CHAPTER 2

MATERIALS AND METHODS

1. Extraction of Plasmodial DNA (Tungpradubkul, 1983)

Materials

1. *Plasmodium falciparum* strain K1 was isolated from blood of a Thai patient in Kanjanaburi Province in 1979. This strain was both chloroquine and pyrimethamine-resistant with concentration of drugs that inhibited growth of the parasites of 1.5×10^{-6} and 5×10^{-5} M, respectively.

This stain of *P. falciparum* used in the experiment was obtained from Sodsri Thaithong, Department of Biology, Chulalongkorn University, and was continuously maintained in the laboratory of Department of Biochemistry, Faculty of Science Mahidol University.

2. Chemicals and Solutions

- distilled water (ice cold)
- 1% Triton x-100 (Sigma company)
- 0.85% NaCl
- 3M Sodium acetate pH4.8
- 0.01 mM Tris-HCl pH 8.0, 0.01mM EDTA, 0.85% NaCl
- TE buffer : 10 mM Tris-HCl, pH 7.4, 1mM EDTA
- Pronase (Calbiochem) proteinase 400 µg/ml of 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 10 mM NaCl, 2% SDS

- Water saturated distilled phenol containing 0.1% 8-hydroxy quinoline
- 3% isoamyl alcohol in chloroform
- RNase "A" (sigma) DNAase-free. 10 mg/ml, heated at 100°C for 10 min just prier to use.
- absolute ethanol, 70% ethanol
- diethyl ether

Method

Twenty ml of *Plasmodium falciparum* infected human red cells approximately 10% parasitemia mainly at mixed population of ring, trophozoite and shizontstage were obtained from 20 culture dishes (10x35 mm) and washed in 0.85% NaCl 2 times by centrifugation at 650g for 5 min at 4°C and supernatant fluid discarded. 1 ml of packed human red cells were lysed in 6ml distilled water and centrifuged at maximum speed in a bench top centrifuge for 15 min at 4°C and supernatant fluid discarded. The pellet was suspended in 6 ml of 1% Triton x-100 in order to solubilize the membrane. The parasite pellet obtained after centrifugation at 650g for 5 min was washed with 1ml of 0.01M Tris-HCl, pH 8, 0.01 M EDTA, 0.85% NaCl and centrifuged again. The washed pellet containing $1x10^9$ parasites was deproteinized by incubation at 37°C for 5 hours in 2 ml of pronase solution.

DNA was then separated from protein by phenol-chloroform extraction as follows. Equal volume of water-saturated phenol was added into DNA solution. The tube was gently mixed by inverting for 2-3 min. One volume of 3% isoamyl-alcohol in chloroform was added and the solution gently mixed as above. The solution was then centrifuged at 650g for 5 min. The lower layer was removed and phenol-

chloroform extraction step was repeated 2 times. The aqueous phase was reextracted for 2 times with 3% isoamyl in chloroform. Phenol and chloroform was removed by extracting with 2 volumes of ether for 5 times. DNA solution was treated with 100 µg/ml DNase-free RNase by incubating at 37°C for 1 hour . The solution was extracted with phenol-chloroform for 2 times and once with chloroform. The aqueous was extracted 3 times with ether. To DNA solution was then added 1/10 volume of 3M sodium acetate.

Two volumes of cold absolute ethanol were then added and the solution was left at -20°C overnight. DNA was collected by centrifugation in a bench top centrifuge at full speed for 10 min, washed once in 70% ethanol and dried at room temperature. The pellet was dissolved in 200 µl of TE buffer, and stored at 4°C. DNA concentration was estimated by measuring absorbance at 260 nm (Shimadzu UV-240 spectrophotometer). 1 OD_{260} unit was equivalent to 50 µl DNA/ml. The DNA concentration should be in the range of 0.1-0.5 µg/µl.

2. Preparation of Plasmid DNA

Materials

1. *Escherichia coli* JM 107 (lac proΔ, end A1, gyr A96, thi, hsd R17, sup E 44, rel A1, F' tra D 36, pro AB^+, lac Z ΔM 15) harbouring plasmid pUN121 or recombinant plasmid.

2. Chemicals and stock solutions

- Luria-Bertani (LB) media, 1 liter containing

Bacto-triptone (Difco)	10 g
Bacto-yeast extract (Difco)	5 g
NaCl (Carlo erba)	5 g

- Amphlicillin (Sigma)

Stock solution of 10 mg/ml solution of the sodium salt of ampicillin in sterilized water, 2.5 ml were added to 1 liter of LB-broth to make a concentration 25 µg/ml and stored at 4°C for only 1-2 weeks.

- Tetracycline (Sigma)

Stock solution of 12.5 mg/ml solution of tetracycline hydrochloride in sterilized water, LB broth was made up to final concentration of 12.5 µg/ml and stored at 4°C for 1-2 weeks.

- Chloramphenical (Sigma)

Stock solution was made up by dissolving solid chloramphenical in absolute ethanol at concentration of 34 mg/ml

- Lysozyme solution (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA, 2 mg/ml lysozyme (Sigma)

- 1% SDS/0.2 N NaOH, prepared just prior to use by mixing equal volume of 0.4 N NaOH and 2% SDS

- 3M Sodium acetate pH 4.8

- 0.05 M Tris-HCl pH8 -0.1 M NaoAc

- TE buffer

- absolute ethanol (Merck), 70% ethanol

- Cesium chloride (Sigma)

- Ethidium bromide (Sigma)

- Isoamyl alcohol (Merck)

Method

2.1 Large scale extraction of Plasmid pUN121 and recombinant plasmid (Birnboim and Doly, 1979)

Escherichia coli JM 107 harbouring plasmid pUN121, or

recombinant plasmid pUNK1-32, pUNK1-34, pUNK1-43, pUNK1-45, pUNK1-51 were grown in 10 ml LB medium containing 25 µg/ml ampicillin (for pUN121) or in 10 ml LB medium containing 12.5 µg/ml tetracycline (for recombinant plasmid) at 37°C with shaking overnight. 1% of activated cells were transfered to 250 ml of LB medium containing 25 µg/ml ampicillin (pUN121) or 12.5 µg/ml tetracycline (recombinant plasmid) in 500 ml flask, and incubated at 37°C with vigorous shaking overnight. Plasmid copy number was amplified by adding 170 µg/ml chloramphenical. The incubation was continued for another 6-14 hours

Bacterial cells were harvested by centrifugation in GSA rotor at 5,000 rpm at 4°C for 4 min. The cell pellet was suspended in 10 ml of freshly prepared solution of lysozyme, for 30 min on ice in order to weaken the cell wall. Then 20 ml of freshly prepared 0.2 M NaOH-1% SDS was added to obtain complete lysis and to denature DNA. The solution was gently mixed and kept at 0°c for 5 min. The cell lysate was then neutralized by the addition of 15ml 3M sodium acetate, pH 4.8, and left on ice for 60 min the solution gently mixed during this time.

Chromosomal DNA, at first denatured by alkali, was renatured to form an insoluble net work whereas closed circular plasmid regained native conformation and remained dissolved in solution. The high concentration of sodium acetate also caused precipitation of high molecular weight RNA and protein-SDS comple . The solution was then centrifugation at 8,000 rpm (Sorval ss-34 rotor) for 15 min. The supernatant fluid contained plasmid DNA and low molecular weight RNA. Plasmid was precipitated by addition of 2 volumes of absolute

ethanol at -20°C for 1 hour. The DNA pellet was obtained from centrifugation at 6000 rpm (Sorval ss-34 rotor) at 4°C for 10 min and washed with 70% ethanol for 2-3 times and dissolved in 2ml TE. The solution was transfered to centrifuge tube (15 ml tube) and TE was added up to 8 ml. Then 8g of CsCl was dissolved in the solution and 0.8 ml of 10mg/ml (H_2O) ethidium bromide were added. The final density of solution should be 1.55 g/ml and the concentration of ethidiumbromide should be approximately 600 µg/ml. The solution and protein aggregate were transfered to 13 ml ultracentrifuge tube (Beckman) and was centrifuged in a Beckman L8-70M ultracentrifuge (rotor 70.1 Ti) at 20°C, 48,000 rpm for 20 hours. The band position was detected under a UV lamp. The upper band consisted of bacterial chromosomal DNA, the middle band consisted of nicked circular plasmid DNA, and the lower band consisted of closed circular plasmid DNA. The lower band was removed through a no.25 hypodermic needle (Terumo). 1-2 ml of closed circular palsmid were collected and an equal volume of isoamylalcohol was added to extract ethidium bromide. The solution was centrifuged at 800g for 5 min at room temperature. Extraction step was repeated about 4-5 times. Two volumes of water were added to dissolve salt, followed by 2 volumes of absolute ethanol. The solution was mixed well and left at -20°C for 1 hour. The plasmid was collected by centrifigation at 800g for 15 min. The DNA pellet was washed with 70% ethanol for 2 times, and dried at room temperature. The pellet was dissolved in 500 µg of TE. The concentration of plasmid DNA was estimated by measuring the absorbance at 260 nm (Shimadzu UV-240 spectrophotometer)

2.2 Small scale extraction of recombinant plasmid (Birnboim and Doly, 1979)

Escherichia coli JM 107 containing recombinant plasmids were grown in 1.5 ml LB-media containing 125 µg/ml tetracycline. The suspension was incubated with shaking at 37°C, overnight. The suspension was transfered into 1.5 ml Eppendorf tube and the cells were pelleted by centrifugation for 2 min in Eppendorf microfuge. 100 µl of 2 mg/ml lysozyme solution was added. The solution was mixed well and incubated on ice for 10 min. 200 µl of freshly prepared 0.2 M NaOH in 1% SDS solution was added into the tube which was then incubated on ice for 5 min. 150 µl of 3M sodium acetate pH 4.8 was added, to neutralize the solution and to precipitate chromosomal DNA. The precipitation was allowed to complete on ice for 60 min. The tube was centrifuged in an Eppendorf centrifuge at 10,000 rpm for 10 min. Plasmid DNA in supernatant liquid was then precipitated with 2 volumes of cold absolute ethanol at -20°C for 1 hour. After centrifugation, the DNA pellet was dissolved with 200 µl of 0.05 M Tris-HCl pH 8.0, 0.1 M sodium acetate. The plasmid was precipitated again in 2 volumes of cold absolute ethanol at -20°C for 30 min. The pellet was washed in 70% ethanol for 2 times. Finally, the plasmid DNA was dissolved in 25-50 µl of TE buffer. The plasmid DNA was analysed on agarose gel electrophoresis. The gel was stained with ethidium bromide (2.5 µg/ml) for 15 min , and DNA bands were visualized under short wave UV light. Plasmid concentration and size was estimated by comparing with known concentration of λDNA markers generated by cutting λDNA with enzyme Hind III .

3. Restriction endonuclease digestion (Maniatis, 1982)

Meterial

1. Buffers : Restriction endonuclease can be divided into three classes, depending on the requirement of ionic strength in buffer system. By following this scheme, only three stock buffers need to prepare as 10x stock solution which can be stored at -20°C. But a few enzyme will not work well in any of these three buffers and the specific buffer should be made up in these situations.

Table 1 10x Buffer for restriction endonuclease digestion

Buffer	NaCl	KCl	Tris-HCl (pH7.5)	Tris-HCl (pH 8.0)	$MgCl_2$	Dithiothreitol (DTT)
low	0 mM	-	10 mM	-	10 mM	1 mM
medium	50 mM	-	10 mM	-	10 mM	1 mM
high	100 mM	-	50 mM	-	10 mM	1 mM
specific*	-	20 mM	-	10 mM	10 mM	1 mM

* buffer for enzyme SmaI

2. Restriction endonuclease enzymes :

EcoRI (prepared by T. Karnthong) was used to digest plasmid pUN121, EcoRI* was used to digest *Plasmodium falciprum* K1 DNA, and others enzymes in table 2 were used to characterize recombinant plasmid

Table 2 Restriction endonuclease enzyme used in this study

Enzyme	Salt	incubation temperature	Recognition sequence
AccI	med	37°c	5'...GT↓$\binom{AG}{CT}$AC...3'
AvaI	med	37°c	5'...G↓PyCGPuG ...3'
BamHI	med	37°c	5'...G↓GATCC ...3'
BstNI	low	60°c	5'...CC↓$\binom{A}{T}$GG ...3'
ClaI	med	37°c	5'...AT↓CGAT ...3'
DdeI	med	37°c	5'...C↓TNAG ...3'
EcoRI	high	37°c	5'...G↓AATTC ...3'
EcoRI*	low	37°c	5'...↓AATT ...3'
HhaI	med	37°c	5'...GCG↓C ...3'
Hind II	med	37°c	5'...GTPY↓PuAC ...3'
Hind III	med	37°c	5'...A↓AGCTT ...3'
Hinf I	med	37°c	5'...G↓ANTC ...3'
kpn I	low	37°c	5'...GGTAC↓C ...3'
Nae I	med	37°c	5'...GCC↓GGC ...3'
Nde I	high	37°c	5'...GA↓TATG ...3'
NRuI	high	37°c	5'...TCG↓CGA ...3'
Pst I	med	37°c	5'...CTCGA↓G ...3'
PvuII	med	37°c	5'...GAG↓CTG ...3'
SacII	low	37°c	5'...CCGC↓GG ...3'
Sal I	high	37°c	5'...G↓TCGAC ...3'
Sau961	low	37°c	5'...G↓TCGAC ...3'
Sma I	specific	37°c	5'...CCC↓GGG ...3'
Sst I	low	37°c	5'...GAGCT↓C ...3'
XbaI	high	37°c	5'...T↓CTAGA ...3'
XhoI	high	37°c	5'...C↓TCGAG ...3'

All enzymes were stored in buffer containing 50% glycerol at -20°c. In the reaction mixture, enzyme was not more than 10% of total volume because a high glycerol concentration inhibited enzyme activity. One unit of enzyme was defined as the amount required to completely digest 1 µg of lamda DNA in 1 hour in the recommended buffer at 37°c

3. DNA :

- *Plasmodium falciparum* K1 DNA from section 1
- plasmid pUN 121 from section 2.1
- recombinant plasmids from section 2.1 and 2.2

4. Loading dye :

0.1 % bromophenol blue (Merck)

40% Ficoll (Phamacia)

5 mM EDTA

stored at 4°c

Method

3.1 *Plasmodium falciparum* and pUN 121 digestion for cloning

Ten µg of *P. falciparum* KI DNA was digested with EcoRI*. This enzyme was EcoRI but digested under modified condition. This restriction mixture, 40 µl, contained 10 µg DNA in 4 µg 10x low salt buffer and 12 µl enzyme EcoRI (15% glycerol in the mixture) and 24 µl sterile double distilled water. The mixture was mixed well and incubated at 37°c for 2 hours to obtain complete digestion. The enzyme was then removed by phenol-chloroform-ether extraction and DNA precipitated by ethanol precipitation. The DNA pellet was washed once with 70% ethanol, redissolved in 20 µl TE buffer and analysed by gel electrophoresis on

0.7% agarose. DNA digested with EcoRI* was used in cloning experiment.

Purified pUN 121 vector was digested with enzyme EcoRI. The reaction mixture, 50 µl, contained 10 µg DNA, 5 mg of 10x high salt buffer, and 20 units of enzyme EcoRI. The mixture was incubated at 37°c for 1 hour. After completion, the digested plasmid was phenolized and precipitated. The DNA pellet was washed once in 70% ethanol, then dissolved in 30 µl TE buffer and characterized by agarose gel electrophoresis.

3.2 Recombinant plasmid digestion for Southern hybridization

Southern blot hybridization was used to select highly repetitive DNA from recombinant clone previously selected by colony hybridization. The chosen recombinant plasmids were extracted (see section 2.2) and digested with enzyme Bam HI in order to open the closed circular form so that the size of inserted DNA could be determined by comparing with DNA size markers. The reaction mixture, 20 µl, contained 0.5 µg of recombinant plasmid DNA, 2 µl of 10 x medium salt buffer, and 2 units of Bam HI. The mixture was incubated at 37°c for 1 hour. The reaction was stopped by the addition of loading dye, prior to loading onto 0.7% agarose gel.

3.3 Plasmodial genomic DNA digestion for Southern hybridization

In order to locate recombinant plasmid pUNKI-34 and pUNKI-45 on *Plasmodium falciparum* genomic DNA, *P. falciparum* KI DNA was cut by various restriction enzymes and the recombinant plasmids were used as probes. The digestion mixture, 30 µl, contained 1 µg *P. falciparum* strain KI DNA, 3 µl of 10x high salt buffer for EcoRl and Sal I reaction, or 3 µl of 10x medium salt for Bam HI and Hind III reaction. Enzymes

were of 10 units. Incubation was at 37°c for 3 hours. The reaction was directly stopped by the addition of 4 µl of loading dye. The solution was mixed well and analysed by 0.7% agarose gel-electrophoresis. The DNA in agarose gel was the processed for Southern hybridization experiment (see section 6.2)

3.4 Recombinant DNA digestion for mapping

Three hundred ng of pUNK1-34 and pUNK 1-45 was cut with various restriction enzymes for restriction mapping using 2 µl of 10x salt buffer, 1-2 units of 2 restriction enzymes in a total volume of 20 µl. Bam HI or Hind III was used in every digestion experiment and various other enzymes were used to cut together with Bam HI or HindIII. The mixture was analysed on 0.7% agarose gel electrophoresis.

4. Ligation between pUN 121 and *Plasmodium falciparum* K1 DNA

Meterials and stock solutions

1. DNA :

   pUN 121 digested with EcoRI

   P. falciparum DNA digested with EcoRI*

2. 10 x ligation buffer contained :

   6 mM potasium chloride (UNIVAR)

   10 mM tris-HCl pH 7.5 (Sigma)

   10 mM $MgCl_2$ (H & W ; JT-Baker)

   11 mM DTT (Sigma)

   1 mM ATP (Sigma)

   100 µg/ml nuclease-free bovine serum albumin (BRL)

   1 mM spermidine which was already neutralized to pH 7 (Sigma)

3. T_4 DNA ligase (BRL)

4. Double distilled water

Method

The cohesive ends of plasmid pUN 121 and *P. falciparum* K1 DNA (from section 3.1) were ligated in the ratio of 1:1 using enzyme T4 ligase. The reaction mixture, 20 µl, contained 600 ng each of EcoRI digested pUN 121 and EcoRI* digested *P. falciparum* K1 DNA, After incubated the DNA at 60°c for 15 min. and quick cooling on ice, 1 µl of 10x ligation buffer and 2 units of T_4 ligase enzyme were added. The reaction was carried out at 14°c for 16-18 hour. Self ligation of EcoRI digested pUN121 was used as control. The DNA patterns of self ligated DNA were determined before transformation.

5. Transformation (Hanahan, 1983)

Materials

1. Bacteria : *E. coli* JM 107

2. Media : - SOB medium : 1 liter containing
2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$
- SOC medium : 1 liter containing SOB plus 0.02 M glucose
- LB-agar : 1 liter containg
bacto-triptone (Difco) 10 g
bacto-yeast extract (Difco) 5 g
NaCl 5 g
bacto agar (Difco) 15 g

- 12.5 µl/ml tetracycline (Sigma)

3. Solution
- DMSO (dimethyl sulfoxide)
- DTT : 2.25 M DTT in 40 mM potassium acetate pH 6.0
- TFB : 100 mM rubidium chloride (Sigma), 45 mM $MnCl_2$ $4H_2O$, 10 mM $CaCl_2$ $2H_2O$, 3 mM hexamine cobalt chloride (Fluka) and 10 mM morpholinoethane sulfonic acid (Sigma)

4. DNA : pUN 121-*P. falciparum* K1 ligated product (from section 4)

Method

In principle competent *E. coli* JM 107 cells were obtained by treatment with divalent cations, DMSO for cation solvations, DTT and hexamine cobalt, to enhance contact between DNA and the phospholipopoly-saccharide of cell surface.

E. coli JM 107 was activated in 1 ml SOB medium at 37°c with shaking overnight. 0.5 ml of activated cells were inoculated into 50ml SOB medium. The flask was incubated at 37°c with shaking at 37°c for 3-4 hours(until OD = 0.3-0.4). The log phase cells were tranferred into 4 Falcon 2059 tubes, and the cells were collected by centrifugation at 3,000 rpm, 4°c in a Hettich-M 25 centrifuge for 10 min. The pellet was suspended in 4ml/tube of cold TFB solution after incubation on ice for 10 min. The pellet was collected by centrifugation at 3,000 rpm, 4°c in a Hettich-M 25 centrifuge for 10 min again, and resuspended in 1 ml of TFB solution. Following addition of 35 µl DMSO, the solution

was left on ice for 5 min. Thirty five µl of 2.25 M DTT in 40 mM potassium acetate pH 6.0 were added and the solution was left on ice for 10 min before 35 µl of DMSO were added again. The solution was cooled on ice for 5 min.

Two-hundred ten µl of competent cells (10^8-10^9 cells) were placed into a cold centrifuge tube and 100 ng of ligated product (from section 4) were added. The solution was mixed well and left on ice for 30 min. The mixture was incubated at 42°c for 90 seconds exactly and ice cooled for 2 min, 800 µl of SOC medium were then added and the solution was incubated at 37°c for 1 hour. The transformed cells were then seperated on LB-agar plate containing 12.5 µg/ml tetracycline and incubated at 37°c overnight.

6. Screening for repetitive clone of *Plasmodium falciparum*

Three steps were used to screen repetitive clones of *P. falciparum*, namely, colony hybridization, repeat colony hybridization, and Southern blot hybridization. Total genomic DNA of *P. falciparum* K1 was used as probe.

6.1 Colonyhybridization (Grunstein and Hogness, 1975)

6.1.1 Cell lysis

Materials

1. Media
   - LB agar containing 12.5 µg/ml tetracycline
   - LB agar containing 100 µg/ml chloramphenicol
2. Solution
   - 0.5 M NaOH

- 1 M tris-HCl, pH 7.4
- 0.5 M tris-HCl, pH 7.4, + 1.5 M NaCl

3. Nitrocellulose filter (BA 85, Scheicher and Schuell)
4. Sterile toothpicks
5. Recombinant clones (from section 5)

Method

The recombinant clones grown at low density on 12.5 µg/ml tetracycline-LB plate (section 5) were picked by sterile toothpicks and stabbed onto two sets of nitro cellulose placed over tetracycline-LB plate. *E. coli* JM 107 containing plasmid pBRT-14 was used as positive control and *E. coli* JM 107 containing plasmid pBR 322 was used as negative control. One set was the working set and other was kept as master set. Each plate was incubated at 37°c overnight.

Recombinant clones from section 5, which were grown at high colony density, were replicated onto two sets of nitrocellulose filters, and then placed on tetracycline-LB plate at 37°c for overnight incubation.

The masterplates were kept at 4°c and other filters were transferred to chloramphenicol-LB plate to increase plasmid copy number.

Steps for cell lysis were as follows. The filters were removed and placed (colonies uppermost) on 0.75 ml of 0.5 M NaOH for 5 min, to lyse the cells and denatured DNA. Then the filters were blotted dry on 3MM paper(Whatman). This step was repeated for complete lysis of cells. The filters were neutralized twice by transfer to 0.75 ml of 1 M Tris-HCl pH 7.4 for 5 min, and blotted dry. The filters were then put on 0.75 ml of 0.5 M tris-HCl pH 7.4 - 1.5 M NaCl, twice for 5 min each, to fix DNA on the filter. After that the filters were

subsequently washed with 95% ethanol, chloroform and distilled water using colony hybridization filter device (MV 082/0 ; Schleicher & Schuell). Then the filters were baked at 80°c for 2 hours and stored at 4°c in sealed plastic bags until required for section 6.1.3.

6.1.2 Nick Translation of DNA probe (Rigby, 1977)

Materials

1. α ^{32}PdATP (Amersham; triethylammonium salt, 6,000 Ci/m mol 10 µCi/ml or Dupont ; tetra (triethylammonium) salt, 5,000 Ci/m mol , 20 µCi/ml
2. Nick translation buffer ; 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 µg/ml nuclease-free BSA, 1 m MDTT
3. 10 µmol/µl of dCTP, dGTP, dTTP
4. 0.6 µmol/µl dATP
5. DNA probe ; 300-400 ng K1 genomic DNA
6. 1 ng/µl of DNase I (Sigma)
7. *E.coli* polymerase I : 1 unit (BRL)
8. 250 mM EDTA, pH 8.0
9. Distilled water
10. TE buffer
11. GF/A filter paper (Whatman)
12. 10% TCA, 5% TCA (Trichloroacetic acid)
13. 95% ethanol
14. Sepadex G-50 (Pharmacia)

Method

The reaction mixture, 25 µl, contained 400 ng of K1 genomic DNA, 2.5 µl nick translation buffer, 0.6 µmol dATP, 10 µmol of each

dGTP, dCTP, dATP, 40 µCi of (α-^{32}P) ATP, 2 µg of DNase I and 1U of *E. coli* polymerase I. The solution was incubated at 15°C until maximum incorporation of (α-^{32}P) dATP into the DNA was obtained.

Kinetics of the reaction was followed by periodic measurement of radioactivity of 1 µl reaction mixture spotted on GF/A filter paper soaked in 10% TCA. The filter was washed with 10 ml of 5% TCA followed by 95% ethanol and then placed into a scintillation vial containing 10 ml distilled water and counted in a liquid scintillation counter (Beckman LS-100). Percentage of incorporation could be determined by comparison with the radioactivity of 1 µl of reaction mixture spotted on untreated GF/A filter paper. When maximum incorporation was obtained, the reaction was stopped by addition of an equal volume of 250 mM EDTA, pH 8.0, and heating at 65°C for 10 min. The labelled DNA was separated from free deoxynucleotide by loading onto a 1 ml column filled with Sephadex G-50, 50 µl of TE buffer were added, and the column centrifuged. The eluate was collected.

6.1.3 DNA hybridization

Materials and stock solutions

1. Prehybridization (PHB) or hybridization (HB) solution contained
   - 50% formamide (99% Fluka)
   - 5 x SSC (1x standard saline citrate was 15 mM NaCl, 15 mM tri-sodium citrate pH 7.5)
   - 5x Denhardt's solution
2. 50 x Denhardt solution contained
   - 10% Ficoll 400 (Pharmacia)

- 1 % polyvinyl pyrolidone (Calbiochem)
- 1 % bovine serum albumin (BSA Fraction V) (Sigma)
- 50 mM EDTA
- 200 mM Tris-HCl pH 7.5

sterilized by filtration and stored frozen

3. Salmon sperm DNA (sonicated)

- 100 µg Salmon sperm DNA/ ml PHB solution heated at 100°c for 10 min and quick cooled on ice just prior to each use.

4. DNA probe

- K1 DNA from section 6.1.2, denatured just prior to use by heating at 100°C for 10 min, and quick cooled on ice.

Method

The nitrocellulose filter was prehybridized with 5-7 ml of PHB solution containing 100 µg/ml denatured salmon sperm DNA. All air bubbles in the bag were removed as much as possible. The bag was sealed with plastic sealer and incubated at 30°c for 16-18 hours.

The PHB solution was extruded and fresh HB (the same as PHB) solution containing heat denatured sonicated salmon sperm DNA was added followed by addition of denatured DNA probe (10^6 cpm/bag with specific activity of 1-5 x 10^7 cpm/µg). The volume of hybridization solution used was small as possible. All air bubbles were removed and the bag was then resealed and incubated at 30°c for 48 hours.

6.1.4 Washing to remove non-hybridized probe

Materials and stock solutions

1. 20 x ssc (3M NaCl, 0.3 M Trisodium citrate pH 7.0)
2. 10% SDS

Method

All of the HB solution was removed and the filter was washed twice with 3x ssc at room temperature with shaking for 10 min. The filter was then washed with shaking in 0.1x ssc 0.1% SDS at 50°c for 15 min for 4 times. The filter was air dried on 3MM paper and put into sealed-plastic bags.

6.1.5 Autoradiography and development of films

Materials

1. x-ray film (Fuji-RX 100)
2. developer (Kodak x-ray developer)
3. fixer (Kodak x-ray fixer)

Method

The washed filter was exposed to x-ray film in an x-ray cassette with intensifying screen, and left at -70°C for appropriate time. The signal was observed after washing the exposed film in developer and fixer as follows.

The x-ray cassette was removed from -70°C freezer and allowed to warm up for 30 min. The film was removed from the cassette in the darkroom, rinsed brifely in water and immersed in developer 2-3 min until the signal was seen. The film was then washed in water and immersed in fixer until the film was clear. The film was finally washed in water for about 1 hour before air drying at room temperature.

6.2 Southern blot hybridization

DNA in agarose gel from section 3.2 and 3.3 were treated

and transfered to nylon membrane after the gel was stained with ethidiumbromide (2.5 µg/ml) and photographed.

Materials and stock solutions

1. nylon membrane (Dupont) cut to dimension of gel
2. 20x ssc (3M NaCl, 0.3 M trisodium citrate, pH 7.0)
3. 0.25 M HCl
4. 0.5 M NaOH, 0.5 M NaCl
5. 1M Tris-HCl pH 8.0, 1.5 M NaCl
6. Whatman 3 MM filter paper

Method

6.2.1 Gel treatment

The gel was shaken in plastic box containing 0.25 M HCl solution to depurinate DNA, at room temperature for 10 min, this was done, twice. The gel was then rinsed in distilled water. DNA was denatured by shaking the gel in plastic box containing 200 ml 0.5 M NaOH, 1.5 M NaCl solution for 15 min, this was done twice. The gel was neutralized by shaking in 200 ml of 1 M Tris-HCl pH 8-1.5 M NaCl solution for 15 min, this was done twice.

6.2.2 Nylon membrane treatment

Nylon membrane was cut to a size 0.5 cm larger than the gel. After soaking the membrane in sterile distilled water, the membrane was soaked with 20 x ssc for 30 min.

6.2.3 Transfer of DNA to membrane

Three cm pile of Whatman 3 MM paper was soaked with 20 x ssc, and laid on Saran Wrap. The gel was then laid on top of the paper and all air bubbles between the gel and the paper were removed. A piece of treated nylon membrane was placed on top of the gel and all trapped air bubbles were removed. Five to six cm pile of whatman 3 MM paper, cut to a size 0.5 cm -larger than the gel was placed on top of the nylon membrane. The blotting set was completely covered with Saran Wrap to prevent evaporation of the solution, and a weight was placed on the blotting set. The transfer step was complete after 3-4 hour (figure 3). The nylon membrane was then removed and shaken in 0.4 N NaOH for 30-60 min and washed in 2x ssc-0.2 M Tris-HCl, pH 8, for 5-10 min. The gel was stained with 2.5 µg/ml ethidiumbromide to check for completeness of transfer. The filter was air dried on Whatman 3 MM paper and kept in sealed-plastic bag at 4°C until used.

6.2.4 Nick translation of DNA probe and hybridization

For Southern blot of DNA from section 3.2, K1 genomic DNA was used as probe (see section 6.1.1). For digested *Plasmodium falciparum* DNA from section 3.3, plasmids pUNK1-34 and pUNK1-45 were used as probe. The protocol was as in section 6.1.1 but the amount of recombinant DNA in the mixture was 200-250 ng and α-^{32}P dATP was 20 µCi

Prehybridization and hybridization was as in section 6.1.3

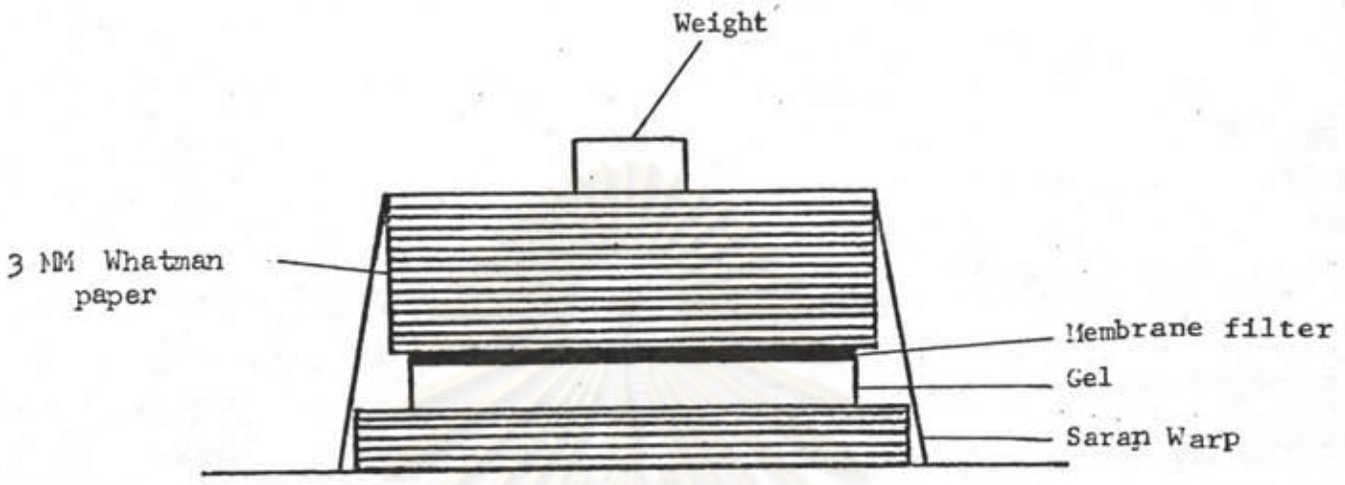

Fig. 3 Diagram of Southern transfer set

7. Test of sensitivity and cross-hybridization of recombinant DNA by dot blot hybridization

Materials and stock solutions

1. Recombinant plasmid DNA (from section 2.1) : pUNKI-32, pUNKI-34, pUNKI-43, pUNKI-45, pUNKI-51

2. Human, *Anophelis dirus* strain A, *Plasmodium vivax*, *P. knowlesi*, *P. chabaudi* and *P. cynomolgi* DNA

3. Chemicals

- 0.5 N HCl
- 0.6 N NaOH
- 2 M ammonium acetate
- 1 M Tris-HCl, pH 7.4

Methods

7.1 DNA treatment

Each DNA preparation was partially depurinated with 0.25N HCl at room temperature for 5 min , and denatured in 0.3N NaOH on ice for 5 min. The solution was neutralized in 1 M ammonium acetate and heat denatured at 100°c for 10 min followed by quick cool for 5 min. The solution was diluted to 1 ng/µl and 5 ng/µl in 1 M ammonium acetate.

7.2 Nylon treatment and dot blotting

Nylon membrane was cut to appropriate size and soaked in 0.4 N Tris-HCl, pH 8.0, for 30 min. Then DNA solutions from section 7.1 were spotted on nylon membrane using Minifold I (Schleicher-Schuell). In cross-hybridization test experiment, 50 ng and 5 ng of recombinant

plasmids, Rep 20 and pBRKI-14 were used, but amounts of the other DNA were 5ng and 1 ng. In sensitivity test experiment, 10 ng and 2 ng of recombinant plasmid, Rep 20 and pBRKI-14 were used and 50 ng, 5 ng of other plasmodial DNA, mosquito and human were dotted. The membrane was dried at room temperature.

7.3 Nick translation and hybridization

One hundred ng of Plasmodial, human and *Anophelis dirus* DNA were mixed and used in nick translation reaction, and 40 µCi of α-^{32}P dATP was added. This mixed probe was used in crosshybridization test experiment.

Four hundred ng of K1 genomic DNA was used as probe for sensitivity test of recombinant DNA and 40 µCi of α-^{32}P dATP was added in nick translation mixture. Hybridization was as in section 6.1.3.

8. Detecting *Plasmodium falciparum* in blood samples

Materials and stock solutions

1. *In vitro* culture of *P. falciparum* (a mixed population of ring, trophozoite and shizont forms) containing 1 x 10^9 parasites/ ml pack RBC corresponding to 10% parasitemia (10^{10} RBC/ ml pack RBC).

2. Recombinant DNA from section 2.1 : pUNK1-34, pUNK1-45 and pUNK1-14 (from K. Chansiri)

3. *P. falciparum* DNA from section 1.

4. Chemicals :

- 0.6 N NaOH
- 2 M Ammonium acetate
- Pronase solution (100 nM Tris pH 10, 10 mM EDTA,

0.4% Triton x-100 + 200 µg/ml Pronase (Calbiochem) incubated at 37°C, 10 min , before use.

5. Nylon membrane (Dupont)

Method

8.1 DNA treatment

Twenty µl of *in vitro* cultured *P. falciparum* were incubated with 20 µl of pronase solution at 37°C for 1-2 hours. After pronase digestion, DNA was denatured by addition of NaOH to final concentration 0.3 N on ice for 10 min. The solution was neutralized with an equal volume of 2M ammonium acetate and then heated at 100°C for 10 min and quick cooled for 10 min. The mixture was centrifuged at 15,000 g for 1 min and the pellet discarded. The supernatant was then applied onto nylon membrane that had been soaked for 30 min with 0.4 M tris-HCl, pH 8.0, using a Manifold II vacuum filtration apparatus (Schleicher & Schuell). The filter was air dried approximately 10 min , before prehybridization and hybridization step.

P. falciparum DNA from section1 and recombinant DNA were treated as in section 7.1 and dotted on the same nylon membrane as positive controls, 100 ng of human and other Plasmodial DNA were used as negative controls.

8.2 Nick translation and hybridization

Six hundred ng of recombinant plasmid (pUNK1-34 or pUNK1-45) or pBRK1-14 was nicked in translated using 80 µCi of α- ^{32}P dATP. The protocol was as in section 6.1.1 and hybridization protocol was as in section 6.1.3.

9. Detecting sporozoite and oocyst in *Anopheles dirus*

Materials and stock solutions.

1. Infected *An. dirus* were obtained from Armed Forces Research Institute of Medical Sciences (AFRIMS), following 8-9 days, 10-14 days and 15-23 days of feeding with infected blood. The appearance of sporozoite and oocyst forms were seen in least 80-100 % of glands and guts.

2. *P. falciparum* sporozoites removed from *An. dirus* salivary gland.

3. Uninfected *An. dirus* for negative control (from AFRIMS).

4. *P. falciparum* K1 from section 1

5. Plasmid pUNK1-45 from section 2.1

6. Chemicals

- Pronase buffer (100 mM Tris pH 10, 10 mM EDTA, 0.4% Triton x 100)

- 4% SDS-4 mg/ml Pronase K (Calbiochem) incubated at 37°C for 15 min before use.

- 0.5 N HCl

- 0.6 N NaOH

- 2M Ammonium acetate

7. Dry ice.

Method

9.1 DNA treatment

Three infected *An. dirus* mosquitoes from 2 groups were dissected and separated head-thorax and abdomen part placed in

Eppendorf tubes. The samples were homogenized in Eppendorf tubes using a Kontes pellet pestle on dry ice. Pronase buffer was added and incubated at 37°C for 30 min by shaking in water-bath. DNA was depurinated with 0.25 N HCl and denatured with 0.3 N NaOH, and then neutralized with 1M ammonium acetate on ice for 5 min. The treated DNA was heated at 100°C for 10 min and quick cooled for 5 min. The solution was centrifuged at 10,000 g for 5 min and supernatant collected. The supernatant was dotted onto treated nylon membrane (see section 7.2) using Manifold II (Schleicher & Schuell). The level of dotting DNA was 3 infected mosquitoes, 1 infected, ½ infected and ¼ infected mosquito.

P. falciparum sporozoite, removed from salivary gland of *An. dirus* was digested with pronase solution as described in section 8.1, and the extracted DNA was applied onto nylon membrane.

Uninfected *An. dirus* mosquitoes were treated as described for infected samples.

Treated *P. falciparum* DNA and plasmid pUN K1-45 DNA were spotted on nylon membrane as positive controls.

9.2 Nick translation and hybridization

Six hundred ng of plasmid pUNK1-45 was nick translated using 80 µCi of α-^{32}P dATP in the reaction as described in section 6.1.1 . Hybridization was as in section 6.1.3.