QUICK BLOTS AND NONRADIOACTIVE DETECTION SYSTEMS: IMPROVEMENTS ON METHODS FOR DNA HYBRIDIZATIONS USING MOSQUITOES

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1990

ACKNOWLEDGEMENT

The author would like to acknowledge the one who makes all things possible, "for from him and through him and to him are all things. To him be the glory forever! Amen." (Romans 11:36)

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KEY TO ABBREVIATIONS AND SYMBOLS

°C	degrees Centigrade
DNA	deoxyribonucleic acid
EDTA	ethylene-diamine-tetraacetic acid
kbp	kilobase pair(s)
LF	LA FRANCE (Dial Corp.)
M	molar
mg	milligram(s)
min	minute(s)
ml	milliliter(s)
mm	millimeter
mM	millimolar
μg	microgram(s)
μl	microliter(s)
NFDM	nonfat dry milk
ng	nanogram(s)
nm	nanometers
pg	picograms
QB	quick blot
RNA	ribonucleic acid
s	second(s)
SSC	saline sodium citrate
SDS	sodium dodecyl sulfate

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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December, 1990

Chairman: Dr. Jack A. Seawright
Major Department: Entomology and Nematology

A DNA (deoxyribonucleic acid) probe was isolated which exhibited specificity for two mosquito species, Culex nigripalpus Theobald and Culex salinarius Coquillett. The nucleotide sequence of another probe specific for Anopheles quadrimaculatus Say species A was determined in order to identify sequences which conferred specificity to the probe and to assist in the production of synthetic oligonucleotide probes. Probes exhibiting specificity for Anopheles nuneztovari Gabaldon were isolated in a primary screening, and other mosquito species-specific probes were partially characterized.

A new technique for preparing targets for hybridization of nucleic acid probes, called the quick blot protocol, was developed. It allowed rapid preparation of multiple (10 or more) equivalent sample-containing filters, called quick blots. Samples were applied uniformly in an orderly arrangement on the filters. The quick blot protocol was used to prepare targets for hybridization with mosquito species-specific DNA probes. Using quick blots, detection of radiolabeled probes was compared with detection of probes prepared with three nonradioactive detection systems. A method was developed for the differential detection of two nonradioactive probes hybridized simultaneously to a quick blot. Finally, the use of synthetic oligonucleotide probes with quick blots was demonstrated.

INTRODUCTION

The need for nonmorphological methods to identify specimens occurs when specimens of related species cannot be distinguished by morphology. These cryptic species probably have arisen from recent speciation events and may therefore provide valuable models for the study of evolutionary processes.

In the case of mosquitoes, it is valuable to have methods for readily identifying cryptic species which differ in their abilities to serve as vectors for a parasite of humans. An example is the Anopheles gambiae Giles species complex, a group of at least six species which are indistinguishable morphologically. These species differ in their significance as vectors of malaria, and under certain conditions can be distinguished by cytological (Coluzzi & Sabatini, 1967) and isoenzyme (Hunt & Coetzee, 1986) analyses. But perhaps the easiest way to distinguish three members of this complex is with a DNA probe (Collins et al., 1988). However, this single probe will not distinguish all the known members of the complex.

There are two major areas of concern in the efforts to make probe technologies useful. One is the isolation of probes with the desired traits, and the other is the

development of methods that can best detect the hybridized probe molecules.

The specificity of base pairing in the annealing, or hybridization, of separated strands of nucleic acid has allowed the development of DNA probe technologies. These have proven invaluable for the detection of pathogens of humans (Hyypia et al., 1989), viruses and viroids infecting plants (McInnes & Symons, 1989a), and human genetic disorders (Sutherland & Mulley, 1989). The specificity of DNA probes has also been used to identify species of mosquitoes (Cockburn et al., 1988; Cockburn, 1990). Methods for using nucleic acid probes usually involve preparation of a suitable target, in which the nucleic acid to be probed is immobilized in a denatured form on a glass or membrane filter support. Denatured probe is then given a chance to anneal with the target nucleic acid in a hybridization step, in conditions conducive to duplex formation. From this point on, the focus will be on DNA probes, for even though RNA probes could be of value in tissue- or age-specific detection, they have not been as widely used as DNA probes and are unstable.

The two main parameters that relate to the value of a given DNA probe are specificity (selectivity) and sensitivity. These parameters are determined in part by the

nucleotide sequence of the probe, but also depend greatly on the conditions used to anneal the probe to the target, and the characteristics of the detection system used to visualize the hybridized (bound) probe.

There are several techniques used for preparing the targets for nucleic acid probes. Nucleic acids may be extracted from tissues, and applied to a filter to form a slot blot (Wahl et al., 1987) or a dot blot (Costanzi & Gillespie, 1987). Or, an organism or isolated tissue may be used directly to form a squash blot (Cockburn, 1990; Keating et al., 1989; Kirkpatrick et al., 1987; Tchen et al., 1985). This is done by using enough force while squashing the material against the filter that some DNA is freed from the cells and becomes bound to the filter. The squash blot method is useful in the preparation of two equivalent filters containing the DNA of individual mosquitoes (Cockburn, 1990).

This study focused on the isolation, characterization, and use of mosquito species-specific DNA probes. The specific objectives were: (1) isolation of new DNA probes showing specificity for certain Culex and Anopheles species (especially the vector of St. Louis encephalitis virus, Culex nigripalpus), (2) characterization of the nucleotide sequence(s) conferring specificity in one or more probes,

(3) development of a fast, reliable system for the preparation of multiple targets for mosquito DNA probes using individual mosquitoes, (4) assessment of the usefulness of commercially available nonradioactive detection systems when applied to systems for hybridization of mosquito DNA probes (including a comparison to radioactive detection methods), (5) assessment of the value of synthetic oligonucleotides as mosquito species-specific probes.

Central to this study were repetitive DNA probes shown previously to exhibit specificity for the four known members of the A. quadrimaculatus species complex (Cockburn, 1990). These probes will be useful in assessing the potential of the members of the complex to serve as vectors of malaria. Probes Arp2, Brp1, and Crp1 hybridized primarily to DNA from A. quadrimaculatus species A, B, and C, respectively. However, Arp2 hybridized slightly to species B DNA. This probe was chosen as a model for the characterization of mosquito species-specific probes, in part because of the possibility of using its nucleotide sequence to prepare oligonucleotide probes which might exhibit improved specificity. Also, restriction analysis of probe Arp2 indicated that it was probably composed of multiple identical (or very similar) repeat sequences (personal

communication, A. F. Cockburn, United States Department of Agriculture (USDA)) which could be detected by nucleotide sequencing of suitable subclones.

A new method, called the quick blot (QB) protocol, is described for preparing hybridization targets using mosquitoes. The QB protocol was used to produce ten equivalent sets of nucleic acid targets on filters, called quick blots (QBs), for use in nucleic acid hybridization assays. It has been found useful in the analysis of individual mosquitoes, with up to 96 individuals per filter. The filters were used successfully as DNA hybridization targets for mosquito species-specific DNA probes. The main advantages that the QB protocol offers over previous methods include: the uniformity of sample application, the orderly arrangement of samples on the filters, the ability to produce multiple identical sample-containing filters, and the rapidity with which numerous samples can be processed.

Specific detection of DNA probes hybridized to QBs was achieved with nonradioactive labeling and detection systems. These results were compared to those obtained with radiolabeled probes.

Nucleotide sequence data were obtained from plasmids containing mosquito species-specific DNA and used to specify the synthesis of oligonucleotides. These oligonucleotides

were tested for their usefulness as species-specific probes to QBs, and advantages of these synthetic probes were demonstrated. Thus, QBs may be used as targets for hybridization of nucleic acid probes; nonradioactive detection systems may be used to advantage with QBs in some situations; and synthetic DNA probes can offer advantages over conventional genomic clones.

MATERIALS AND METHODS

General Molecular Methods

Gels were 0.5-1.0% agarose (Sigma), buffered and run in 1X TBE (89mM Tris-borate, 89mM boric acid, 2mM EDTA) at less than 5.5 volts per centimeter. Fragments were sized using a *Hind* III digest of bacteriophage lambda or 1 kbp ladder fragments (Bethesda Research Laboratories, Life Technologies, Inc. (BRL)) as markers.

Plasmids were prepared by a modification of the alkaline-lysis method of Birnboim & Doly (1979) and cesium chloride purification, or by the boiling method (Holmes & Quigley, 1981). Insect genomic DNA was prepared by the method of Cockburn & Seawright (1988). Standard methods were used for restriction analysis of plasmid and genomic DNA, except that restriction enzymes were used in excess of the manufacturer's (BRL) recommendations. Nucleic acids were quantified by ultraviolet absorption at 260 nm.

Double-stranded DNA was radiolabeled by nick translation (Nick Translation System, BRL) with ^{32}P-dCTP, and unincorporated label was removed by size exclusion chromatography (using Bio-Gel P-60, BioRad). Oligonucleotides were radiolabeled with ^{32}P-ATP and T4 polynucleotide kinase. Unincorporated nucleotides were

removed by size exclusion chromatography (using Bio-Rad Bio-Spin 30 columns).

Unless otherwise noted, filters were subjected to the following treatments after application of the target DNA. Prior to prehybridization, nitrocellulose filters were baked for 20-45 min at 80°C under vacuum (vacuum-baked), and nylon filters were subjected to treatment with 300 nm ultraviolet (UV) light (1-2 min on the glass surface of a Chromato-Vue Transilluminator, Ultraviolet Products, Model TM-36). Filters were prehybridized in 1% NFDM (nonfat dry milk), 0.2% SDS at 55°C for at least 30 min, and hybridized with (denatured) probe in 30% formamide, 5X SSPE (20X SSPE is 3.6M NaCl, 0.2M NaH_2PO_4 pH 7.4, 20mM EDTA), 1% NFDM, 0.2% SDS at 55°C overnight.

Prehybridization of blots for oligonucleotide probes was in buffer (6X SSPE, 0.3% SDS, 1.0% NFDM) for one hour at 65°C. Hybridization with labeled oligonucleotide probe was performed by adding the probe samples to the bags containing the filters and prehybridization buffer, resealing, and incubating for 24 hours at 37°C. Following hybridization, the filters were washed four times for 15 min each wash in 4X SSPE at 65°C. All films used for autoradiography and chemiluminescent detection (see below) were Kodak X-AR with Kodak intensifying screens.

Excess probe annealing to highly repetitive DNA can provide a higher sensitivity than a probe annealing to moderately repetitive or single-copy sequences, if whole genomic DNA serves as the target. In this study, excess probe (0.5-1.0 μg per filter) was used in each hybridization experiment to ensure that detection of bound probe was not limited by probe concentration in the hybridization step.

Two distinct terms are used to describe spurious detection: background and nonspecific detection. The term background is used to refer to apparent signal development in areas of the target filter (or its image on film) not corresponding to locations where nucleic acid was applied. The term nonspecific detection (or nonspecific signal) is used to denote the appearance on the filter (or film) of signal in areas where nucleic acid was applied but where no probe was expected to be localized (based on the known specificity of the probe).

The DH5-α and JM103 strains of Escherichia coli were the hosts for all plasmids, and the DH5-α and HB101 strains were the hosts for all transformations. Bacteria were grown on Luria-Bertani culture medium with 30 μg/ml kanamycin or 50 μg/ml ampicillin. Bacteria were transformed by standard methods (Hanahan, 1983) and screened for plasmids of appropriate size, using agarose gel electrophoresis.

Oligonucleotide probes were synthesized at the ICBR DNA Synthesis Facility, Gainesville, Florida.

Sources of Mosquitoes and Probes

Specimens of the following mosquito species used in this study were supplied by the mosquito rearing facility at the Medical and Veterinary Entomology Research Laboratory, USDA, Gainesville, Florida: *Aedes taeniorhynchus* (Wiedemann), *Anopheles albimanus* Wiedemann, *A. quadrimaculatus* species A (ORLANDO strain), *Culex quinquefasciatus* Say, and *C. salinarius* Coquillett. Specimens of *Anopheles crucians* Wiedemann, *Coquillettidea perturbans* (Walker), and *Culex nigripalpus* Theobald were supplied by Mr. O. R. Willis (USDA), and were collected in Alachua County, Florida. *A. quadrimaculatus* species B, C, and D mosquitoes were supplied by P. E. Kaiser and S. E. Mitchell (USDA).

Probes pA2, pBrp1-S1, pCrp1-S1, pCrp-S2, and pCrp-S3 were supplied by A. F. Cockburn and were derived by subcloning of phage Arp2, Brp1, and Crp1 (Cockburn, 1990) *Sal* I fragments into plasmid pK19. It was demonstrated previously (Cockburn, 1990) that the probes Arp2, Brp1, and Crp1 probes exhibited sufficient specificity for *A. quadrimaculatus* species A, B, and C, respectively, to allow differentiation of specimens of all four members of the *A.*

quadrimaculatus species complex. The insert in pA2 was 2.8 kbp, much smaller than the 12 kbp Sal I fragment in phage Arp2. The sequence organization of this clone (see below) suggests that a deletion occurred by recombination between one or more internal repeats. Probe pKA2 was derived by transferring the (Sal I) insert of pA2 into pK19 using Hind III and EcoR I. Probe pAf1-S1 was prepared by subcloning a 3.4 kbp Sal I fragment from an Anopheles freeborni Aitken-specific phage probe (A. F. Cockburn, USDA) into pK19. The derivation of other probes and subclones is detailed in the appropriate sections below. All plasmid probes contained vector pK19 unless otherwise indicated.

Squash Blots and Dot Blots

Squash blots for hybridization of mosquito species-specific DNA probes were prepared as described previously (Cockburn, 1990). A damp blotting filter was covered with mosquitoes arranged in a grid pattern. A second filter was placed on top of the mosquitoes, and a metal rod was rolled over the filters to thoroughly squash the mosquitoes in between. The resulting sandwiches were laid on filter paper soaked with denaturing solution (0.5M NaOH, 1.5M NaCl) for about 5 min per side, then transferred to paper saturated with neutralizing solution (1.5M NaCl, 1M Tris, pH 8.0) for 5 min per side. The two filters were separated, and

subjected to either the UV fixation (nylon filters) or vacuum-baking (nitrocellulose filters) steps as described above.

Dot blots were prepared using standard methods (Costanzi & Gillespie, 1987). The dot blot is a way to prepare hybridization targets using purified DNA. The DNA can be diluted serially and applied to a blotting filter to provide spots containing different amounts of bound DNA. Application of sample solutions to the filter is simple when using an apparatus called the dot blot manifold (Table 2).

Isolation of the Culex-specific probe, pCx1

Attempts were made to isolate a DNA probe specific for C. nigripalpus by the method of Cockburn & Mitchell (1989). Two variations of this approach were tried, using a phage vector and a plasmid vector.

Recombinant phage was prepared by ligation of C. nigripalpus DNA cut with EcoR I and Xba I with LambdaGEM-4 EcoR I-Xba I Arms (Promega). Ligated DNA was packaged (Gigapack Gold, Stratagene) for screening. Library screening was performed according to Cockburn (1990), using duplicate plaque lifts hybridized separately to C. nigripalpus and C. salinarius genomic DNA. Phage were grown on E. coli strain P2392.

Plasmid subclones were obtained from recombinant LambdaGEM-4 according to the directions supplied by the manufacturer (Promega). Inserts in the LambdaGEM-4 vector were contained within the pGEM-4 plasmid which is included as part of the vector; plasmid subclones were easily derived by cutting the purified recombinant LambdaGEM-4 DNA with Spe I, ligating, and transforming suitable host bacteria.

Plasmid libraries containing C. nigripalpus genomic DNA in pK19 were prepared using double digests with Hind III and Xba I, EcoR I and Pst I, or EcoR I and Hind III. A plasmid library was also prepared with C. salinarius DNA in pK19 using a Hind III and EcoR I double digest. Transformants from the ligation mixtures were grown on kanamycin-containing medium. Colony lifts were prepared according to the method of Buluwela et al. (1989), and served as targets for differential hybridization to C. nigripalpus, C. salinarius, and C. quinquefasciatus genomic DNA.

Isolation of Anopheles nuneztovari-specific Probes

Isolation of Anopheles nuneztovari-specific probes was accomplished according to the methods of Cockburn & Mitchell (1989) and Cockburn (1990), using an A. nuneztovari library in phage EMBL 3A supplied by A. F. Cockburn. Genomic DNAs from A. nuneztovari and Anopheles oswaldoi (Peryassu),

supplied by L. P. Lounibos and J. Conn, were used for the primary differential hybridization screening.

DNA Sequencing

Subcloning strategies were designed for the selection of deletion subclones generated by restriction enzymes, and to allow the use of the standard universal (forward) and reverse primers.

Sequencing of pKA2 was aided by subcloning of *Nsi* I fragments from the insert. The recipient vector (pK19) DNA was cut with *Pst* I and phosphatased, and pKA2 DNA was cut with *Nsi* I. These two digests were ligated, and transformants were selected and used for sequencing. Using this approach, each subclone was expected to contain a single *Nsi* I fragment from the insert. The subclones were called pKA2-N1, pKA2-N2, etc.

Nucleotide sequence data were also obtained from other plasmid clones. These were the *A. quadrimaculatus* species B-specific probe pBrp1-S1, the *A. freeborni*-specific probe pAf1-S1, the *Culex*-specific probe pCx1, and three plasmid subclones of the *A. quadrimaculatus* species C-specific probe (pCrp1-S1, pCrp1-S2, and pCrp1-S3, comprising the total insert in the parental recombinant phage probe). Sequencing of the unrearranged phage Arp2 insert was

accomplished by preparing Nsi I subclones in pK19. These were designated pArp2-N1, pArp2-N2, etc.

Sequencing reactions were performed on boiling-method preparations of 1-5 μg of plasmid DNA extracted from 2-ml bacterial cultures grown up overnight. Primer annealing was performed on alkali-denatured plasmid DNA. Sequenase version 2.0 (U.S. Biochemical Corp.) was used for sequencing by the chain-terminating method (Sanger, 1977) with manufacturer-supplied reaction solutions and procedures. Reaction products were labeled with ^{35}S dATP in buffers containing Mg^{++} ions. Sequencing reactions were run on 0.2-0.9 mm wedge gels (4% acrylamide [19:1 linear to bis, LKB], 8M urea, 1X TBE) at 55°C, 1750 volts on a Macrophor (LKB) or Sequigen (BioRad) electrophoresis unit. Gels were rinsed for 10-20 min in 10% acetic acid before drying in a forced-air oven at 80°C. Gels run on the Macrophor were bonded to the running plate, and others were transferred to filter paper prior to drying and autoradiography.

Sequence analysis was done on the Multiple Sequence Editor (Massachusetts Institute of Technology) and the Genetics Computer Group Software Package (Devereux et al., 1984) version 6.1, both running on a MicroVAX II computer. Nucleotide sequence searches were performed using the European Molecular Biology Laboratory (EMBL) version 22

(modified; February, 1990) and Genbank version 63 (March, 1990) databases.

Nonradioactive Detection Systems

Overview of Nonradioactive Detection Systems

Three different nonradioactive labeling and detection methods were used in this study: the SA-AP (streptavidin-alkaline phosphatase) method (GENE-TECT protocol, Clontech Laboratories, with BRL reagents); the ECL (enhanced chemiluminescence) method (ECL kit, Amersham Corporation); and the Genius method (Genius Nonradioactive DNA Labeling and Detection Kit, Boehringer Mannheim Biochemicals).

The nonradioactive labeling and detection kits were used essentially as recommended by the suppliers, except where otherwise noted. The SA-AP kit used biotinylation of probe DNA via nick translation, and detection of hybridized probe by binding of streptavidin-alkaline phosphatase, followed by an enzyme-catalyzed color reaction. The ECL probes were prepared by covalent binding of peroxidase to the DNA, and detection of hybridized ECL probes was achieved by a chemiluminescent reaction using X-ray film. The Genius kit used random primed incorporation of the steriodal hapten digoxigenin into probe DNA. Following hybridization, Genius probes were detected by enzyme-linked immunoassay using an

antibody conjugate (α-digoxygenin-alkaline phosphatase conjugate), and the same color reaction used with the SA-AP method.

Preparation and Use of Biotinylated Probes

The preparation of biotinylated probes was achieved by nick translation of double-stranded template DNA for the incorporation of biotinylated nucleotides. The BRL Nick Translation System (BRL) reagents were used, according to the recommendations for the Biotin-21-dUTP Labeling System (Clontech Laboratories). Unincorporated nucleotides were removed by gel exclusion chromatography (using BIO-GEL P-60, BioRad).

Unless otherwise noted, prehybridization and hybridization conditions for use of biotinylated probes were as described in the section on general molecular methods, above.

Detection of hybridized biotinylated probes was accomplished according to the directions in the GENE-TECT protocol (GENE-TECT Detection System, Clontech Laboratories). All detection steps were performed at room temperature. Filters were first washed 30 min in 3% NFDM (blocking step). Then they were incubated for 25 min with SA-AP (streptavidin-alkaline phosphatase), in a solution made by adding 2.5 μl SA-AP conjugate (BRL) per ml Buffer A

(0.2M NaCl, 0.05% Triton-X-100, 0.1M Tris, pH 7.5). The filters were then washed 3 times with Buffer A, 10 min each wash, then once for 10 min with Buffer C (0.1M NaCl, 50mM $MgCl_2$, 0.1M Tris, pH 9.5). Then the filters were incubated in the color solution (Buffer C with chromogenic substrates) in reduced illumination until signals were developed properly. The color reaction was terminated with 1mM EDTA.

Preparation and Use of ECL Probes

The directions supplied by the manufacturer of the ECL kit (ECL Version 2, Amersham) were followed in the preparation and use of ECL probes, including the prehybridization and hybridization steps, except that SSPE was substituted for SSC in the wash solutions (see below). Double-stranded DNA to be labeled was precipitated and resuspended in deionized water at a concentration of 10 ng/ml. The DNA was boiled for 5 min, then immediately cooled on ice for 5 min. An equivalent amount of DNA labeling reagent and then glutaraldehyde solution were added to the DNA and mixed thoroughly. The solution was consolidated by spinning briefly (5 s) in a microcentrifuge, then incubated for 10 min at 37°C. The labeled probe was stored in 50% glycerol at -20°C until used.

The supplied hybridization buffer was used for both prehybridization (at least 10 min at 40-42°C) and hybridization (overnight at 40-42°C) after adding NaCl to 0.5M.

Following hybridization of probes according to the ECL protocol, filters were removed from the hybridization medium and washed twice (20 min each wash) at 40-42°C with primary wash buffer (6M urea, 14mM SDS, 0.5X SSPE). Then the filters were washed twice (5 min each wash) at room temperature with 2X SSPE. Equal volumes of detection solutions 1 and 2 were mixed, and the filters were incubated in this detection buffer 1 min at room temperature. Filters were wrapped in plastic wrap and exposed to x-ray film in the dark, with the side of the filter which received the DNA during application of target DNA facing the film. The film was developed after a 1 min exposure, followed by longer exposures as needed.

Preparation and Use of Genius Probes

The directions supplied by the manufacturer of the Genius kit (Genius Nonradioactive DNA Labeling and Detection Kit, Boehringer Mannheim Biochemicals) were followed in the preparation and use of Genius probes, except that labeled probes were precipitated with NaCl rather than LiCl, and SSPE was substituted for SSC in the hybridization steps. Genius probes were prepared by the random primed

incorporation of digoxygenin-tagged nucleotides, and detected by immunoassay.

In the preparation of a Genius probe, linearized, purified, heat-denatured probe DNA was mixed with the supplied hexanucleotide mixture, dNTP labeling mixture, and Klenow enzyme according to the instructions provided with the kit, and incubated for at least 60 min at 37°C. The reaction was stopped by addition of 1mM EDTA. The unincorporated tagged nucleotide was removed by ethanol precipitation, the probe DNA was resuspended, and was stored at -20°C until used in a hybridization reaction.

The Genius prehybridization and hybridization buffer was composed of 5X SSPE, 5% of the supplied blocking reagent, 50% formamide, 0.1% sodium N-lauroylsarcosine, and 0.02% SDS. The temperature used for prehybridization and hybridization, 42°C, was that recommended for buffer with 50% formamide. Filters were hybridized overnight, then washed twice for 5 min each wash at room temperature in 2X SSPE, 0.1% SDS. Next the filters were washed twice for 15 min each wash at 68°C in 0.1X SSPE, 0.1% SDS. Detection was performed immediately following these washing steps.

All steps in the Genius detection protocol were performed at room temperature. The Genius detection was begun by washing filters for 1 min in Genius buffer 1 (150mM

NaCl, 100mM Tris, pH 7.5), then for 30 min in buffer 1 in which had been dissolved 0.5% of the blocking agent. A brief (1 min) rinse of the filters in buffer 1 was followed by incubation for 30 min in a solution of antibody-conjugate, prepared as a 1:5000 dilution of the supplied antibody-conjugate in buffer 1. Unbound antibody-conjugate was removed with 2 washes, each for 15 min, in buffer 1. Next the filters were incubated for 2 min in buffer 3 (100mM NaCl, 50mM $MgCl_2$, 100mM Tris, pH 9.5), and finally in the color solution (buffer 3 plus chromogenic substrates) under reduced illumination until signals were properly developed. The color reaction was stopped with 1mM EDTA.

MOSQUITO SPECIES-SPECIFIC DNA PROBES

Isolation Methods and the Relevance of Genome Organization

Mosquito species-specific DNA probes were isolated by the method of Cockburn (1990). The method involves a search for repetitive DNA clones from a library using differential screening. The clones each contain a small piece of genomic DNA. Two genomic DNA probes are used to screen clones for the presence of a species-specific DNA insert. One (homologous) probe is genomic DNA from the same species used to prepare the library. The other (heterologous) probe is genomic DNA from a different species. Only clones containing a DNA sequence repeated many times in the genomic DNA probe hybridize at detectable levels. To isolate a clone from the C. nigripalpus libraries, DNA from the closely related species C. salinarius or C. quinquefasciatus was used as the heterologous probe. To isolate a clone from the A. nuneztovari library, DNA from A. oswaldoi was used as the heterologous probe.

The cloning strategy, including the choice of vector, used in the preparation of DNA libraries to be screened for probes determines the size (or range of sizes) of inserts from the organism's DNA that end up in the clones. The average size of the inserts in the library can affect the

outcome of the screening by differential hybridization, due to peculiarities of genome organization.

The organization of the genomes of anopheline and culicine mosquitoes is known to differ (Cockburn & Mitchell, 1989). Both anopheline and culicine genomes contain regions of repetitive DNA, but there are longer stretches of interveniong nonrepetitive DNA between the repeats in anopheline genomes, as compared to culicine genomes.

Species-specific probes can be isolated for Anopheles species rather easily by differential hybridization, using phage vectors that typically contain 10-15-kbp inserts (Cockburn & Mitchell, 1989). The separation of repetitive DNA in the Anopheles genomes allows large inserts to retain species specificity when the insert contains only a single species-specific repeat. The different interspersion pattern of culicine genomes, however, causes large inserts to be more likely to show cross-hybridization to heterologous DNA used in differential screening, due to the presence of nonspecific repetitive DNA scattered throughout the genome.

One way to enhance the possibility of isolating a species-specific repetitive DNA probe from Culex DNA is to use a vector which favors small inserts. This decreases the chance that a clone carrying species-specific repetitive DNA also contains a portion of nonspecific repetitive DNA. That

was the rationale for using the LambdaGEM-4 and plasmid vectors with double-digested genomic DNA in the attempts to isolate a Culex-specific probe. The double-digested genomic DNA used for preparation of libraries was mostly in the 100 base pair size range, and the LambdaGEM-4 vector excluded inserts greater than 4.1 kbp. Cloning of small inserts thus favored the isolation of a species-specific DNA probe from Culex, using the differential hybridization method.

Isolation of Probe pCx1

In an attempt to isolate a C. nigripalpus-specific probe by differential hybridization of genomic DNA from C. nigripalpus and C. salinarius to recombinant LambdaGEM-4, about 5000 recombinant phage containing C. nigripalpus DNA were screened. From this primary screen, 10 plaques were picked which gave some degree of differential signals. In no case was the degree of hybridization to C. salinarius genomic DNA negligible. However, two of the clones which gave the best differential signals were chosen for further characterization, because it was thought that they might contain species-specific DNA along with nonspecific sequences. The recombinant pGEM-4 plasmid was recovered from the two clones (the plasmid is part of the phage vector LambdaGEM-4), and the insert in both clones was found to be about 1 kbp, but slightly different in size.

The DNA from the two recombinant pGEM-4 clones was cut separately with 10 different restriction enzymes, each of which cut the insert DNA into several fragments. These digests were run on gels, and blotted to hybridization filters to obtain equivalent targets that were hybridized separately to C. nigripalpus and C. salinarius genomic DNAs. The results of autoradiographic detection revealed that none of the fragments hybridized differentially to the degree necessary to distinguish the two species. Accordingly, work with these clones was terminated.

Probe pCx1 was isolated from a plasmid library of C. nigripalpus Hind III/EcoR I fragments which was screened with C. quinquefasciatus and C. nigripalpus genomic DNAs. The insert in pCx1 was about 10 kbp. Squash blots with radiolabeled pCx1 provided detection of C. nigripalpus and C. salinarius, compared to negligible signals to C. quinquefasciatus and all other mosquito species used in this study.

About 5000 colonies containing C. nigripalpus insert DNA, and about 1000 colonies with C. salinarius insert DNA, were screened for species specific sequences by differential hybridization to C. nigripalpus and C. salinarius genomic DNAs. None were found to display specificity sufficient for a species-specific probe.

Isolation of Anopheles nuneztovari-specific Probes

A partial Sau3A I library of A. nuneztovari fragments (about 15 kbp insert size) in phage EMBL 3A was obtained from A. F. Cockburn.

In an initial screen for A. nuneztovari-specific probes, nine plaques were isolated which gave good differential signals in hybridization to A. nuneztovari and A. oswaldoi genomic DNAs. These phage will be evaluated to determine if they can distinguish these and other species of the A. nuneztovari complex.

Mapping and Sequencing of Anopheles quadrimaculatus- and Anopheles freeborni-specific Probes

Physical mapping was performed with the clone pAf1-S1, using single and double digests. This resulted in the localization of unique EcoR I, Hind III, Sst I, and Kpn I sites located at about 250, 500, 600, and 1400 base pairs, respectively, from the Xba I site in the vector. This analysis also revealed the presence of four Pst I sites, and the absence of sites for Acc I, BamH I, Sal I, and Xba I, in the insert. Many (more than 10) Sau3A I sites were detected in the insert, with several clustered within 200 base pairs of the Pst I site in the vector. Deletion subclones were

constructed using the four unique restriction sites found in the insert.

Analysis of the nucleotide sequence data obtained from probes pBrp1-S1 (Figure 1), pAf1-S1 (Figure 3), and the Crp plasmids (Figure 2) did not reveal any repeat structures which might be important in conferring species specificity. Of the three Crp plasmids, only pCrp1-S2 and pCrp1-S3 were found to retain the specificity of the phage Crp probe, in tests with quick blots (see below).

Comparisons of all the sequence data obtained in this study to the data contained in the EMBL and Genbank databases revealed no significant findings (no contiguous regions of mosquito DNA longer than about 20 nucleotides were similar to sequences stored in the database), with the following exceptions. A small portion of sequence at one end of the pAf1-S1 clone was found to show considerable homology to several ribosomal sequences from plant and animal sources, suggesting that the elimination of this small part of the probe insert could result in increased specificity. Comparisons of the sequence from pCrp1-S3 to cytochrome P-450s from several sources may not be significant, as the extent of similarity was not great; however, this finding will be pursued further.

The fact that no repeat sequences are reported here for the pBrp1-S1, pCrp1-S1, pCrp1-S2, pCrp1-S3, and pAf1-S1 probes does not indicate that the sequences conferring species specificity to the probes were not found. Such sequences may be present in the data, but the small amount of sequence data obtained from these clones is just a start in the effort to characterize them at the molecular level. The sequences important for species specificity in these clones may not be small repeats (as is the case for the Arp2 probe), and the repetitive sequences providing specificity may not become apparent even with the entire sequences in hand, especially if only one repeat is contained in a given clone. If this happens, subcloning and additional specificity testing could narrow down the region conferring specificity, and the species-specific subclones could be used as tools to probe the genome directly.

Enough nucleotide sequence data were obtained from the *Nsi* I subclones of pKA2 to allow recognition of conserved internal repeats (Figures 4 and 11). This allowed the specification of synthetic oligonucleotides. As the insert in pKA2 was known to be rearranged with respect to that in phage Arp2, sequence data were also obtained from subclones prepared directly from phage Arp2 (Figures 5 and 11). The latter data were thought to reflect more accurately the

actual sequence in the A. quadrimaculatus species A genome. In the sequence data obtained from the phage Arp2 subclones, it was found that in most every 200 base pair stretch of contiguous sequence there were from one to five copies of a given sequence motif, and two or three of the different motifs, represented.

The differences between the sequence data obtained from phage Arp2 and that from the pKA2 subclones suggest that the sequence obtained from the phage Arp2 does not correspond to the same regions of the mosquito genome as the sequence obtained from the pKA2 subclones. However, the striking conservation of consensus sequences in the data from both sources (Figure 11) suggests that the pKA2 insert accurately preserves at least some of the sequences found in the A. quadrimaculatus species A genome. It also suggests a mechanism that explains the shortening of the phage Arp2 insert in the subcloning step that generated pA2 (and thus pKA2) from phage Arp2: the multiple conserved consensus sequences in the Arp2 insert provided a suitable substrate for an internal recombination event in the bacterial host that resulted in a large deletion. This type of event would leave the majority of sequences within the pA2 insert intact with respect to the corresponding regions in the phage Arp2 insert.

Forward Primer. 181 Nucleotides.

```
  1  GACGTCCAGC TGCCGCTTCC TTCGTCTGcC GGCGTCGGAG TGACTTGTTG
 51  GACGACGACG TCGGGCCGTT GCgcTCcCGC CAGCCGACGC TCACGCTGGT
101  GTACCGCATG AAGTTCCGCC ACGCGTTGGG CGTGGACTTC GCCATggccA
151  GGGTCTgcTT GTTCGACTAA TAGgCCAACC T
```

Reverse Primer. 275 Nucleotides.

```
  1  ATCTCAGCTG ACTGCATAGT TTAGACGATT AACGTTGACT CGACCAAACA
 51  ACGTCATGCA AACCAGCAAC TTTTGGTTGc CGTCGAATTT CCACCTCACA
101  TGGCAAAAGA GTGGACAGTC CTCGTTGTGT CGCTACGGTC AGCTACAATG
151  GCGCTcCCGT TAGAAGCCGA CCGCCGCCCA CATTCGTTCT TCTTAAAGAT
201  CGTCTTTATT AAAaGAACAC GCCGGTCCGT GGCGGTCAAA CCTAATGTGT
251  ACTGCCACTA TTTtCCTGGC CAGAA
```

Figure 1. Nucleotide Sequence from the A. quadrimaculatus Species B-specific Probe, pBrp1-S1. Lower case nucleotides indicate uncertainty in the data at those positions.

pCrp1-S1. Forward Primer. 264 Nucleotides.

```
  1  AGCCAGCTGG ACGTCCAGTT GCCTAGTTCT CTTGCTTCTT GTCGTGTGAT
 51  AGCCGNGCGA TTCGGTAATC GGCGCGTTGC CTACGGCANC GTGCTACCGT
101  GCCCGTTTGT CACCTAGGCA GCACATGCAG TCTTACAGTA GCACCAAaCG
151  GCTTACCAAA TGACGGGCTA GAGGCTATAC CTTGCGATAA CAGACTCTAA
201  CGATGATACG ATGGCGTTGC CAgGATgcAg GAAGCTCTtA aTGACAGTCA
251  CCAAGACACA CACG
```

pCrp1-S1. Reverse Primer. 201 Nucleotides.

```
  1  CTCAGCTGGT AAGCTGCTTA AAGATGnGGC GTAGCCGGGT GCCTGTCGGG
 51  GTCTGCCTGT TGGGCGTCAT ACTGCATGTT GTTTCACGTT ACATCTTTTT
101  GTGTTGTGAT AAACTTCAAC AACCCTTGTC TTAgTTGGCn AcGGataTTt
151  CCATTAAGTG ACGTGAGTTt CATGTTGTTT tCCCGTATAT tGgAaTTGTa
201  A
```

pCrp1-S2. Forward Primer. 168 Nucleotides.

```
  1  GGCGGCGGGT GTAAGCAAGA AGAATTTCTA GCAGAAATAA TTTtCTtGcT
 51  GcCgGCCAGG CACCGCcCAG TTTGGATTAC ACATGACGGT GATAaAAAGG
101  ACCGGtCTGC CGGTCGCCGg TACAATgGcC ATCGCGTCTG ATACTTGGCG
151  CGTATAATCG nACTCGGA
```

Figure 2. Nucleotide Sequence from the A. quadrimaculatus Species C-specific Probes pCrp1-S1, pCrp1-S2, and pCrp1-S3. Lower case nucleotides indicate uncertainty in the data at those positions, and N (or n) indicates the occurrence of a nucleotide of unknown identity.

pCrp1S-S2. Reverse Primer. 90 Nucleotides.

```
  1  TATCGGTTGG ACACGAGCAG AGCAAGGTGC GTGGATCGAC GGgCGGCTGG
 51  TGAGGCTGTG CCGAGCTGCG CGAAAAGCTT CGGTATCACg
```

pCrp1-S3. Reverse Primer. 124 Nucleotides.

```
  1  GAGCGTACGG CTGAACGACA TTTTCTACTG AGATATGACC AAACTTGTTT
 51  GAATCCTTTC TTTGCTTTGC GTAGCTTCTG AGCTACGCTC CCAAACAATT
101  GCTCACTGCT AATGAaAGAA AAaG
```

Figure 2--continued.

Forward Primer. 177 Nucleotides.

```
  1  GATCCGGGGT AGTCCACTAT AACACAAACA AACAACCAAA GGTCAGGAAT
 51  GAGTAAATGG AGGTGCGTTG GGCTAGCTTG CCAACCGAAA CATAAGGAAT
101  GAGTACATGG AGTTGAGTTT GGTTTCCAAT CTACTATAAG GAAGCAAAAA
151  ACTTTACCTT AAATGAATTC TGCGTCA
```

Reverse Primer. 282 Nucleotides.

```
  1  GTGTTGGATT GCTAGGAGGC GCTTgCgACC CCCAAATaCC ACGTTCGTAA
 51  TGGATCGgAT GTcCGTACnC TGCGGATCGA CAAGTGCACC GCgGCCTtGC
101  ACgCcCGGGG GnCCACCGAC nggGCTGAAT gTCGCCCCGG TCTATTGAGT
151  TCAACGGGTT TGTTCCCCTA GGCAGTTTAC GTACTCTTTG ACTCTCTATT
201  CAGAGTGCTT TnAACTTtCC TCACGGTACT TGTTCGCTAT CGGCTCATGG
251  TGGTATTAGC TTAGAaGGAG TTCTCcACTT AG
```

Reverse primer. 160 Nucleotides.

```
  1  GCAAAAAACT TTACCTAAAT GAATTCTGCG TCATATCATG GGTGTTCTAG
 51  TCAAGTGGCC AAGATAACCA AGAGGTGCAG CAAATTACAA ATGAGAAGTT
101  GAGTATGCCT TCTCATATgA TAACCCTCTA ACAAAGTCAA TGACGCAAAT
151  CAACATTGGA
```

Figure 3. Nucleotide Sequence from the Anopheles freeborni-specific Probe pAf1-S1. Lower case nucleotides indicate uncertainty in the data at those positions, and N (or n) indicates the occurrence of a nucleotide of unknown identity.

pKA2-N1. Forward Primer. 85 Nucleotides.

1 **TGCATACACC AATAGATGCA ATNAGTTTNG AG**TATGTTCT ATGATAGGTT

51 TGTTAACAGA TGCCTAGATA TGGCATGTAT TCATA

pKA2-N1. Reverse Primer. 294 Nucleotides.

1 **GCATATAGCT GGTGCTAGTT T**TTANANAGT GGNAGAACAT GGGAAATCTG

51 TGAAGCAAAC CAAGTCACAG GACAGACTCC GAAACTGATG GCATCTATTG

101 GGCTACGCAT GGAAAACCCG CTT**TTTGCAT ATAGCTGGTG CTAGTTT**TGG

151 ATATATNNTT GGGAATACGN CTG**TTTGCGT ATAGCTGGTG CTAGTTT**GGA

201 ACTGTGACAC AATTCAATCT GTTAGCAATC ATAGGACATA CTCAACTATG

251 GCATGATCGG TGTACGATGA AC**gCTATTGC TAGCTGGTGT CTAG**

Figure 4. Nucleotide Sequence from NsiI Subclones of Plasmid pKA2. Lower case nucleotides indicate uncertainty in the data at those positions, and N (or n) indicates the occurrence of a nucleotide of unknown identity. Internal repeats (conserved or consensus sequences) used to specify the production of synthetic oligonucleotides (Figure 11) are indicated as follows: **SEQUENCE 1**; SEQUENCE 2; SEQUENCE 3; and **SEQUENCE 4**. Note the overlaps of some of the repeats at their ends.

pKA2-N2. Data from Forward & Reverse Primers. 267 Nucleotides.

```
  1  CGCTGTTTGC ATATAACTAG TGCTAGATTT GGATATATGG CACAAATGTC
 51  AAATCTGTTA GCAAATCAAT CATAGGACAT ACTTCAAACT CATGGCATCT
101  ATTGGTGTAC GCATGGTAAT CCGCTGTTTG CATATAGCTG GTGCTAGTTT
151  GAGATATATG GCACAAATGT GATCAATTGT CATATCTAGG CATCTGTTAG
201  CAAACCAATC ATAGGACATA CTCCAAACTC ATTGCATCTA TTGGTGTATG
251  CAGGTCGACT CTAGAGG
```

pKA2-N3. Data from Forward & Reverse Primers. 113 Nucleotides.

```
  1  CGCTGTTTGC ATATAGCTGG TGCTAGTTTG AGATATATGG CAAAAATGTC
 51  AAATCTGTTA GCAAACCAAT CATAGGACAT ACTCCAAACT CATTGCATCT
101  ATTGGTGTAT GCA
```

Figure 4--continued.

pArp2-N1. Reverse Primer. 135 Nucleotides.

1 CAAGCTTGCN TNCCTGCATA CACCAATANA TGCAATGAGT TTGGAGTATG

51 TCCTATGATT GGTTTGCTAA CAGATTTGAA ATTTGTGTCA CAGTTCCAAA

101 ACCAGCACCA GCCATATGCA AACAGCGTAT TCCCA

pArp2-N3. Reverse Primer. 262 Nucleotides.

1 TAGCTGGTGC TAGTTTTTTA TATATGGCAA ACATGTCAAA TCTGTTAACA

51 AACCAATCAC AGGACATACT CCAAACTCAT GGCATCTATT GGTCTACGcC

101 ATGAAAACCg CcGcTTTTTG CATATAGCTG GTGCTAGTTT TGGATATATG

151 CTTGGGAATN nNTGTTTGCG TATANTGGTG CTAGTTTNNN AaCTGTGACA

201 CAAATTTCAA AtctGattaG CAaATCAATC ATAGGACATA CTCAaACTAT

251 GGCATGTATC GG

pArp2-N5. Reverse Primer. 94 Nucleotides.

1 CTATGATTGA TTTGCTAAAA GATTTGACAT TTGTGcCCAT ATATCCAAAA

51 CTAGCNCCGG CTATAACCAa ACAGCGTATT TCCATGCAGG TCGA

Figure 5. Nucleotide Sequence from Subclones of Phage Arp2. Lower case nucleotides indicate uncertainty in the data at those positions, and N (or n) indicates the occurrence of a nucleotide of unknown identity. Internal repeats are identified (see Legend for Figure 4).

QUICK BLOTS

Experiments Leading to the Quick Blot Protocol

In attempts to use nonradioactive detection systems with mosquito species-specific probes, it was found that an improved method for preparing targets from a series of individual mosquitoes was needed. Table 1 provides a summary of the experiments that led to the development of this method.

Experiment 1 (Table 1) demonstrated the effectiveness of SA-AP detection, using mosquito genomic DNA as both the target and the probe, with dot blots. It revealed that 10 ng of target DNA could be detected with homologous probe, even when background was very high.

Experiments 2 and 3 (Table 1) suggested that the high background seen in experiment 1 could be reduced by substituting nitrocellulose filters for nylon without sacrificing sensitivity. Using SA-AP detection with nitrocellulose filters, a sample of 10 pg of target DNA was detected on dot blots. Experiment 4 was performed in order to determine if results differed when NFDM was substituted for BSA (bovine serum albumin) in the SA-AP detection protocol. Either ingredient could be used without effect on the sensitivity of detection or the level of background.

Thus, NFDM was used in place of BSA in all subsequent SA-AP detections.

In experiment 5 (Table 1) and in other experiments (Tables 1 and 3), the recombinant plasmid pKA2 was used as a probe. Using nick translation and autoradiography for labeling and detection, respectively, it was found that radiolabeled pKA2 provided specific detection of A. quadrimaculatus species A, without showing significant detection of A. quadrimaculatus species B, C, and D, A. crucians, A. albimanus, A. aegypti, A. taeniorhynchus, C. quinquefasciatus, and C. perturbans.

Experiments 5 and 6 (Table 1) showed that an unmodified SA-AP detection protocol could not be used for species identification of mosquitoes using the squash blot protocol (Cockburn, 1990) with species-specific probes, due to nonspecific detection. The problem was thought to be caused either by residual streptavidin-binding substance (perhaps biotin) or alkaline phosphatase activity in the target areas on the filters. Results of experiment 7 (Table 1) suggested that the former was the cause, since no signals were formed upon equivalent detection when both the SA-AP enzyme complex and biotinylated probe were omitted. Thus, improvements to the SA-AP detection system were needed, which would allow specific detection of mosquitoes using species-specific

probes. It was thought that the streptavidin-binding substance might be removed or neutralized by certain treatments of the filters following sample application but prior to the prehybridization step. These treatments are hereafter referred to as post-application treatments or post-application washes, as they were applied to blots after the binding of sample (target) DNA to the filters.

Experiments 8 and 9 (Table 1) suggested that an unmodified ECL detection protocol would not be useful for mosquito identification using species-specific probes in either the dot blot or squash blot systems, due to a relatively high level of nonspecific detection. Experiment 10 showed that the ECL system was functioning in the detection of non-mosquito control DNAs in the dot blot system. Hence it was obvious that alterations would have to be made before the ECL detection system could be used with species-specific mosquito DNA probes. Experiment 11 (Table 1) was designed to find out whether the ECL detection protocol would produce signals on nitrocellulose squash blots of mosquitoes in the absence of hybridized probes. Indeed it did, suggesting that residual peroxidase activity in the target areas may have contributed to nonspecific detection. It was thought that certain post-application wash

conditions might be found to inactivate this activity on filters before prehybridization steps were performed.

In order to discover post-application treatments of the filters which would eliminate the barriers to species-specific nonradioactive detection of mosquito squash blots using DNA probes, various washes of the filters were tested. In experiments 12a and 12b (Table 1), squash blots were washed separately for 45 min at room temperature in the following solutions : 10% SDS, 8M urea, 0.5 M HCl, 10% meat tenderizer in 1X SSPE, 10% LA FRANCE (whitener/brightener powder containing protease, The Dial Corporation), 8M urea plus 10% SDS, or 8M urea followed by 10% SDS. In these experiments, the washing step was followed by detection steps, and the prehybridization and hybridization steps were omitted.

When the ECL detection protocol was applied to these unprobed squash blots (experiment 12a, Table 1), all the washes were found to be useful in greatly reducing background levels. However, various levels of nonspecific detection were observed. The lowest signals occured on nylon filters which received the urea and the urea-then-SDS treatments, and on nitrocellulose filters which received the LA FRANCE or urea-then-SDS treatments (see notes on Table 1 for more experimental details).

Another set of squash blots treated as described above was subjected to the SA-AP detection protocol (experiment 12b, Table 1). High background levels were observed on all of the nylon filters, whereas all nitrocellulose filters showed very low background. The nylon and nitrocellulose filters which received the LA FRANCE treatments showed negligible signals, apart from background, whereas all other filters had a moderate level of nonspecific signals.

Experiments 12a and 12b (Table 1) revealed that specific treatments of squash blots were effective for reducing background and/or nonspecific detection. The results suggested that nitrocellulose squash blots washed with LA FRANCE could be used with DNA probes to provide specific detection of mosquitoes with either the SA-AP or the ECL systems.

First Attempts at Making Quick Blots

Although experiments 12a and 12b (Table 1) demonstrated the potential usefulness of post-application treatments for improving the specificity of nonradioactive detection with squash blots, it was obvious that further improvements could be made in signal-to-noise ratio, as well as in standardization of sample application. It was thought that an adaptation of the 96-well dot blot manifold might allow the use of batch-processing techniques and ensure uniform

sample application. Further improvement in detection might also be achieved with this apparatus by using a selective barrier for excluding cuticle and large pieces of tissue from binding to the filters. A dot blot manifold was used essentially as in a standard dot blot protocol, but with the important modification of placing a filter paper above the blotting filter instead of below it.

Experiments 13a and 13b (Table 1) represent the first attempts at implementing the QB protocol based on some of the ideas described above. In experiment 13a, a thick filter paper pad was used as a blocker. In experiment 13b, a thin tissue was used. The filters were probed with radiolabeled pKA2, and detection was by autoradiography. Detection of DNA was superior when the thin tissue was used as the blocker. The thicker paper apparently blocked the DNA from reaching the blotting filter. After this experiment all other QBs were produced by using the thin tissue.

Table 1. Experiments Performed to Develop the Quick Blot Protocol.

EXPT	FILTER/SAMPLE	PREHYB/HYB	PROBE	DETECTION	RESULTS
1	NY dot blot of of AqA DNA	std	AqA -BIOTIN	SA-AP	10 ng det unequivocal; very high bg
2	NC dot blot of biotinylated #147 DNA	std	NONE	SA-AP	100 pg det unequivocal; 10 pg barely distinguish-able; very low bg
3	NC dot blot of #147 DNA (unlabeled)	std	#147 -BIOTIN	SA-AP	same as for expt 2
4	same as for expt 2	std	NONE	SA-AP (BSA)	same as for expt 2
5	NC squash blots with Ac, AqA, Cq, and Cs	std	pKA2 -BIOTIN	SA-AP	nonspecific det of all samples; very low bg
6	same as for expt 5	std	NONE	SA-AP	same as for expt 5
7	NC squash blots with AqA, Cs, and Cq	NONE	NONE	SA-AP (without SA-AP reagent)	no det
8	Hy-ECL dot blot (with AqA, Cn, Cq, and Cs)	ECL	pKA2	ECL	nonspecific det; very low bg
9	NC squash blots with AqA, Cs, and At	ECL	pKA2 -ECL	ECL	nonspecific det (signals for all three species); very low bg

Table 1--continued.

EXPT	FILTER/SAMPLE	PREHYB/HYB	PROBE	DETECTION	RESULTS
10	NY dot blot and Hy-ECL dot blot (both with lambda DNA dilution series)	ECL	lambda -ECL	ECL	10 pg det unequivocal with NC and NY filters; 1 pg det (barely) on NY
11	NC squash blot with AqA, Cs, and At	NONE	NONE	ECL	Nonspecific det; low bg
12a	NC and NY squash blots with AqA, Cs, and At (VARIOUS POST-APP WASHES: SDS, urea, HCl, mt, LF, urea-SDS, urea-then-SDS)	ECL	NONE	ECL	Nonspecific det with very low bg, on all filters, but very faint signals only on NY filters which received the urea and the urea-then-SDS treatments, and lowest signals among the NC filters which were treated with urea-then-SDS or LF

Table 1--continued.

EXPT	FILTER/SAMPLE	PREHYB/HYB	PROBE	DETECTION	RESULTS
12b	same as for expt 12a	std	NONE	SA-AP	All NY filters showed high bg, and all NC filters showed very low bg. Nonspecific signals seen on all targets on all filters EXCEPT that virtually no signals seen on both NC and NY filters which received the LF washes
13a	NY QB but with filter paper blocker; AqA	std	pKA2 -32P	AR	only 36 (out of 94) spots show a signal
13b	NY QB (with tissue paper blocker); AqA	std	pKA2 -32P	AR	all (of 94) spots show a signal

Notes for Table 1. EXPT = experiment (number). PREHYB/HYB = type of prehybridization and hybridization conditions used. AqA = A. quadrimaculatus species A (its DNA, when used in probe column); Ac = A. crucians; Cq = C. quinquefasciatus; Cs = C. salinarius; Cn = C. nigripalpus, At = A. taeniorhynchus. bg = background level of signal development (i.e., signal intensity where target DNA was not applied to the filter). #147 DNA = Hsp70 deletion subclone from A. albimanus in pUC19, from Mark Benedict. pKA2 = plasmid pK19 with part of the mosquito DNA insert from phage Arp2 (Cockburn, 1990). lambda = phage lambda DNA.

Notes for Table 1--continued. Std = standard prehybridization (1% NFDM, 0.2% SDS, 55°C for at least one-half hour) and hybridization (30% formamide, 5X SSPE, 1% NFDM, 0.2% SDS, plus probe) conditions. SA-AP (BSA) = the GENE-TECT detection protocol, Clontech Laboratories, Inc., with SSPE substituted for SSC (saline sodium citrate). SA-AP = the GENE-TECT detection protocol, but with NFDM substituted for BSA, and SSPE used instead of SSC. NC = BA-85 nitrocellulose filter, Schleicher & Schuell. QB = Quick Blot. NY = ZetaProbe nylon filter, Bio-Rad. Hy-ECL = HyBond ECL filter (nitrocellulose; Amersham). NONE = no treatment, or no probe. BIOTIN = biotinylated probe prepared by nick translation. ECL = ECL hybridization buffer (for prehybridization and hybridization steps), as supplied plus 0.5M NaCl, or ECL probe prepared according to protocol provided by manufacturer (Amersham). pg = picogram(s). ng = nanogram(s). det = detection. no det = no signals or only very faint (barely observable) signals. Dot blots = standard dot blot procedure using dilution series of purified DNA applied to the filters, with separate spots containing different amounts of the same DNA. Squash blots prepared as described by Cockburn (1990). POST-APP = post-application treatments, or washes of filters after target DNA samples were applied (all for 45 min at room temperature): SDS = 10% SDS; urea = 8M urea; HCl = 0.5M HCl; mt = 10% meat tenderizer (Tone's Meat Tenderizer, Tone Bros., Inc.); LF = 10% LA FRANCE (whitener/brightener powder containing protease, Dial Corporation); urea-SDS = 8M urea, 10% SDS; urea-then-SDS = first wash with 8M urea, second wash with 10% SDS. Nitrocellulose filters were subjected to standard vacuum-baking procedures following application of samples, and nylon filters were subjected to standard UV-fixation (ultraviolet light treatment) following application of samples (except where otherwise noted). However, results of equivalent experiments in which these fixation steps were omitted suggested that non-fixed filters of both types yield signals equal in intensity to fixed filters.

Steps in the Quick Blot Protocol

This section describes the steps in the QB protocol in detail. A list of materials and apparatus used in the preparation of QBs is given in Table 2, and a picture of the apparatus is shown in Figure 6.

Mosquitoes (larvae, pupae, or adults) were placed individually into the wells of a 96-well microtitration plate. Denaturing buffer (Table 2) was then added. For standard sized wells of 10 mm deep and 13 mm in diameter, a maximum of 200 μl per well of denaturing buffer was used.

The DNA was then released from the tissues by grinding with the steel pegs of the Replaclone for about three min in the orientation that allowed all of the Replaclone pegs to be inserted into wells of the plate. The progress of this grinding step was checked visually at 30 s intervals by inspecting the coloration of the sample buffer and noting whether any large tissue fragments were attached to the proximal part of the steel pegs. A given mosquito species or life stage produced a characteristic (usually slightly brownish or yellowish) coloration of the buffer when grinding was sufficient. In about 5% of adult mosquito samples, tissue fragments required being pushed back into the buffer by using a pin or fine forceps.

The plate was incubated for 30 min at room temperature, and then neutralization buffer (Table 2) was added and mixed thoroughly in the sample wells using the Replaclone (or micro-pipette). The volume of neutralization buffer added per well was one-fourth of the volume of denaturing buffer added previously. Use of a multi-channel pipette for all transfers of solutions significantly decreased the time and effort required to complete the protocol, but was not required.

A blotting filter (nitrocellulose or nylon) was cut to size and wet in water. The base of the dot blot manifold was positioned for convenient access to the vacuum source, and then the middle block of the manifold was set in position over the base. Next, the wetted filter was placed over the top of the middle block of the manifold so that it was more or less centered over the sample application areas. The two corners of the filter were trimmed where the metal pegs arose from the manifold, so that the entire filter was flat.

A tissue was used to keep larger pieces of cuticle and other debris off the blotting filter. The tissue was wetted, then placed over the surface of the filter by starting from one edge or corner. In this way, large bubbles did not become trapped between the tissue and the filter.

The top portion of the manifold was clamped tightly over the tissue, filter, and lower portions of the apparatus. Vacuum was applied (usually with a trap and valve mechanism so that a low level of suction was applied). Then samples from the microtiter plate were applied to the dot blot manifold wells, preserving the relative orientation of sample locations between the microtiter plate wells and the manifold wells. Once the samples had been aspirated through the membrane, wash buffer (Table 2; about 350 μl per well) was added to the wells to wash portions of the samples remaining on the walls of the manifold wells onto the filter. When all the buffer had been washed through the filter, the vacuum was removed from the manifold, the manifold was disassembled, and the filter removed.

By splitting the sample solutions into several aliquots, a given set of samples was used to produce multiple equivalent filters. Duplicate filters were prepared in the dot blot manifold, samples being applied from the same microtiter well plate, until the total sample volume had been used. In this way, many (up to 10) equivalent filters were produced with a single set of samples.

Nitrocellulose filters containing samples were vacuum-baked. Then the filters were used as hybridization targets with DNA probes. Nylon filters were either air-dried or UV-fixed and then air-dried.

Table 2. Materials and Apparatus for the Quick Blot Protocol.

Plastic Microtitration Plate, 96-well (flat-bottom wells; many sources)

96-Place Microsample Filtration Manifold (Dot blot manifold; Schleicher & Schuell)

Micro-Pipette (Multi-channel preferred; many sources)

Pipette Tips (many sources)

Replaclone (96-prong model; L.A.O. Enterprises)

Filters for Nucleic Acid Blotting (nitrocellulose, such as BA-85 from Schleicher & Schuell; or nylon, such as Zeta-Probe from Bio-Rad), cut to 12 x 8 cm size

Laboratory Tissues (such as Kimwipes from Kimberly-Clark, or

Stirling Light Duty Wipes from Stirling Converting Company, Inc.)

Vacuum Source (sink aspirator or pump)

Buffers

Denaturing Buffer: 0.5M NaOH, 1.5M NaCl

Neutralization Buffer: 3N sodium acetate, 2N acetic acid

Wash Buffer: 2X SSPE

Figure 6. Apparatus used to prepare quick blots. The 96 steel pegs of the Replaclone (left) fit into the wells of the microtiter plate (lower right) when grinding the mosquitoes. The dot blot manifold is shown with blotting filter in place, overlaid with a tissue to prevent bits of cuticle and cell debris from adhering to the blotting filter. Before samples are applied to the blotting filter, the top portion of the manifold is clamped into place, and the vacuum source is attached. The optional multichannel pipette speeds transfer of solutions.

Experiments to Optimize Use of Quick Blots with Mosquito Species-specific Probes

Table 3 summarizes the results of experiments performed to evaluate and refine the QB protocol for use in identification of mosquito species by DNA hybridization using species-specific probes. These experiments were required for the optimization of results when using various detection systems for species-specific DNA probes with mosquito QBs.

Experiments 14 and 15 (Table 3) were performed to evaluate the effectiveness of various post-application treatments of QBs probed with either one or two probes. In experiment 14, various post-application washes of the filters were tested to maintain conditions that would reduce the level of nonspecific detection. In experiment 14, the probe was omitted from one set of filters which were treated with the same washes, and it was found that washes that contained a whitener/brightener with protease (LA FRANCE) were effective at improving the specificity of detection (filters C, D, G, and H in Figure 7). Even though specific signal strengths were decreased somewhat by the use of LA FRANCE, the overall effects were desirable due to a dramatic reduction in nonspecific detection (compare filters E and F with filters G and H in Figure 7).

In experiment 15 (Table 3), standard ECL prehybridization was used, and ECL detection was performed before SA-AP detection. The pCx1-ECL probe, when used alone or in combination with a biotinylated probe, gave at least a medium level of specific detection and a low level of nonspecific detection with any of the post-application treatments. When SA-AP detection of biotinylated pKA2 was performed after ECL detection, very strong specific detection was achieved. This occurred after post-application washes, with both the urea-SDS-LF and the LF-then-urea-SDS treatments (see notes to Tables 1 and 3 for more details on post-application washes). These results suggested that some aspect of the ECL prehybridization and/or detection was enhancing the results of SA-AP detection, since SA-AP detection of biotinylated pKA2 using standard prehybridization resulted in nonspecific detection (experiment 5, Table 1) and/or unacceptably high background (experiment 15, Table 3).

Regarding experiments 14 and 15, there was considerable variation in intensity between spots from different mosquitoes. Since this was seen with spots which received the same amount of homogenate (starting with a single mosquito for each homogenate), the variation was probably due to one or a combination of the following: a variable

amount of DNA was released from each mosquito which was ground by this method, or a variable amount of target repetitive DNA sequences in the genomes of individual mosquitoes. Another result of these experiments which was seen consistently in the QB results was the concentration of signal in a small spot in the center of the circular area where samples were applied. The latter effect was probably due to tangential flow toward the center of the wells in the dot blot apparatus during preparation of QBs. Also, identical filters were given the post-application treatments described, but using a wash temperature of 45°C. The results were virtually identical to those obtained for similar filters which received the post-application treatments at room temperature.

Experiments 16a and 16b (Table 3) were performed to confirm the utility of a combination of biotinylated and ECL probes to QBs used in a single hybridization experiment, when using stepwise LA FRANCE and urea-SDS post-application treatments and ECL prehybridization. The two probes were labeled reciprocally in the experiments, in hopes of distinguishing effects from the detection system from effects resulting only from properties of the particular probes. Although some of the latter effects were manifested, the results prove the utility of the conditions used for the

specific detection of these hybridized probes in a sequential application of detection protocols.

In experiments 16c through 16f (Table 3), the same post-application and prehybridization treatments were used as in experiments 16a and 16b, but the filters were dot blots instead of QBs, so that a rough quantitation of the sensitivity of specific detection could be obtained. Detection levels were in the range of 1-10 ng for ECL, SA-AP, and autoradiographic detection.

Experiment 17 (Table 3) was performed to assess quantitatively the levels of detection possible when ECL-labeled and biotinylated probes were used in a single hybridization step. A standard dot blot strip was used for this experiment. It is not known why the sensitivity of detection of the ECL-labeled probe was lower than that found when only a single probe was used in the hybridization step (experiment 10, Table 1). However, the results suggested that detection in the 10 ng (or higher) range should be sufficient for properly scoring results of various detection systems using mosquito QBs. Also, this experiment showed that when using a biotinylated probe in ECL prehybridization and hybridization conditions, detection levels were lowered considerably as compared to the levels obtained when standard prehybridization and hybridization conditions

(i.e., those used with radiolabeled probes) were used (experiments 2 through 4, Table 1).

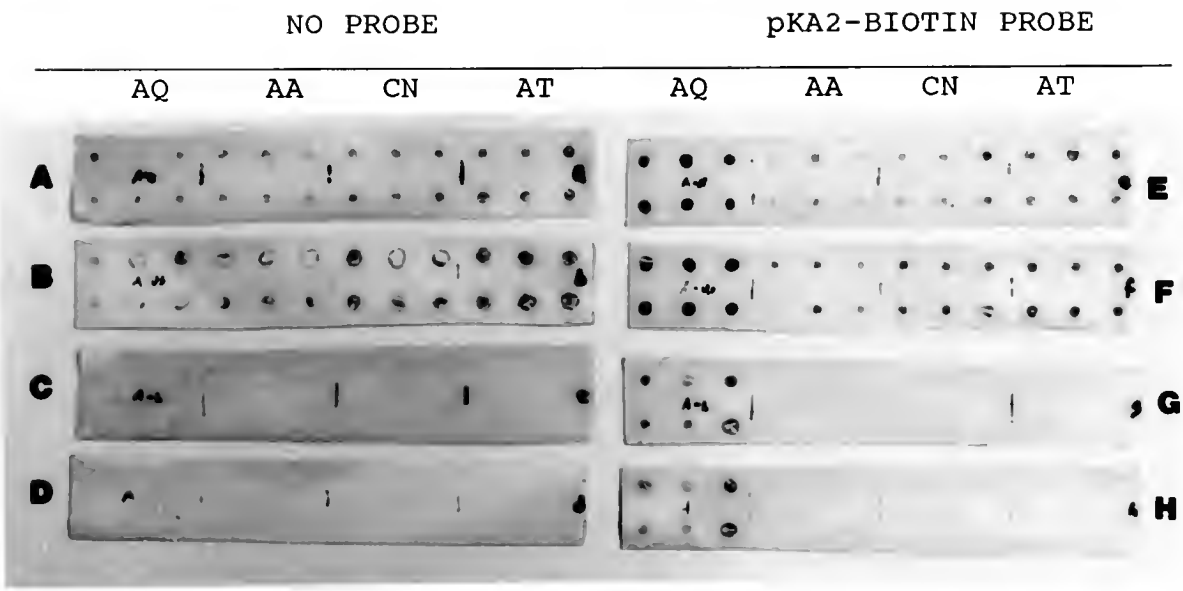
Experiment 18 (Table 3) revealed the level of detection attainable with radiolabeled probe hybridized to homologous target DNA in a dot blot. Detection levels in the ng range were achieved consistently in several experiments using these conditions, and detection in the pg range has been observed on occasion.

Figure 8 reveals the effects of using different filter types and DNA binding conditions on the results of SA-AP detection (experiment 19, Table 3). UV fixation of nylon filters did not in itself affect the detection levels (Figure 8 B and C) when QBs were subjected to SA-AP detection. A degradation of specificity resulted when alkaline binding was used in preparing a QB with a nylon filter (Figure 8 A), as compared to that obtained when the other conditions were used.

Experiments 20a and 20b (Table 3) were designed to further test whether UV fixation of nylon QBs would improve the detection of hybridized species-specific DNA. Radiolabeled probe was detected by autoradiography, and the QB which did not receive the UV fixation yielded signals equivalent to those produced from the UV-fixed filter.

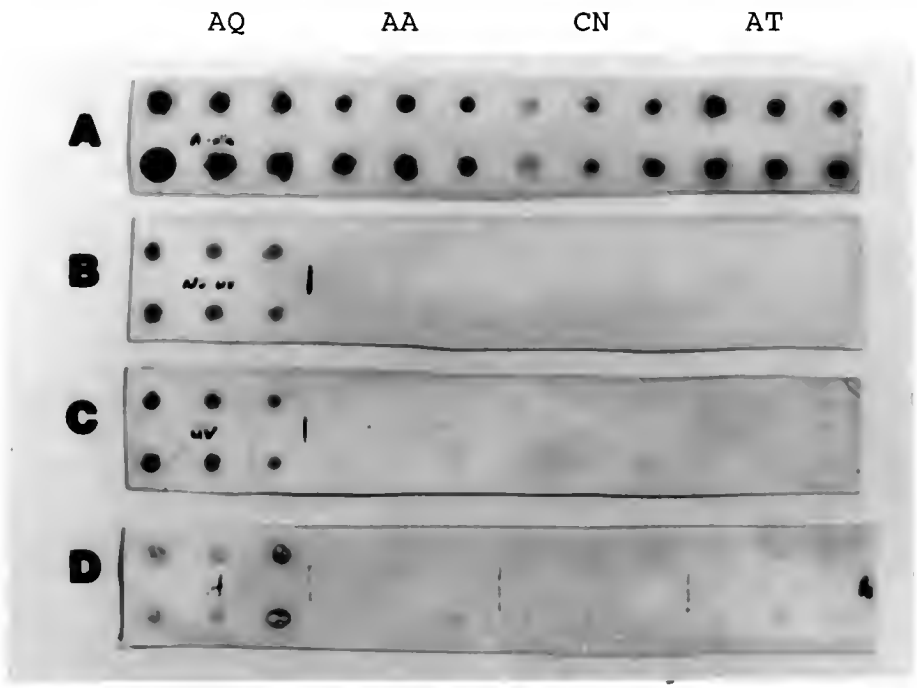
Three different nonradioactive detection systems were used separately with QBs (experiment 21, Table 3; Figure 9). Whereas reliable specific detection was obtained with the ECL and SA-AP systems, the Genius system detection was quite variable. Many of the experiments in Tables 1 and 3 were performed with the Genius system, which was used as suggested by the supplier, except for substituting SSPE for SSC, and changing the formamide concentration in the hybridization buffer to 50% for hybridization at 42°C. Figure 9 B is typical of the results with the Genius system, as a patchy distribution of high background often interfered with interpretation.

Experiment 22 (Table 3), shown in Figure 10, confirmed some of the results of experiments 15, 16, and 17. This proves that probes with different specificities can be used in a single hybridization step and detected differentially with a sequential application of nonradioactive detection methods.



A,E: no wash
B,F: urea-SDS
C,G: LF (LA FRANCE)
D,H: urea-SDS-LF

Figure 7. Effects of various post-application treatments of quick blots on the specificity of SA-AP detection. Quick blots were prepared using nitrocellulose filters, with each spot receiving one-tenth of the solution in which a single mosquito was macerated. Six (two rows of three) spots per filter contained DNA from different individuals of a single mosquito species, as follows: AQ = A. quadrimaculatus species A; AA = A. albimanus; CN = C. nigripalpus; AT = A. taeniorhynchus. Certain filters received treatments between target DNA application and prehybridization. These treatments were called post-application washes (or treatments), and were performed at room temperature for 45 min. Filters A and E received no post-application wash. Filters B and F received a post-application wash of urea-SDS (8M urea, 10% SDS). Filters C and G received a post-application wash of 10% LA FRANCE (Dial Corporation). Filters D and H received a post-application wash of urea-SDS-LF (8M urea, 10% SDS, 10% LA FRANCE). Filters were prehybridized and hybridized (with or without biotinylated probe pKA2, as indicated) according to the GENE-TECT protocol (Clontech Laboratories, using BRL reagents, except that NFDM was substituted for BSA and SSPE was substituted for SSC).



A: NYLON: alkaline binding
B: NYLON: no UV
C: NYLON: UV-fixed
D: NITROCELLULOSE: baked

Figure 8. Effects of filter type and DNA-binding conditions on SA-AP detection of probe pKA2 hybridized to quick blots. All four filters were subjected to the urea-SDS-LF post-application treatment before being subjected to prehybridization and hybridization with biotinylated pKA2 probe (see legend to Figure 7 for details of post-application treatment, prehybridization and hybridization conditions, and abbreviations). The method used for fixing the DNA to the filters during sample application was varied. Filter A was prepared according to the normal QB protocol, except that the samples in the denaturing buffer were applied to the blotting filter without being mixed with neutralization buffer (Table 2). Nylon filters B and C were prepared according to the normal QB protocol, except that no vacuum-baking step was performed, and filter C was treated with UV light after sample application. Nitrocellulose filter D was prepared according to the unmodified QB protocol.

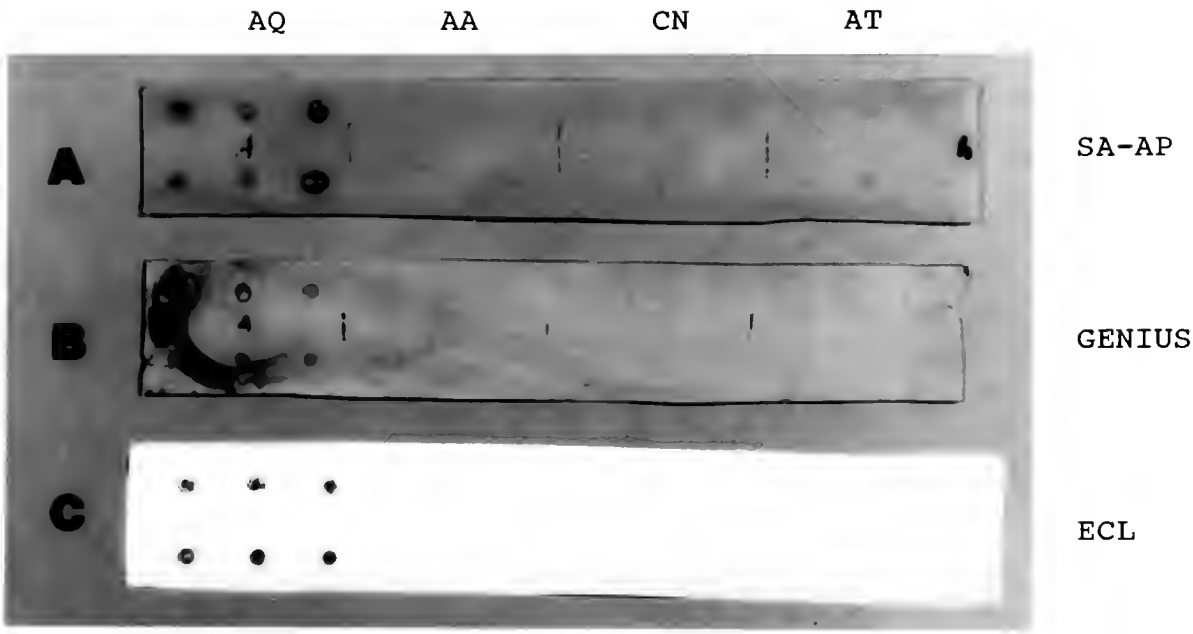


Figure 9. Different nonradioactive systems used for detection of probe pKA2 hybridized to quick blots. All three nitrocellulose filters received the urea-SDS-LF post-application treatment before being hybridized with pKA2 probe. See legend to Figure 7 for details on the post-application treatment and abbreviations). Probe DNA was labeled according to the different methods appropriate for detection by the SA-AP, Genius, and ECL protocols. Prehybridization and hybridization conditions were as follows: for the SA-AP and ECL filters, conditions were as for the filters on which SA-AP and ECL detection was performed in the experiments described in Table 3; for the Genius filter, conditions were as suggested by the manufacturer (Boehringer Mannheim Biochemicals), except for substituting SSPE for SSC, and changing the formamide concentration in the hybridization buffer to 50% for hybridization at 42°C.

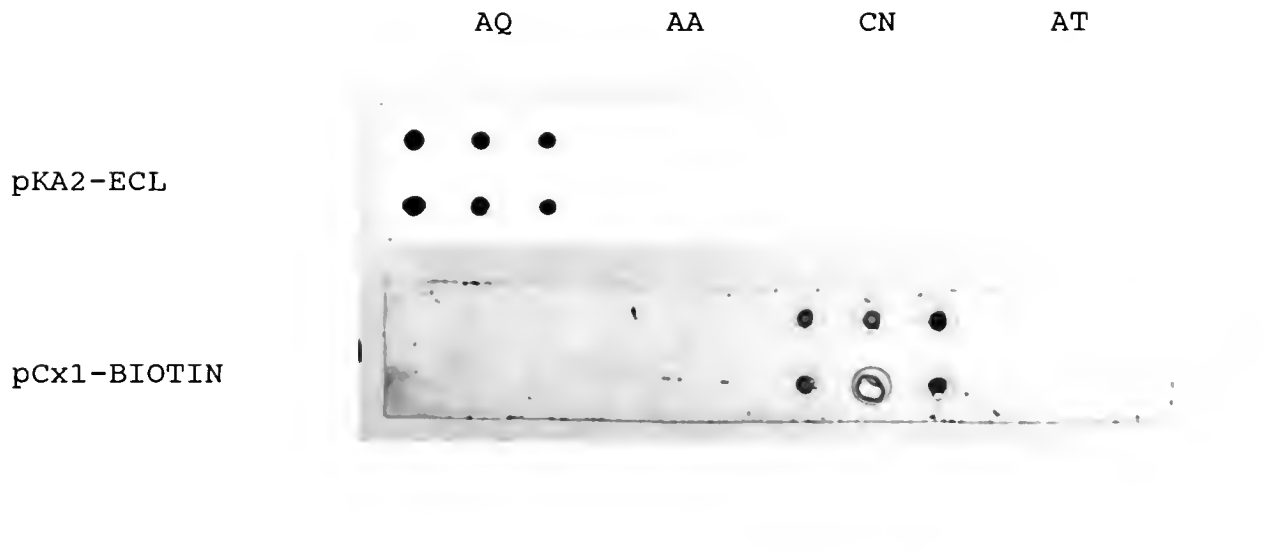


Figure 10. Sequential use of nonradioactive detection systems following a single hybridization of two probes to a quick blot. A quick blot was prepared in the same way as filters D and H of Figure 7 (standard quick blot protocol, with urea-SDS-LF post-application treatment; see Figure 7 legend for experimental details, and for abbreviations). Two probes, biotinylated pCx1 and ECL-labeled pKA2, were hybridized to this filter, using the ECL prehybridization and hybridization conditions (ECL hybridization solution supplied in the kit plus 0.5M NaCl). The bound pKA2 probe was detected first, using X-ray film according to the ECL protocol, then the pCx1 probe was detected using the SA-AP detection protocol.

Table 3. Experiments Performed to Evaluate and Optimize the Quick Blot Protocol.

EXPT	FILTER/SAMPLE	PREHYB/HYB	PROBE	DETECTION	RESULTS
14	NC QB with AqA, Aa, Cn, and At, WITH VARIOUS POST-APP TREATMENTS:				
	none	std	NONE	SA-AP	light to med nonspecific det at all spots; no bg
	urea-SDS	std	NONE	SA-AP	variable (light to strong) nonspecific det at all spots; no bg
	LF	std	NONE	SA-AP	no signals; no bg
	urea-SDS-LF	std	NONE	SA-AP	no signals; no bg
	none	std	pKA2 -BIOTIN	SA-AP	very strong specific det; light to med nonspecific det; no bg
	urea-SDS	std	pKA2 -BIOTIN	SA-AP	very strong specific det; light to med nonspecific det; no bg

Table 3--continued.

EXPT	FILTER/SAMPLE	PREHYB/HYB	PROBE	DETECTION	RESULTS
14 (continued)					
	LF	std	pKA2 -BIOTIN	SA-AP	light to med specific det; no nonspecific det; no bg
	urea-SDS-LF	std	pKA2 -BIOTIN	SA-AP	light to med specific det; no nonspecific det; no bg
15	NC QB; Aa, At, AqA, Cq, Cs WITH VARIOUS POST-APP TREATMENTS:				
	LF	ECL	pCx1-ECL	ECL	med level of specific det, but light nonspecific det of all other spots; med bg
	urea-SDS	ECL	pCx1-ECL	ECL	med level of specific det, but light nonspecific det of all other spots; med bg
	urea-SDS-LF	ECL	pCx1-ECL	ECL	med level of specific det, but light nonspecific det of all other spots; med bg

Table 3--continued.

EXPT	FILTER/SAMPLE	PREHYB/HYB	PROBE	DETECTION	RESULTS
15 (continued)					
	LF-then-urea-SDS	ECL	pCx1-ECL	ECL	ECL: med level of specific det, but light nonspecific det of all other spots; med bg
	LF	std	pKA2 -BIOTIN	SA-AP	med nonspecific det on all spots; high uneven bg (perhaps some specific det but high bg makes interpretation difficult)
	urea-SDS	std	pKA2 -BIOTIN	SA-AP	med nonspecific det on all spots; high uneven bg (perhaps some specific det but high bg makes interpretation difficult)

Table 3--continued.

EXPT	FILTER/SAMPLE	PREHYB/HYB	PROBE	DETECTION	RESULTS
15 (continued)					
	urea-SDS-LF	std	pKA2 -BIOTIN	SA-AP	med nonspecific det on all spots; high uneven bg (perhaps some specific det but high bg makes interpretation difficult)
	LF-then-urea-SDS	std	pKA2 -BIOTIN	SA-AP	some nonspecific signals but high bg made interpretation difficult
	urea-SDS-LF	ECL	pKA2 -BIOTIN and pCx1-ECL	ECL then SA-AP	ECL: good specific det; but also very light nonspecific det; very low bg SA-AP: very strong specific det; no bg except blotches due to filter overlap

Table 3--continued.

EXPT	FILTER/SAMPLE	PREHYB/HYB	PROBE	DETECTION	RESULTS
15 (continued)	LF-then-urea-SDS	ECL	pKA2 -BIOTIN and pCx1-ECL	ECL then SA-AP	ECL: good specific det; but also very light nonspecific det; very low bg SA-AP: very strong specific det; no bg, except for blotches due to filter overlap
16a	NC QB with Aa, At, AqA, Cq and Cs. WITH POST-APP TREATMENT: LF-then-urea-SDS	ECL	pCx1-ECL and pKA2 -BIOTIN	ECL then SA-AP	ECL: specific (Cs) signals somewhat higher than others but nonspecific signals med; med bg SA-AP: specific signals med, and all other spots show very faint signals; low bg (UNEQUIVOCAL DET.)

Table 3--continued.

EXPT	FILTER/SAMPLE	PREHYB/HYB	PROBE	DETECTION	RESULTS
16b	NC QB with Aa, At, AqA, Cq and Cs. WITH POST-APP TREATMENT: LF-then-urea-SDS	ECL	pCx1 -BIOTIN and pKA2-ECL	ECL then SA-AP	ECL: strong specific (AqA) SA-AP det; nonspecific signals and bg undetectable except at very long exposures SA-AP: signals to Cs strong, signals to Cq weak; no other signals (no nonspecific signals); no bg
16c	NC dot blots with L-HI and pKA2 WITH POST-APP TREATMENT: LF-then-urea-SDS	ECL	L-HI-ECL and pK19 -BIOTIN	ECL then SA-AP	ECL: specific (L-HI) det at 10 ng with 20 sec exp, to 1 ng with 10 min exp SA-AP: specific det at 10 ng (and barely seen at 1 ng); no nonspecific det; very low bg

Table 3--continued.

EXPT	FILTER/SAMPLE	PREHYB/HYB	PROBE	DETECTION	RESULTS
16d	NC dot blots with L-HI and pKA2 WITH POST-APP TREATMENT: LF-then-urea-SDS	ECL	L-HI -BIOTIN and pK19-ECL	ECL then SA-AP	ECL: specific (pK19) det at 10 ng with 20 sec exp, to 1 ng with 10 min exp SA-AP: specific det at 10 ng no nonspecific det; very low bg
16e	NC dot blots with L-HI and pKA2 WITH POST-APP TREATMENT: LF-then-urea-SDS	ECL	L-HI -32-P	AR	3 day RT exp: specific det faint but unequivocal at 1 ng
16f	NC dot blots with L-HI and pKA2 WITH POST-APP TREATMENT: LF-then-urea-SDS	ECL	pK19 -32-P	AR	3 day RT exp: specific det faint but unequivocal at 1 ng
17	NC dot blots with L-HI and pKA2 (NO POST-APP!!)	ECL	pK19-ECL and L-HI -BIOTIN	ECL then SA-AP	ECL: specific det at 10 ng (5 min exp), at 1 ng at longer (40 min) exp but with increased bg SA-AP: specific (but light) det at 10 ng; light bg

Table 3--continued.

EXPT	FILTER/SAMPLE	PREHYB/HYB	PROBE	DETECTION	RESULTS
18	NC dot blots with L-HI and pKA2 (NO POST-APP!!)	std	L-HI -32-P	AR	specific det at 1 ng; light bg
19	QB with AqA, Aa, Cn, and At. WITH POST-APP TREATMENT: urea-SDS-LF WITH DIFFERENT FILTERS AND/OR BINDING CONDITIONS:	std	pKA2 -BIOTIN	SA-AP	
	NY: alkaline binding				nonspecific det variable: light to strong det at all spots; low bg
	NY: no UV binding				med specific det; no nonspecific det; low bg
	NY: UV binding				med specific det; no nonspecific det; low bg
	NC				light to med specific det; no nonspecific det; low bg
20a	NY QB; AqA	std	pKA2 -32P	AR	all spots where sample applied show clear signal

Table 3--continued.

EXPT	FILTER/SAMPLE	PREHYB/HYB	PROBE	DETECTION	RESULTS
20b	NY QB; AqA UV-fixed	std	pKA2 -32P	AR	same as for expt 20a
21	NC QB with AqA, Aa, Cn, and At. WITH POST-APP TREATMENT: urea-SDS-LF WITH DIFFERENT NONRADIOACTIVE DETECTIONS:				
		std	pKA2 -BIOTIN	SA-AP	light to med specific det; faint to no nonspecific det; med bg
		Gen-2	pKA2 -GENIUS	Genius	light specific det; faint to no nonspecific det; heavy bg in areas that tend to obscure signals
		ECL	pKA2 -ECL	ECL	med specific det; no nonspecific det; no bg
22	NC QB with AqA. Aa, Cn, and At. WITH POST-APP TREATMENT: urea-SDS-LF	ECL	pKA2 -ECL and pCx1 -BIOTIN	ECL then SA-AP	ECL: strong specific det; no nonspecific det; low bg SA-AP: strong specific det; no nonspecific det; low bg

Table 3--continued.

EXPT	FILTER/SAMPLE	PREHYB/HYB	PROBE	DETECTION	RESULTS
23	NC QB with AqA, AqB, AqC, AqD, Aa, and Cn. WITH POST-APP TREATMENT: urea-SDS-LF	oligo	Arp-1 through Arp-4 oligo probes (32P) (used separately)	AR	Arp-1 and Arp-4 probes: strong specific det; no nonspecific det; no bg. Arp-2 probe: strong specific det; light nonspecific det; high bg Arp-3 probe: faint specific det; no nonspecific det; no bg

Notes for Table 3. see notes for Table 1, and the following. AqB = A. quadrimaculatus species B. AqC = A. quadrimaculatus species C. AqD = A. quadrimaculatus species D. AR = autoradiography; L-HI = phage lambda DNA restricted with Hind III. Aa = A. albimanus. Gen-2 = GENIUS prehybridization/hybridization mix modified for use at 42°C by including in the recommended mix 50% formamide, and substituting SSPE for SSC. med = medium. Postapplication treatments were performed as described in the notes on Table 1, plus: urea-SDS-LF = 8M urea, 10% SDS, 10% LA FRANCE; LF-then-urea-SDS = first wash with 10% LA FRANCE, then second wash with 8M urea, 10% SDS.

SYNTHETIC OLIGONUCLEOTIDE PROBES

Four oligonucleotides were synthesized based on nucleotide sequence data obtained from *Nsi* I deletion subclones derived from pKA2, in an attempt to (1) obtain greater specificity in identification of A. quadrimaculatus species A, as compared to that obtained by using pKA2 as a probe, and (2) demonstrate that synthetic oligonucleotides can provide valuable tools for identification of cryptic mosquito species. Sequence data obtained from the *Nsi* I subclones of pKA2 (which were used to specify the oligonucleotide sequences) were compared to sequence obtained from subclones of phage Arp2 (Figure 11), since the latter was thought more likely to preserve the sequences present in the mosquito genome.

Figure 11 shows the sequence of the synthetic oligonucleotides compared to sequence data obtained from subclones of phage Arp2 and plasmid pKA2. Here, differences between an oligonucleotide sequence and the sequence from a given phage subclone do not necessarily reveal the result of molecular rearrangement in the pKA2 subclones. Rather, they probably reflect small differences between the many repeat elements found within the mosquito genome. The large

differences among the four sequence motifs detected in the pKA2 subclones (and represented in the oligonucleotide sequences) contrast with the similarities among the sequences of a specific motif, whether from pKA2 or Arp2 subclones.

In the limited sequence data obtained from the pKA2 and pArp2 subclones, the motifs 1 and 3, and the motifs 2 and 4, were found to be adjacent or overlapping in at least six instances per combination. Motifs 1 and 2 were also found nonoverlapping, in at least four and three instances, respectively (Figures 4 and 5).

The oligonucleotides were radiolabeled and used as probes to quick blots prepared with A. quadrimaculatus species A, B, C, and D, A. albimanus, and C. nigripalpus (Table 3, experiment 23). The results of autoradiographic detection of hybridized oligonucleotide probes is shown in Figure 12. A long exposure of ten days (at -80°C with intensifying screens) revealed that the specificity of oligonucleotide probes Arp-1 and Arp-4 (and probably also Arp-2) was greater than that of the phage Arp2 probe (Cockburn, 1990). These results, when considered in light of the sequence data obtained from the plasmid subclones, suggested that the sequence elements present in A. quadrimaculatus species A DNA which conferred species

specificity to the phage Arp2 probe are short, nonidentical (but very similar) repeats, and that there exist three or more distinct motifs which contribute to this specificity. These sequence motifs are not tandem repeats, and are present in some cases in inverted orientations with respect to one another.

There are several possible advantages in using oligonucleotides over cloned DNAs for the preparation of hybridization probes. Usually a cloned insert will be a much longer segment of DNA than the oligonucleotide, so there is more chance for a degeneracy in specificity to be manifested by some portion of the cloned DNA sequence. Thus, the oligonucleotide may provide increased specificity if it lacks nonspecific sequences found in the cloned DNA. Another advantage of synthetic oligonucleotide probes is that since their chemical structure is completely defined, new lots of the probe may be produced at any facility set up for synthesis of oligonucleotides. Incompletely characterized DNA probes contained in plasmid vectors must be prepared using suitable (bacterial) host strains and sufficient amounts of recombinant plasmid. A third possible advantage of oligonucleotides over recombinant plasmids for use as probes is their purity. Probe DNAs propagated in recombinant plasmids must be purified to remove bacterial nucleic acids,

proteins, and lipids. While these purification steps are usually adequate for most applications, DNA modifying enzymes (such as those used in the labeling of hybridization probes) are often inhibited by trace contaminants. This is not a problem with synthetic oligonucleotides, which are typically free of contaminants.

Figure 11. Synthetic oligonucleotide sequences compared to phage Arp2 and plasmid pKA2 subclone sequences. The sequences of the four oligonucleotides used in this study are each shown above sequence information obtained from subclones of the insert from phage Arp2 and the subclones of plasmid pKA2. The sequences of the oligonucleotides were determined from sequence data obtained from the pKA2 subclones, and are shown with their 5' ends to the left. Since there was some doubt about whether the pKA2 sequence accurately represented sequence from the phage (Arp2) insert, subclones from the phage (including those listed in Figure 11 as subclones 1, 3, and 5) were sequenced and found to be similar to the sequences of the oligonucleotides. Since the insert in phage Arp2 was isolated from genomic DNA of A. quadrimaculatus species A (Cockburn, 1990), this figure reveals the close similarity of the sequences of the oligonucleotides to the repeats in the mosquito genome. oligo = oligonucleotide.

```
TTTGCATATAGCTGGTG-CTAG-TTT
.................-....-...
.........G.......-..G.-...
         .........-....-...
....GT......C..N.-....-...
.....G....-N.....-....-...
   ..............-....-...
..................-....-...
.....G............-....-...
GC.ATTGC.........T....
..........A..A...-....A...
..................-....-...
..................-....-...
```

Oligonucleotide Arp-1
pArp2-N3
pArp2-N1
pArp2-N3
pArp2-N5
pArp2-N3
pKA2-N1
pKA2-N1
pKA2-N1
pKA2-N1
pKA2-N2
pKA2-N2
pKA2-N3

```
GCAAACCAA-TCATAGGACATACTC
.........-...............
A.........-...C...........
.....T...-...............
.....T...-......
A......T.-......A........
.........G...C......G....
TGTT.G...-...............
.....T...-..............T
.........-...............
.........-...............
```

Oligonucleotide Arp-2
pArp2-N1
pArp2-N3
pArp2-N3
pArp2-N5
pKA2-N1
pKA2-N1
pKA2-N1
pKA2-N2
pKA2-N2
pKA2-N3

```
TTTGAGATATATGG-CACAAATGTGATCAATT
...TG.........G.........C.AATC..
...TTT........-..A.C....C.AATC.G
...TG..AC.G..A-.......T.C.AATC.G
....G-.........-.........---...A.
..............-..................
..............-..A......---...A.
```

Oligo. Arp-3
pArp2-N5
pArp2-N3
pArp2-N1
pKA2-N2
pKA2-N2
pKA2-N3

```
CTCCAAACTCATTGCATCTATTGGTGTATGCAG
..................N..............
............G............C..C..CA
...N.....N.....................
..T.........G................C...T
................................
................................
```

Oligo. Arp-4
pArp2-N1
pArp2-N3
pKA2-N1
pKA2-N2
pKA2-N2
pKA2-N3

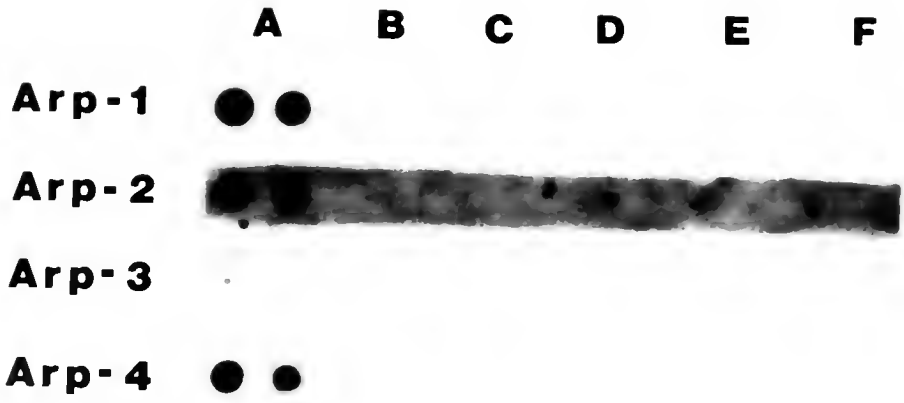


Figure 12. Results of using radiolabeled oligonucleotide probes with quick blots for species-specific detection. Quick blots were prepared, with each filter containing two spots from each of six species. Each spot received one-tenth of the solution in which a single insect was macerated. A, B, C, D = A. quadrimaculatus species A, B, C, and D, respectively. E = A. albimanus. F = C. nigripalpus. The figure shows the results of autoradiographic detection (ten days at -80°C with intensifying screens).

CONCLUSIONS AND SUMMARY

Discussion of the Efforts to Isolate a Culex-specific Probe

The difficulty encountered in isolating a C. nigripalpus-specific DNA probe indicates that the vast majority of species-specific repetitive DNA in the genome of C. nigripalpus, if this exists, is closely linked with nonspecific DNA sequences. One study (Cockburn & Mitchell, 1989) indicated that the level of repetitive DNA interspersion in C. quinquefasciatus DNA was higher than that found in anopheline DNA, although lower than that found for Aedes aegypti (Linnaeus) DNA. Even if some repetitive DNA is clustered within the C. nigripalpus genome, these clustered repeats may not be species-specific. Indeed, the results of the attempts to isolate a C. nigripalpus-specific probe indicate a paucity or lack of species-specific sequences in the genome.

The fact that the pCx1 probe could be isolated from a C. nigripalpus library by screening with C. quinquefasciatus DNA, and the observation that the insert in pCx1 is large, indicate that the conclusions above regarding C. nigripalpus versus C. salinarius do not apply when comparing the genomes of C. nigripalpus and C. quinquefasciatus. These results support the close phyletic relationship of C. nigripalpus

and C. salinarius, with C. quinquefasciatus a more distant relative. They also show that the techniques used are capable of isolating differentially repeated sequences when they exist.

Significance of Synthetic Oligonucleotide Probes and Characterization of Other Mosquito Species-specific Probes

The ease with which potential species-specific synthetic oligonucleotide probes were specified from the sequence data obtained from the pKA2 subclones, and the success in using the synthetic oligonucleotides as species-specific probes, indicate that this approach to obtaining probes from clones thought to contain numerous repeats due to a paucity of restriction sites is a valuable one. The improved specificity of the synthetic oligonucleotide probes showed these can be valuable tools for mosquito species identification.

It may not be possible to identify repeat sequences in clones containing only one (or a portion of one) repeat. This could be one reason why repeats were not identified in the sequence data obtained for pAf1-S1, pBrp1-S1, pCrp1-S2, and pCrp1-S3. Nevertheless, in some cases testing of the hybridization specificity of synthetic oligonucleotides specified by sequence data from such clones may provide species-specific probes as well as the localization of

repetitive species-specific sequences. For example, since pCrp1-S2 and pCrp1-S3 retain the specificity of Crp1, it is likely that a species-specific repeat spans the genomic region corresponding to the junction of these clones. Thus, oligonucleotides could be synthesized based on the sequence data obtained for these two clones, and tested for hybridization specificity. In this case, restriction analysis could be used to define which of the ends composed this junction.

The physical map of pAf1-S1, and the sequence data obtained from pAf1-S1 and the various A. quadrimaculatus-specific probes, provide a foundation for further characterization of the sequences which confer species specificity in these clones. The data are also valuable for providing a beginning of a more in-depth study of repetitive DNA of mosquitoes, which might include transposons or other interesting mobile genetic elements. There is a possibility that the pCrp1-S2, pCrp1-S3, or other clones may contain such mobile elements. The discovery of mobile genetic elements in mosquitoes could provide valuable tools for genetic engineering of these organisms.

Significance of the Quick Blot Protocol and Nonradioactive Detections

There are several methods available for preparing targets for nucleic acid hybridization experiments, and the decision of which method to use in a particular situation should be based on a number of considerations. These include the specific goals of the experiment and the advantages afforded by use of a particular method of preparing the target(s). In the simplest of cases, where a single probe is to be used with a single type of target, a slot blot, dot blot, or squash blot may be appropriate. It may be more advantageous to use a quick blot, however, for an experiment requiring a single sample of tissue to be probed separately with many probes, or for an experiment requiring many samples to be probed.

Most of the features of quick blots are available in one or more of the other types of blots, but none of the others provides the unique combination of traits of quick blots. Also, the ability to prepare multiple sets of equivalent targets with a given set of samples, with little additional effort, is a feature that is shared by the quick blot, dot blot, and slot blot protocols, but not possible with squash blots. The QB protocol can be used to prepare sets of nucleic acid samples in a form suitable for various

types of nucleic acid analysis. The nucleic acids could potentially be derived from any of a wide range of tissues from various animals, including insects and other arthropods, soft tissue samples from various non-arthropod animals, and plants. It is well suited for analysis of nucleic acids extracted from entire insects in the 1-20 mg size range, or body parts or isolated tissues from larger individuals. We have used the QB protocol to analyze DNA from individual mosquitoes.

The availability of nonradioactive detection systems has allowed nucleic acid hybridizations to be carried out in laboratories not equipped for handling radioactive reagents. Many agencies or groups not able or willing to comply with regulations or safety requirements relating to radioisotopes thus have the opportunity to use DNA probes in their basic or diagnostic research.

Nonradioactive detection systems have been used in many ways with various types of blots (McInnes & Symons, 1989b), but their potential usefulness with quick blots is especially great in those situations where nonradioactive detection must be used with multiple probes to a given set of samples. This is due to the ease of preparation of multiple equivalent blots with the quick blot protocol. These multiple blots can serve as targets for probes of

different specificities. This study has shown the feasibility of using quick blots to screen any number of mosquitoes with as many as ten different probes. The probes may be species-specific, allowing the detection of different mosquito species, or some may be pathogen-specific, allowing the detection of particular mosquito-transmitted diseases among the samples. Simple treatments of blots were described which effectively reduce nonspecific background. The procedure presented in this study for the nonradioactive detection of two probes hybridized simultaneously would allow a species-specific probe and a pathogen-specific probe to be used in the same hybridization step.

Nucleic acid hybridization probes will be used in increasing ways in basic and applied research, as they allow rapid, accurate, and often extremely sensitive detection of nucleotide sequences. In particular, it is expected that these techniques will become more important in the development of animal and plant breeding programs, and in the diagnosis and treatment of many types of diseases. The advances in DNA probe techniques described here are part of a trend to moving DNA probes beyond the laboratory and into the field. The advances may eventually allow field epidemiologists and others to possess field kits which can identify a putative vector, show what it is infected with, and show what it has fed on, in a few simple steps.

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BIOGRAPHICAL SKETCH

I was born in Columbus, Ohio, on June 7, 1959. I moved to Florida when I was 11 years old, and received the B.S. in Microbiology & Cell Science from University of Florida in December, 1981. I received the M.S. in Microbiology from The Florida State University in August, 1984.

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