

## MOLECULAR BIOLOGICAL APPROACHES TO THE STUDY OF VECTORS IN RELATION TO MALARIA CONTROL

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*To a large extent, control of malaria vectors relies on the elimination of breeding sites and the application of chemical agents. There are increasing problems associated with the use of synthetic insecticides for vector control, including the evolution of resistance, the high cost of developing and registering new insecticides and an awareness of pollution from insecticide residues. These factors have stimulated interest in the application of molecular biology to the study of mosquito vectors of malaria; focussing primarily on two aspects. First, the improvement of existing control measures through the development of simplified DNA probe systems suitable for identification of vectors of malaria. The development of synthetic, non-radioactive DNA probes suitable for the identification of species in the *Anopheles gambiae* complex is described with the aim of defining a simplified methodology which is suitable for entomologist in the field. The second aspect to be considered is the development of completely novel strategies through the genetic manipulation of insect vectors of malaria in order to alter their ability to transmit the disease. The major requirements for producing transgenic mosquitoes are outlined together with the progress which has been made to date and discussed in relation to the prospects which this type of approach has for the future control of malaria.*

Key words: molecular biology – DNA probes – vectors – malaria control

Despite enormous efforts over many years, malaria is an increasingly important health problem in the world today. The incidence of this disease is increasing due largely to the development of insecticide resistance by the mosquito vectors and by the appearance of drug resistance in the malaria parasite. These factors are exacerbated by migration of increasing numbers of people from non-endemic areas to regions where malaria is prevalent. Until now, control of malaria on a global scale has relied on the application of chemical insecticides to limit Anopheline vector populations. The appearance of insecticide resistance, coupled with rapidly escalating costs for developing new insecticidal compounds, and the increasing

awareness of the detrimental effect insecticides have on the environment has stimulated interest in alternative methods for malaria control. What is required is the development and evaluation of a new generation of methodologies which will have a profound and long-lasting effect on malaria transmission. Clearly, biotechnology and molecular biology can play a central role in the search for, and production of, such new tools. The development of potential antimalarial vaccines and biotechnologically produced larvicidal compounds are obvious examples of the power of the approach. Perhaps, surprisingly, this technology has not been applied to the Anopheline vectors of malaria until very recently. Advances in the molecular analysis of vector-parasite relationships and vector molecular biology now make such an approach very attractive especially as past experience has shown that vector control is an effective way of disrupting malaria transmission.

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This investigation received financial support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Disease. We would also like to thank the Wolfson Foundation, Wellcome Trust, Lister Institute of Preventive Medicine, Medical Research Council and Liverpool University for financial assistance. JMC is a Wellcome Trust Senior Research Fellow in Basic Biomedical Sciences and PE is a Lister Institute Research Fellow.

Two applications of molecular biology will be considered in relation to vector biology and

malaria control. The first is the utilization of DNA probe technology to improve vector identification techniques. The aim here is to enhance the efficiency of existing control measures by providing accurate data about vector populations. The second is a completely revolutionary approach to malaria control through genetic manipulation of Anophelines so as to disrupt the parasite transmission cycle. There are many other areas where molecular biology will have a profound impact on our understanding of the insect vectors of malaria. However, these two examples serve to emphasize the power of this technology and they are discussed further below.

#### DNA PROBES FOR THE IDENTIFICATION OF MALARIA VECTORS

Many insect vectors of malaria are members of sibling species complexes. Sibling species are morphologically identical but are reproductively distinct. It is important to distinguish these species in control programmes as the different species within a complex may exhibit differences in ecology, vectorial capacity and response to control measures (White, 1982). There are a number of techniques which are available for the identification of vector specimens. Of particular value for this purpose have been isoenzyme analysis (Mahon et al., 1976) and cytogenetics (Gatti et al., 1982). Both of these techniques, however, suffer from a number of drawbacks including the complexity of the technique, the experience required to accurately determine the species of the specimen and the small number of specimens which can be analyzed by an individual in a given time. In some cases the technique may also be limited to a specific stage of the mosquito life-cycle. These problems have led us to investigate the use of DNA probe technology for vector identification. We have set out with the express aim of developing a methodology which is cheap, accurate and easy to use. Essentially two approaches may be taken when developing identification techniques based on DNA technology. The first, which may be termed an orientated or directed approach, involves the cloning of a DNA sequence which has a high probability of varying between the species within a complex. An example of such a sequence is the ribosomal DNA. There are multiple copies of these genes in all organisms; each copy of the gene cluster is separated by spacer regions. It is the DNA sequence of these spacer regions which commonly varies signifi-

cantly between closely related species. Probe systems can therefore be generated which take advantage of this variation to identify each specimen. A number of researchers have utilized this approach and in one instance have modified it to utilize the polymerase chain reaction for vector identification (Paskewitz & Collins, 1990). We have not taken this approach because it does not lend itself to being greatly simplified, as it relies on variation in a sequence which is present in all specimens being studied.

We have adopted an alternative 'shotgun' approach. This involves the selection of any piece of DNA from the genome of the insect which is present in one of the species of interest but not in the others. Such a probe lends itself to being used in a very simple assay as it gives a yes-no answer, i.e. the target DNA sequence is either absent or present and thus gives a simple and unambiguous answer. Thus, in the ideal situation, a series of such probes, one for each of the species within a complex, will allow each specimen to be identified.

Using this approach we have developed DNA probes for the identification of species in the *Anopheles gambiae* complex (Diptera, Culicidae) which includes the major vectors of malaria in Africa. Six species within the *An. gambiae* complex of mosquitoes have been identified on the basis of mating incompatibility (Davidson et al., 1967) – *An. gambiae*, *An. arabiensis*, *An. quadriannulatus*, *An. melas*, *An. merus* and *An. bwambe*. The first two are the most important vectors and are widely distributed throughout tropical Africa. Differential screening of clone banks generated from the DNA of each of those species has allowed the isolation of a number of cloned DNA probes which may be used for the identification of five of the six species in the complex (Gale & Crampton 1987, a, b, 1988). Subsequent work has been directed towards refining these DNA probes and simplifying the methods for using them so that they become available for entomologists in the field.

One of the major refinements has involved simplification of the sample preparation technique so that now all that is required is to squash a portion of the specimen, usually the head, directly onto a nylon filter membrane (Hill et al., 1991a). A further refinement has been to derive short oligonucleotide DNA probes from

the original cloned probes (Hill et al., 1991b). Such oligo-probes have a number of advantages for vector identification. These advantages include being cheap and easy to synthesize chemically; they may be tagged during synthesis so that they may be used with a variety of reporter molecules other than radiochemicals; and the kinetics of hybridization using single-stranded oligo-probes are such that the identification process can be greatly shortened (Crampton et al., 1989).

In addition to the use of oligo-probes, the conditions for hybridizing the probes have been greatly modified. The aim here has been to reduce the reliance on laboratory equipment and to reduce the complexity of the hybridisation solutions so that they are both cheap and very stable. It is now possible to carry out the whole process at room temperature and with a very simple hybridisation solution which can be premixed as a dry powder and can be stored for long periods at room temperature (Hill et al., 1991c).

A major drawback of DNA probe systems is that they have previously relied on the use of radiochemical labelling as a means for detecting hybridisation to a target. Major advances in the utilization of non-radioactive reporter molecules has now made it possible to incorporate these techniques into a simplified vector identification protocol. A comparison of the available non-radioactive labelling and detection techniques has shown that the E-link plus™ system produced by ICI does not suffer from background problems, is the most sensitive and the cheapest (2-4 cents/specimen) of the current alternative systems (Hill et al., 1991a). In addition, it may be used with squash-blotted specimens (ie the DNA does not have to be isolated or purified from each specimen) and the result can be detected either as a colour change on the filter or by photographic detection of chemiluminescence. Further refinements of the detection system are underway to shorten and simplify this aspect of the process.

The system which has evolved is easy and quick to use and, above all, is cheap enough to allow many specimens to be identified at minimal cost. Indeed, the methodology in its present form is being assessed by a number of laboratories in Africa by comparing it with existing identification techniques.

An important aspect of this technology is that the method may be used with any probes

which have already been developed, such as those for the *An. dirus* complex (Panyim et al., 1988), or *An. farauti* (Booth et al., 1991). In addition, probes which would provide other useful epidemiological data, such as those for *Plasmodium falciparum* detection, blood meal analysis, or insecticide resistance determination could also be incorporated into this methodology. Hopefully, with further improvements and simplification of the technique and the development of probes for a range of malaria vector species complexes, DNA probe technology may shortly realize its full potential in the field of vector identification.

#### GENETIC MANIPULATION OF MALARIA VECTORS

Control of insect populations by genetic means is not new. A number of vector control programmes have utilized the mass release of sterile males, the generation of cytogenetically induced sterility through translocation and the use of mating behaviour to transfer lethal or sterilizing agents between members of the populations. The strategy of sterile male release has proved to be particularly effective for the eradication of screw-worm fly from the southern USA (Krafsur et al., 1987). However, such strategies of autocidal population control have proved prohibitively expensive since they involve the repeated mass release of treated males (McDonald et al., 1977).

The advent of recombinant DNA and transgenic techniques now provides the means for the controlled genetic manipulation of malaria vector genomes by the direct introduction of DNA into the germline of these insects. Two particular advantages of using transgenic technology over classical genetics for future manipulation are evident and should be emphasized. One is the potential to exploit genes and gene constructs across species barriers and the other, the ability to introduce particular, defined sequences without the genome disruption of a conventional cross.

Transgenic technology in relation to malaria vectors may have potential application for population suppression. Perhaps, more interestingly, it may also provide a means for altering the vectorial capacity of insect populations. For example, it may be possible to produce strains which are refractory for the pathogen, strains of the vector which have reduced vector competence or reproduction potential, or to increase the susceptibility of the vector to ex-

isting control strategies. These are applications for the future. More immediately, this type of technology, and molecular biology in general, may be used as an exquisite analytical tool to begin to dissect the complex relationships between the insect vector and the malaria parasite which it transmits.

In order to be able to carry out this type of genetic manipulation in malaria vectors, a number of areas of basic research need to be developed. The areas to be considered include: i) Genome complexity and organization analysis; ii) Methods for introducing DNA into mosquito embryos and cells; iii) DNA vector systems for use in Anopheline mosquitoes; iv) The definition of mosquito or heterologous genes for introduction, disruption or manipulation in the insect. Each of these areas will be considered in turn in order to indicate what has been achieved in relation to what still needs to be done.

#### *Genome organization and complexity*

If the genome of malaria vectors is to be genetically manipulated in a controlled and directed fashion it is important to understand the size of the genome which is to be manipulated. Genome organization, that is the nature and dispersion pattern of repetitive sequences and how they are organized in relation to the coding sequences, is also important. This is because it will have a profound influence on the types of manipulation which can be envisaged and the approaches to be adopted to identify and clone sequences of interest.

Until recently, very little was known about the size and organization of mosquito genomes. Black & Rai (1988) have analyzed the genomic DNA from four species of mosquito, *An. quadrimaculatus*, *C. pipiens*, *Ae. albopictus* and *Ae. triseriatus*. More recently, Cockburn & Mitchell (1989) have shown that the genomes of Anopheline mosquitoes are generally relatively small and exhibit what is called a long period interspersed pattern (LPI) of repetitive sequences (the euchromatic DNA consists of long, single-copy sequences interrupted by a few moderately repeated sequences). This LPI type of genome organization in Anopheline mosquitoes is in marked contrast to that found in the *Ae. aegypti* genome which is both large and much more complex in its organization (Warren & Crampton, 1991). The fact that

*Anopheles* mosquitoes have genomes which are relatively small and an LPI organization means that, in molecular terms, it is easy to work with and that many of the approaches used for analysis of the *Drosophila* genome (which also has an LPI organization pattern) are directly applicable to *Anopheles*.

#### *Methods for introducing DNA into mosquito cells and embryos*

The generation of transgenic mosquitoes requires a means for introducing DNA into the germ line of the insect as well as an efficient DNA vector system which will allow stable integration of the introduced DNA. Microinjection systems have now been developed for a number of mosquitoes including *An. gambiae* (Miller et al., 1987), *Ae. triseriatus* (McGrane et al., 1988) and *Ae. aegypti* (Morris et al., 1989). The systems which have been developed are derived from that employed for *Drosophila* transformation modified to take account of differences in the physiology and development times between *Drosophila* and the mosquito species studied. The DNA which was introduced into the mosquito embryos in these experiments was the pUCHsneo vector/helper system based on the P transposable element from *Drosophila melanogaster* (Steller & Pirrotta, 1985). An antibiotic resistance gene (*neo*) forms part of this DNA vector system. The presence of this gene, if it becomes integrated into the mosquito genome, confers resistance to the synthetic antibiotic G418. Progeny of those individuals which survive microinjection are exposed to G418 so that only those mosquitoes which have integrated the injected DNA may be selected. Experiments with this vector/helper system have served to demonstrate that DNA may be injected into mosquito embryos with survival rates comparable to those in work with *D. melanogaster* (Spradling & Rubin, 1982). Integrations of the introduced P element DNA have been observed in both *Anopheles* and *Aedes* mosquitoes and the integration events appear, in some cases, to be heritable and clearly involve the germline of the mosquitoes involved. Although these events did not result from normal P element transposition, some functional role of the P sequence can not be excluded. However, it is clear from this work, and other experiments involving the transfection of the same DNA into mosquito cells in culture (Lycett et al., 1989), 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 embryos and cultured cells, the major stumbling block is currently the lack of an appropriate DNA vector system for manipulating the mosquito genome.

#### *The search for a mosquito DNA transformation vector system*

As indicated above, the DNA vector systems currently available are not ideal for creating transgenic mosquitoes. Attempts are being made to modify the P system for more general use (O'Brochta, 1990) but alternative elements, such as the Ac element from maize, are also being investigated for their usefulness in the mosquito system. In addition, there is increasing interest in identifying mosquito elements with the properties of mobile genetic elements. Such an element could form the core of a mosquito transformation vector.

A number of approaches have been taken to identify such mobile elements in mosquitoes. One of these is to analyze specific gene systems, such as the ribosomal DNA of mosquitoes, in order to detect variants of these genes arising from the insertion of a mobile element. No such insertions have, as yet, been detected in *Ae. aegypti* rDNA (Gale & Crampton, 1989) but insertion events have been detected in the rDNA of *An. gambiae* and these elements are being fully defined (Paskewitz & Collins, 1989). The elements appear to resemble a particular class of transposable genetic elements (TGE), non-viral retroposons. It is unlikely, however, that these elements will prove useful as transformation vectors because of the ill-defined nature of their mode of transposition.

We have recently adopted an alternative strategy to directly identifying a specific class of TGEs, known as retrotransposons, in the mosquito DNA. The approach relies on utilizing the characteristic biochemical and structural properties of these elements to identify them. This has led to the successful isolation of several retrotransposon-like elements from the *Ae. aegypti* genome (Crampton et al., 1990a, b). More recently we have used the polymerase chain reaction (PCR) to develop a particularly rapid methodology for identifying endogenous retrotransposon-like elements in mosquito DNA (Warren & Crampton, 1991). Once such ele-

ments have been isolated, fully characterized and their ability to transpose autonomously established, they may be engineered to form the core of a transformation vector system.

Any DNA vector system which is to be of practical value will have to incorporate a number of other features. Most important of these are a selectable marker system and specific promoter or enhancer sequences. As indicated above, current DNA vectors incorporate an antibiotic resistance gene which allows for selection of transformed individuals by exposure to G418. However, this system is less than satisfactory because different mosquitoes exhibit a spectrum of sensitivity to G418 and more importantly depends on high levels of expression of the resistance gene in the transformed mosquitoes. The method of choice would be the use of a phenotypic marker, such as eye colour, so that transformed individuals may be identified by direct visual inspection. Such a method requires mutant strains of mosquitoes and the corresponding cloned gene coding for an easily scored phenotype which can be incorporated into a transformation vector. Although eye colour mutants of *An. gambiae* are available, cloning of the genes responsible for these phenotypes has not yet been completed.

Finally, a considerable body of work using the pUCHsneo transformation vector, both in cultured mosquito cells and injected embryos, has indicated that the *Drosophila* heat shock promoter sequence is not entirely satisfactory for driving the expression of genes in mosquitoes (Lycett et al., 1992). In addition, at some stage it will be desirable to express defined genes in mosquitoes in a tissue or stage specific fashion. For this to be envisaged, mosquito stage and tissue specific promoters have to be defined. None are, as yet, available but attempts to characterize the DNA sequences responsible for expressing certain genes in mosquitoes are well underway (James et al., 1989).

All of these different aspects of basic research need to be pursued in parallel and eventually amalgamated to provide a mosquito DNA vector system of real practical value.

#### *Potential target genes for manipulation*

The sections above dealt with the practical problems of introducing DNA and obtaining

transformed mosquitoes. Subsequently, target genes have to be identified that are suitable for manipulation, introduction or disruption and which have potential in relation to disrupting the malaria transmission cycle.

In this respect, there are a number of obvious targets for manipulation including the genes involved in the mosquito immune system, developmental control genes, genes influencing behaviour and others. A more interesting possibility is to manipulate the gene or genes controlling the susceptibility of the vector to the malaria parasite it transmits, ie to alter directly the mosquito's vectorial competence. A number of genetic loci have been identified in mosquitoes which influence their susceptibility to specific parasites. For example, a strain of *An. gambiae* has been selected for refractoriness to malaria and characterized genetically (Collins et al., 1986). However, no gene or gene product has yet been defined or identified at the molecular level which controls susceptibility of the vector to a parasite. 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. Clearly the use of transgenic technology through transposon tagging will assist in the characterization of refractory genes and their products.

An important genotypic characteristic not met by the majority of the genes encoding refractoriness is that any such gene to be introduced into the mosquito would have to be 'dominant' in its activity, ie be able to overcome the expression of the endogenous genes. Such a dominant gene would also ensure that one copy of the introduced gene in the mosquito would be sufficient to alter the phenotype of the insect. It is therefore clear that currently there is no defined gene in the mosquito genome which may be manipulated to alter the ability of the insect to transmit malaria.

In contrast, 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 (Winger et al., 1987). These vaccines attack antigens present on gametes and ookinetes of the malaria parasite and antibodies which recognize these antigens are able to block the

development of the parasite in the mosquito midgut. We are currently assessing the feasibility of creating a transgenic mosquito incorporating an antibody gene which will be expressed in the insect midgut in response to a blood meal and which blocks 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.

The above possibilities are clearly speculative and for the future. Also, it would be naive to assume that the problems would be solved by the introduction of foreign genes into laboratory mosquitoes. There are considerable practical and ethical problems associated with the release of genetically manipulated mosquitoes into natural populations. This has been amply demonstrated by previous studies on genetic control, and the fitness of laboratory stocks is clearly lower than that of individuals from natural populations. Despite these difficulties, genetic transformation has much to offer, both to basic mosquito research and to the development of future control schemes. The possible hazards of releasing this type of manipulated organism are difficult to assess in the absence of experimental evidence. However, this approach has enormous potential which should at least be explored so that an informed assessment may be made when balancing the benefits against the potential problems.

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