

The two genetic screens utilized are illustrated in Figures 2 and 3. In both cases, the EMS treatment was performed according to the method of Lewis and Bacher [25]. Note that the females with the treated X-chromosome used in the F₁ cross need not be virgins because the only male offspring produced by the previous cross are of FM6 genotype for the X-chromosome.

2.4. Flipase expression during early development

Virgin females heterozygous for a new lethal mutation induced on the X-chromosome of genotype *y w*¹¹¹⁸ *P{ry⁺=neoFRT}19A/FM6*, *y*^{31d} *B* were crossed to males of genotype *P{ry⁺=neoFRT}19A/Y; MKRS*, *P{ry⁺=hsFLP}3*, *M(3)76A¹ kar¹ ry² Sb¹/+*. Eggs were collected for three days and the adults transferred into a new vial for an overnight collection. Immediately after removal of the adults, both egg collections were placed in a waterbath for 1hr at 37°C. This treatment is expected to produce flipase activity causing mitotic recombination at the base of the X-chromosomes in region 19A (Figure 4; [26]). Adults of phenotype *v*⁺, non-*B* and *Sb*, were then screened microscopically for *y* or *w* clones in the cuticula and eyes, respectively.

2.5. Localization of males-absent-on-the-X (max)

The *y ct v fos*^{SH} chromosome was used to obtain a coarse localization of the map position of *max*. Since the data indicated that *max* is located between *y* and *ct*, a second mapping experiment was carried out with a *cm ct*⁶ *sn*³ chromosome.

2.6. Western blotting

Proteins were extracted from ten third instar larvae of genotype *Basc*, *y*⁺/*Y*, *Basc*, *y*⁻/*y max* and *y max/Y*, using the color of the mouth hooks and gonad size for identification. Preparation of the extracts, SDS-PAGE and transfer of the proteins to nitrocellulose were performed according to Bopp et al. [27]. Following blocking, the blots were treated with anti-SXL polyclonal serum (diluted 1:500) followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antiserum.

3. RESULTS

From 560 embryos injected with the truncated *msh-1* gene lacking the putative SXL binding site and two poly A signal sites, 204 survived to adulthood and 144 were fertile when crossed to *w*¹¹¹⁸ partners. Five independent transformant lines were established from that cross with insertion sites on the X-chromosome (2), second chromosome (1) and third or fourth chromosome (2). No reduction in viability of females heterozygous or homozygous for the transgene was observed in any of the transformant lines. Proof that the transgene is actually transcribed and translated is provided by the observation that it rescues male flies mutant for *msh-1*^{Y222}. The absence of any effect of the *msh-1*Δ3' transgene in females indicates that the *msh-1* gene cannot be the *msh* gene that is regulated in a sex-specific manner or that it is not the only gene that is regulated in this manner.

As mentioned before, several genetic observations indicate that there are additional genes, that are involved in the process of dosage compensation [21]. We chose to search for these on the X-chromosome. Such a search had not been undertaken because of an operational difficulty: in order to demonstrate that a mutation is male-specific, homozygous mutant females must be shown to survive and their synthesis is not trivial in the absence of mutant males. The mutation *mei-S332* causes a high frequency of nondisjunctions in the second meiotic division [28] and provides, therefore, an optimal tool to generate females with both X-chromosomes derived from their mother.

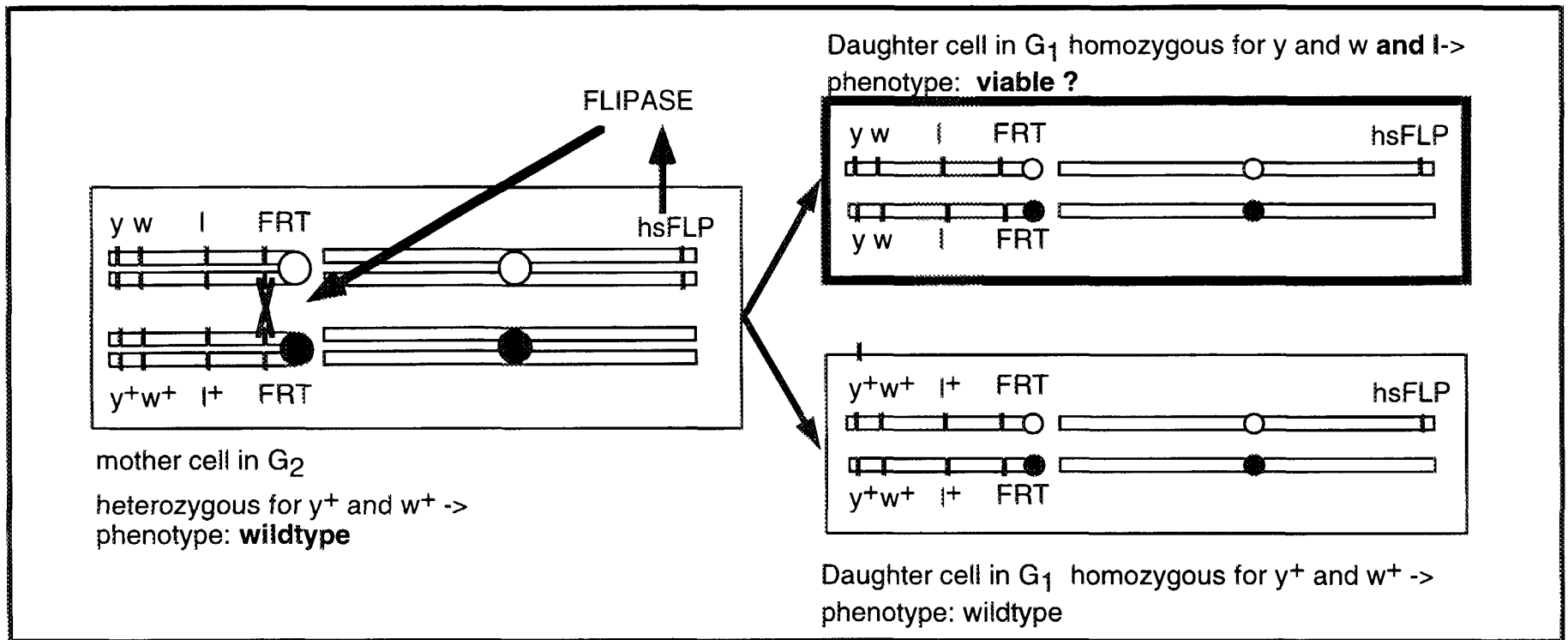


Figure 2: - Genetic crosses to isolate newly induced lethal mutations on the X-chromosome that interfere with male-specific lethality. The mutation *mei-S332* causes a high frequency of nondisjunctions in the second meiotic division (RT II). Two different *mei-S332* chromosomes were used to reduce the possible presence of a homozygous suppressor of the *mei-S332* effect. FM6 and CyO are balancer chromosomes dominantly marked with B for the X-chromosome in the former and dominantly marked with Cy for the second chromosome in the latter case.

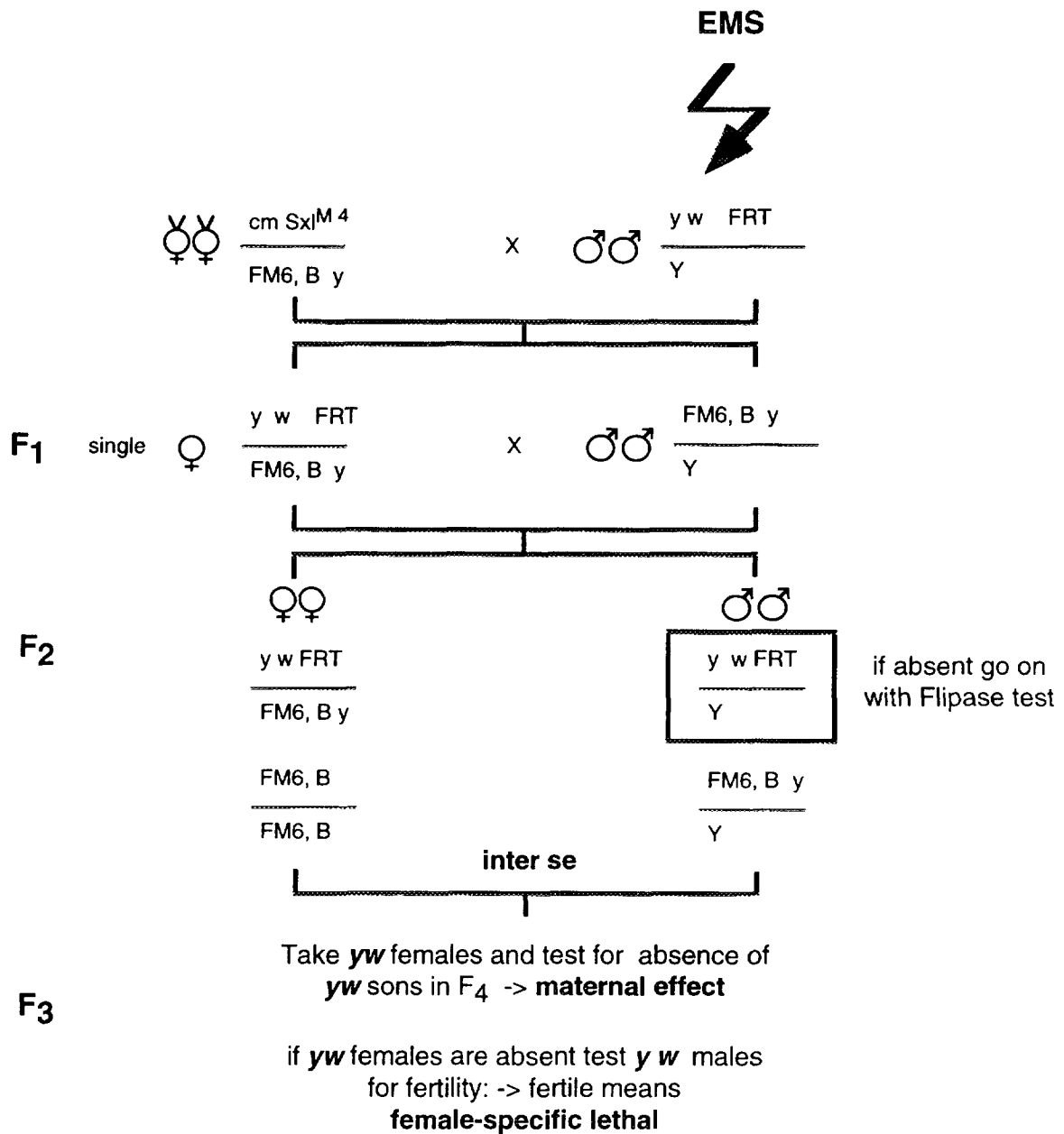


Figure 3: - Genetic crosses to isolate newly induced lethal mutations on the X-chromosome that interfere with male-specific lethality. The FRT stands for the flipase target sequence $P\{ry^+ = neoFRT\}19A$ at the base of the X-chromosome in region 19A. FM6 is balancer chromosome for the X-chromosome dominantly marked with B.

In the first screen some 2400 lines were successfully checked for the presence of females homozygous for the mutagenized X-chromosome (Figure 2). Seven putative male-specific mutations were found. However, six of these mutations have not produced any homozygous mutant (non-disjunctional) females since the original test progeny. One line continues to produce non-disjunctional homozygous females and is the basis for further investigations.

In a first genetic mapping experiment the mutation responsible for the male-specific lethality was placed close to the *cut* gene(*ct*). This mutation, named *males-absent on the X* (*max*), was mapped more precisely at 1-13.8. The lethal phase of males mutant for *max* was determined by isolating, using the color of their mouth hooks, *y max* mutant male larvae from among the progeny of *y max/Basc* females crossed to *Basc/Y* males (*Basc* is a balancer that is wildtype for the *y* gene). The development of the mutant larvae was found to be similar to that previously described for the *msl* mutants [11]. Male larvae mutant for *max* reach the third larval instar, but arrest there for up to two days, form prepupae and die. Nevertheless, one cannot conclude from these results that the *max*⁺ product is not needed in embryogenesis. Homozygous *y max* females are not able to produce sons even if the latter carry a wild type allele-bearing paternal X-chromosome derived by nondisjunction; furthermore such females crossed to wild type males produce few female larvae with *y* mouth hooks. Therefore, we expect to find a strong maternal effect in our subsequent investigations.

Could *max* be lethal in males because it causes SXL function to occur? We tested this possibility by performing a Western blot of crude protein extract of male larvae mutant for *max*. The results showed that there is no SXL protein present in these males and that *max* is not a mutation in a gene which leads to the constitutive expression of *Sxl*.

In our second screen, we tried to circumvent the labor intensive care of the *mei-S332* stocks (Figure 3) by using FRT-FLP system found in yeast and introduced in *Drosophila* by Golic and Lindquist [29] and Golic [30]. We wanted to test female viability in large cell clones homozygous for a X chromosome carrying a lethal factor (Figure 3 and 4). Approximately one quarter of 10,000 successful single crosses seemed to have a newly induced lethal mutation on the treated X chromosome. Two hundred of these lines were exposed to heat shock as described in material and methods. Lines that produced *y w FRT* males and were therefore not carrier of a lethal factor on the X-chromosome served as controls. Unfortunately, no clones that were induced during embryonic development could be found in any of the lines although small clones that were induced later in development were present, indicating the functionality of the flipase system *per se*. Even the extension of the heat shock period to four hours, while causing much higher mortality, did not help in generating early clones.

Some 7,500 lines with no apparent lethal factor on the X-chromosome were used to set up maternal effect test crosses. Approximately 200 of these lines carried a female-sterile mutation, 500 were male-sterile, 100 were female-lethal or just subvital in both sexes and 40 produced no or just low numbers of sons. The last group is of special interest since they resemble the phenotype of the *sonless* (*snl*) mutation [31,32] that was lost several years ago. However most of these lines can be qualified as representing maternal-effect embryonic lethal mutations that allow a zygotic rescue by the paternal chromosome. Since only female zygotes receive a paternal X-chromosome only sons were killed. Nevertheless, this is a very interesting group of mutations that will be the target of further investigations.

4. DISCUSSION

The discovery of a key gene, *Sex-lethal* (*Sxl*), the master regulatory gene that controls both sex determination and dosage compensation in *Drosophila melanogaster*, was the starting point of the belief that highly effective genetic sexing techniques should become available soon for the biological control of pest insects. However, it is becoming increasingly apparent that, in all Dipteran insect species checked to date, an SXL homologous protein is present in *both* sexes and that its function may not be that of a developmental switch in the control of sex differentiation. Therefore, the search for the appropriate genes to be used in genetic sexing approaches is still open.

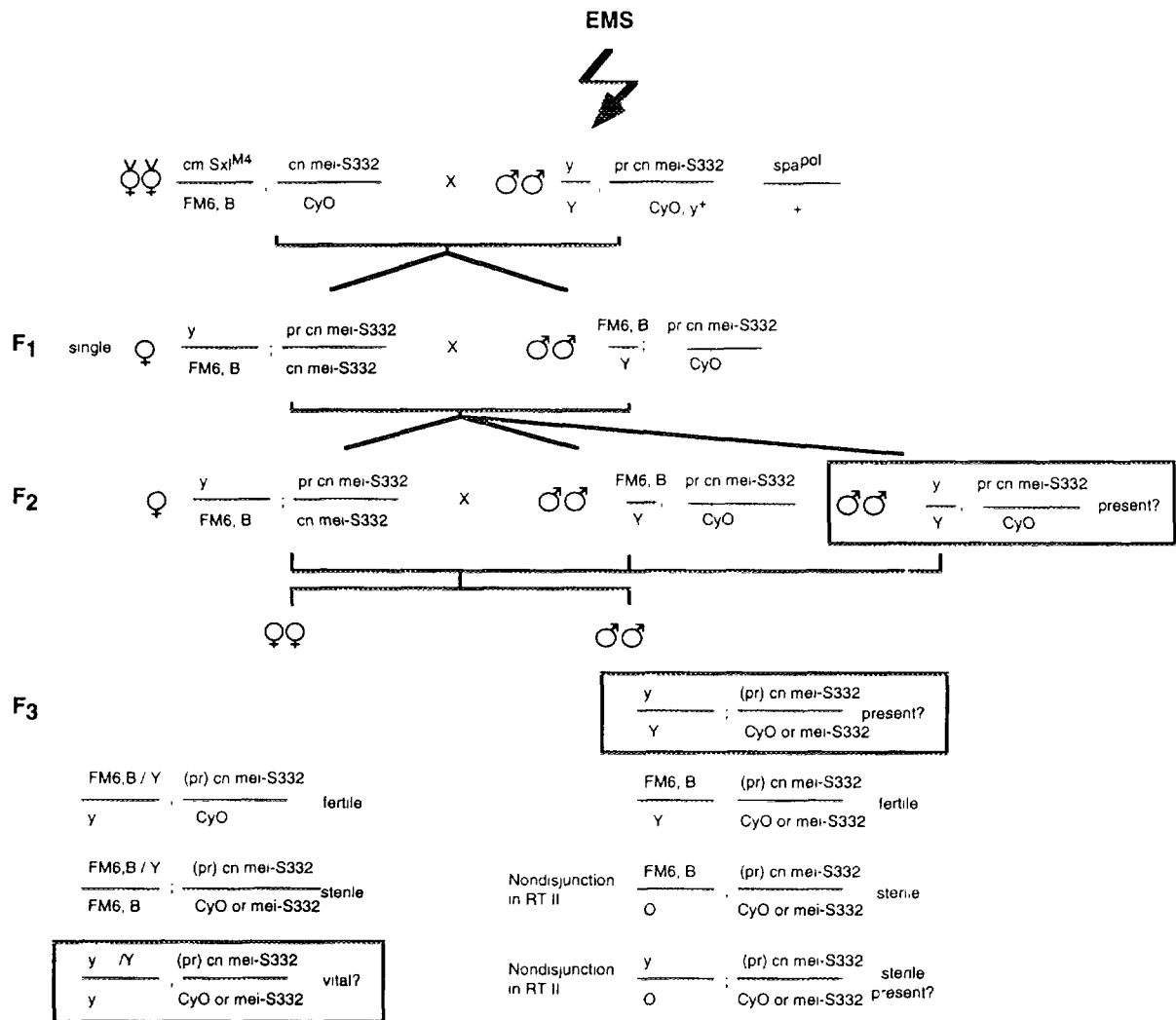


Figure 4: - Genetic system for the production of $y\ w^{1118}\ l$ clones. The scheme represents XX cells and shows chromosome 1 and chromosome 3. One of the homologues of the third chromosome carries the $P\{ry^+ = hsFLP\}3$ (designated as *hsFLP*) that serves as a source of flipase under heat shock condition. The mother cell is shown in the four-strand stage (G_2) of the mitotic cell cycle, and the consequences of mitotic recombination (X) are given on the right. One daughter cell has lost all dominant alleles ($y\ w^{1118}$) and is thus homozygous for $y\ w^{1118}$ and l . The other daughter cell is homozygous for the wildtype alleles.

We believe that the process of dosage compensation in *Drosophila* is intimately related to the general process of transcriptional control and gene regulation and that it should be, therefore, well conserved among different Dipteran species. However, to use its genetic elements as tools for the development of genetic sexing techniques, all attributes of these elements and their functions must be thoroughly understood.

In this paper, we report a further step in our attempts to elucidate the process of the genetic control of dosage compensation. Since this process is regulated sex-specifically, at least one of its genetic components has to be differentially expressed in one of the two sexes. To date, although *mle*, *msl-1* and *msl-3* have been cloned, the data pertaining to the molecular characterization of MLE and MSL-1 have been published. MLE is expressed in both males and females [12]. In contrast, although the *msl-1* gene is transcribed in both sexes, the MSL-1 product is almost absent in females [17]. Whether this difference is due to instability of the unbound protein or whether it is due to sex-specific regulation at the

translational level remains to be determined. Three *msl-1* mRNA's, differing at the 3' untranslated trailer because of the use of three different polyadenylation signals, are produced (Figure 1A). Two of these mRNA's have a consensus SXL binding site. We wished to test if, in females, the SXL protein binds to the two longer forms of mRNA in the nuclei thus inhibiting their transport into the cytoplasm and their translation with the consequence that the amount of MSL-1 protein would be below a certain threshold needed for X-chromosomal binding and hypertranscription. We constructed a transgene with a truncated 3' end such that the putative SXL binding site and two polyadenylation signal sites have been removed (Figure 1A, B and C). Mutant *msl-1* males carrying one copy of the transgene are fully viable; everything else being equal, the level of functional mRNA in these males is sufficient to allow hypertranscription. Since the same amount would be present in females, the simple threshold model is insufficient to explain the sex specific inhibition of hypertranscription in females. However, Western blots have revealed the surprising information that females carrying the transgene have substantially higher levels of MSL-1 protein than control females (Y. Yang, unpubl. results). In any case we cannot rule out that the putative SXL binding site present on two of the *msl-1* transcripts does have a function without performing an important control: additional copies of an *msl-1* gene bearing the SXL consensus binding site and a single polyadenylation site (downstream from the SXL site) must be introduced in females and shown to have little or no effect on the level of MSL-1. At least our results suggests that there are other components of the regulatory mechanisms that are sex-specifically regulated.

Since in past years, large scale mutagenesis screens have been undertaken for these and other purposes, one can wonder why some elements of the dosage compensation process are still missing. Firstly, it is possible that the gene(s) of interest have multiple functions and that their inactivation would result in non sex-specific lethality. This is actually true for a few genes known to be involved in the initiation and maintenance of the *Sxl* gene function that are also involved in vital processes in both sexes (for reviews see [33,34,35]). The genetic identification of such genes would depend on the recovery of a hypomorphic allele. Secondly, as mentioned above, it is difficult to identify genes of interest on the X chromosome. Since this chromosome represents 20% of the haploid genome of *D. melanogaster* or an estimated number of 2000 genes, we decided to perform a mutagenesis screen for mutations interfering either zygotically or maternally with male viability. The results are promising in that we found at least one additional locus that causes male-specific lethality when mutated. Whether this lethality is the result of a defect in dosage compensation has yet to be determined.

ACKNOWLEDGEMENTS

We are grateful to Karama Neal, Thaddeus Ellett and Sunil Gujrathi for technical assistance and to Dr. Maxwell J. Scott for excellent technical advice. We thank Dr. Anne Kerrebroek and Dr. Rolf Nöthiger for providing us with the *mei-S332* fly stocks, Dr. Brigitte Dauwalder for the pCaSpeR4 transformation vector and the helper plasmid pUChs1_Δ 2-3 and Dr. Daniel Bopp for the SXL antisera. We also thank the Bloomington, the Bowling Green and the Umeå Stock Centers for providing us with stocks. This research was supported by a postdoctoral fellowship from the Swiss National Science Foundation to A.H. and by a grant from the NIH (GM15691) to J.C.L.

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