CHAPTER 4

DISCUSSION

One of the objectives of this thesis was to produce a genomic DNA library by cloning into *E. coli* using as vector the plasmid pUN121. The first step was the isolation of DNA from *P. falciparum*, K1 isolate. . The studies of *P. falciparum* DNA digested with various restriction enzymes showed a smeared pattern of DNA fragments along the length of gel with a number of discrete bands superimposed. These discrete bands indicated that *P. falciparum* genome contains repetitive sequences since a single copy sequence would not been able to be seen in the gel (figure 5).

Tungpradubkul *et al* (1983) and Fucharoen (1985) have demonstrated that repeat elements were present in the plasmodial DNA by studies of restriction endonuclease digestion patterns of Plasmodial DNA. However the type and number of discrete patterns depend on the restriction enzyme used. Hind III and EcoRI, A-T recognition enzymes, gave digestion patternsof *P. falciparum* with many discrete bands of smaller fragment size than that of the G-C recognition enzyme such as Bam HI and Sal I. Pollack *et al* (1982) have also described that when *P. falciparum* DNA was digested with G-C recognition enzyme (Msp I and Hpa II), the degree of digestion is limited .

For cloning of genomic DNA, there are four types of DNA used as vectors for cloning, namely plasmids, bacteriophage λ, cosmids and bacteriophage M13. Construction of DNA probes of *P. falciparum* by several laboratories had been used various vectors for cloning. Probes Rep 2 and Rep 20 were selected from *P. falciparum* DNA library, which

had been produced by means of bacteriophage lambda vector (Franzen *et al* 1984, and Oquendo *et al*, 1986) The advantage of cloning by bacteriophage lambda vector is that it can carry insert lengths to a maximum of 20-25 kb. Using plasmid or bacteriophage, in either case fairly large libraries (10^5-10^6 individual recombinants) are likely to required to represent a complex eukaryotic genome (10^9 bp). Franzen *et al* (1984) have demonstrated that a *P. falciparum* DNA library which was constructed by cloning into lambda vector 1059 contained 10-15 kb insert DNA fragments in the recombinant plaques. About 10^5 recombinants were obtained, this is about twentyfold number necessary to cover the entire parasite genome. Rep 2 was subcloned in plasmid pBR 322 and Rep 20 was subcloned into plasmid pBR 325. However large size of insert fragment may cross-hybridize with other DNA.

Zolg *et al* (1987) had recently cloned a Tanzanian isolate of *P. falciparum* DNA using bacteriophage M 13 as vector to obtain 56 clones which give the strongest signals and containing inserts ranging from 147 base pairs to 5.9 kb. Clone 26 contained insert DNA size of 147 base pairs long and contains 6 complete tandem repeat of 21 base pairs each. Cloning by M 13 vectors could facilitate the sequencing of the DNA fragment inserts. Tirawanchai (1983) and Barker *et al* (1986) had cloned *P. falciparum* DNA into plasmid pBR 322 vector to obtained partial genomic DNA libraries. Tirawanchai (1983) obtained 70 clones of total Plasmodial genome and the size of the DNA fragment inserts were mainly about 1 kb. Barker *et al* (1986) did not describe the obtained library size. Cloning by plasmid vector allows us to obtain small size of DNA fragment inserts. In our experiment plasmid pUN 121 was used as cloning vector for positive selection of transformants.

It has ampicillin-resistant gene and tetracycline-resistant gene which does not normally function due to the inhibitor by CI gene product. The EcoRI* restriction fragments of *P. falciparum* DNA were inserted into EcoRI cloning site present with in the CI gene. Therefore, all colonies which can be grown on tetracycline harboured only recombinant plasmids.

The reason for the use of EcoRI* restriction endonuclease on *P. falciparum* DNA was to obtain large numbers of *P. falciparum* DNA fragments. There is a high AT content (80%) in *P. falciparum* DNA and EcoRI* can recognise the sequence of 5'...↓AATT...3'.

To produce EcoRI* reaction, the conditions for EcoRI digestion was changed to low ionic strength and 15% glycerol (figure 6). Ligation contained an excess of plasmid vector pUN121. Enzyme DNA ligase will catalyze the formation of a phosphodiester band between adjacent nucleotides only when one nucleotide contains a 5'-phosphate group and the other a 3'-hydroxyl group. A foreign DNA fragment with 5'-terminal phosphates can be ligated into open circular plasmid DNA and these can then be transferred into *E. coli* by using DMSO method.

There are many factors that can affect the transformation efficiency such as the ratio of vector to passenger DNA, size and molecular form of the recombinant plasmid, the ratios of plasmid to viable cell, bacterial host strains, transformation conditions. The transformation efficiency decline linearly with increasing plasmid size, and relaxed plasmids transform at 75% of the efficiency of their supercoiled forms (Hanahan, 1983). The transformation efficiency is nearly linear over a range of 10^4, from plasmid-to-cell ratios of 1:2000 to 1:1. Increasing the plasmid-to-cell ratios 200-fold, from 1:1 to

200 : 1, only increases the number of transformed cells by six to eight-fold (Hanahan, 1983). Variation of transformation efficiency ($2x10^7$ - $5x10^8$) can be observed among different bacterial strains (Hanahan, 1983). Transformation conditions involved interaction of *E. coli* cells with exogenous DNA in medium containing elevated level of Mg^{2+} and incubation of the cell at 0°C in a solution of Mn^{2+}, Ca^{2+}, Rb^+ or K^+, dimethyl sulfoxide, dithiothreitol and hexamine cobalt (III) and heat pulse. By the condition transformation efficiency increased to 10^7-10^8 colonies/µg of pBR-322 DNA in *E. coli* K12 strains (Hanahan, 1983).

In this thesis transformation efficiency was $1x10^7$ colonies/µg pUN 121 and decreased to $6x10^4$ colonies/µg of ligated product. A library containing 20,000 transformants were obtained. All of Plasmodial genome were represented, based on the calculatian of the Clark and Corban (1976) equation :

$$N = \frac{\ln (1-P)}{\ln (1-f)}$$

where p = The desired probability

f = Fractional proportion of genome in a single recombinant

N = The necessary number of recombinants

DNA content in *P. falciparum* has been showed to be 0.02 pg/nucleus (Gutteridge *et al*, 1971 and Goman *et al*, 1982), equal to genomic size of $1x10^7$ bp. The average size of inserted DNA fragments was 4,000 bp. (figure 14). Assuming that 99% of Plasmodial DNA are represent in a library, the number of bacterial colonies would be 11,500.

About 400 DNA clones were first selected by screening using colony hybridization with K1 genomic DNA probe. This was based on the criterion that clones containing highly repetitive sequence of *P. falciparum* would hybridize large amount of probe, and appearing as dark spots on the autoradiogram, but those containing single copy sequences would give hybridization signal below the level of detection. However such factors as unequal growth rates of each clone and presence of cell components such as protein and RNA might give rise to false signals in colony hybridization. Thus Southern blot hybridization was used to select and analyse 53 recombinants which were selected from duplicate colony hybridizations of the original 400 clones. About 70% of inserted DNA fragments had size range of 0.2 to 4.0 kb with on average size of 3.9 kb. The smallest insert size of the recombinant plasmids was about 200 bp and the largest was about 15.4 kb.

From Southern hybridization 5 clones were then selected based on their high sensitivity in comparison to pBRK1-14. These were pUNK1-32 -34, -43, -45, and -51. The clones pUNK1-34 and -45 were more sensitive than other clones in detecting K1 genomic DNA in dot blot hybridization assays (figure 15). Using pUNK1-34 and 45 as DNA probe,,it was possible to detect 5000 parasites in 20 µl of blood or equal to 0.005% parasitemia (figure 18-19), These probes were less sensitive than pPF-14 which was able to detect 0.0009% parasitemia using 50 µl of patient's blood (Barker *et al*, 1986). However, the sensitivity of pUNK1-34 and -45 can be increased by using a larger blood sample volume, eg. with a 2.5 fold increase in the blood sample volume (50 µl) the assay would probably allow detection of 0.002% parasitemia. This sensitivity level is still slightly less than that of Rep 2 which was able to detect parasitemia of 0.001% in 50 µl blood sample (Franzen, 1984).

Cloned DNA probes pPF-14, Rep 2 and pUNK1-34, -45 had lower sensitivity than total genomic DNA of *P. falciparum* probe described by Pollack *et al* (1985). This genomic DNA was able to detect parasitemia level of 0.0001% in 10 µl of blood. However our results (figure 16) showed that there were cross-hybridizations between total genomic DNA of *P. falciparum* and DNA of *P. chabaudi, P. knowlesi, P. vivax* and *P. cynomolgi* and man. Thus using genomic *P. falciparum* DNA probe could lead to false positive result.

It is worth pointing out that the tests for sensitivity of each probe were done by different techniques. Franzen *et al* (1984) incubated the infected blood for 1 hour at 37°C with proteinase K and 0.2% SDS. Before dotting onto nitrocellulose the incubated blood was phenol extracted, and DNA was precipitated, denatured and neutralized. A positive signal of 25 pg parasite DNA was obtained and 0.1% parasitemia of patient's blood could be detected (determined by microscopy). However sample with equal parasitemia did not show similar intensity, although the authors claim Rep 2 was able to detect 0.001% of *in vitro* culture of *P. falciparum*, no data was shown.

Barker *et al* (1986) used a technique which was more simple than that of Franzen *et al* (1984). Infected blood was incubated with pronase K, and then applied to nitrocellulose filters. The DNA on nitrocellulose was denatured and neutralized before hybridizing with pPF-14 probe. The authors claimed that pPF-14 was able to detect 10 pg of purified *P. falciparum* DNA and 40 parasites per microliter of infected blood. Using a 50 µl sample, this corresponded to a parasitemia of 0.0009%. However a blood sample which contained about 80 parasites did not show any signal.

Mucenski *et al* (1986) demonstrated that a 21 base pairs synthetic oligonucleatide probe of *P. falciparum* detected parasites in blood samples lysed directly on nitrocellulose which was then laid over filter paper saturated with 1xSSC, 4% sarcosyl and pronase K for 1 hour at 37°c, DNA was then denatured for hybridization. The technique was able to detect 0.01% parasitemia in 1 µl spot of infected blood. In the method of Pollack *et al* (1985), ten-microliter samples were lysed directly on nitrocellulose. In our studies, the infected blood was incubated with pronase and the DNA was denatured and neutralized in a tube before spotting onto nylon membranes (Table 4)

Table 4 Comparision of sensitivity of pUNK1-34 and pUNK1-45 with other DNA probes

	Detect parasite in infected blood		Detect extracted *P. falciparum* DNA		Detect oocyst and sporozoite	
	parasitemia (in 50 µl blood)	remark	sensitivity	remark	oocyst	sporozoite
pUNK1-34	0.002*	cross-hybridize with *P. chabaudi*	10 ng		-	1,000
pUNK1-45	0.002*	-	0.1 ng		in ½ mosquito	5,000-1,000
Rep 2	0.001	data not shown	25 pg	not equal intensity in the equal concentration of DNA spot	-	-
Rep 20	-	-	0.1 ng	-	-	-
pPF-14	0.0009	blood containing 0.0016 parasitemia did not show any signal	10 pg	-	-	-
total genomic DNA	0.00002**	cross-hybridize with DNA of *P. chabaudi* *P. knowlesi* *P. vivax* *P. cynomolgi* Man	-	-	-	-

* calculate from 0.005 parasitemia in 20 µl blood

** calculate from 0.0001 parasitemia in 10 µl blood

For field studies, the simplest technique should be adapted. This would involve the direct application of blood onto the nitrocellulose filters, and processing of the samples for hybridization carried out on the filters.

Malaria infection is normally initiated when an infected mosquito, in the act of feeding, injects sporozoites into a susceptible host. A mosquito whose salivary glans contain 60,000 sporozoites would inject about 3,000 sporozoites at each feeding (Shute, 1945). There are different anopheline vector species for *P. falciparum*. Many species of anophelines are efficient vectors only for geographically coindigenous strains of *P. falciparum* (Garnham, 1966). The prevalence of female anopheline mosquitoes with sporozoites in their salivary glands is the parameter for describing the epidemiology of malaria in a particular area. This measurement can establish both vector identity and differences in transmision intensity over space and time (Macdonald, 1957).

The sporozoite infection rate is determined by dissection of individual mosquitoes. However, the experiment can only be performed on freshly captured mosquito and there are less than 0.1% of vector population with sporozoite infection (Warren, 1975). Furthermore it is impossible to identify species of the parasite based on the morphology of sporozotes. Dot blot hybridization of pUNK1-45 to sporozoites and oocysts DNA of *P. falciparum* showed that pUNK1-45 was sensitive and species specific. It did not cross-hybridize with mosquito DNA and other plasmodium DNA, eg. *P. vivax*, *P. chabaudi*, *P. knowlesi* and *P. cynomolgi*. By this techniques 1,000-5,000 sporozoites could be detected (figure 19 and 21). pUNK1-45 was able to detect sporozoites and oocysts in ¼ of infected mosquito (figure 21). This suggests that the probe be used to detect and identify sporozoites and oocyst in extracts of one

single infected mosquito. Moreover seven samples of infected salivary glands were probed with pUNK1-45. It was found that five of these samples could be detected at the dilution of 1:10. The double antibody enzyme-linked immunosorbant assay (ELISA) and immunoradiometric assay (IRMA) using monoclonal antibody that recognize a repetitive epitope on the circumssporozoite proteins of sporozoite were able to detect 100-500 sporozoites in infected mosquito, but these techniques cannot detect oocysts.

Plasmid pUNK1-34 can also be used as DNA probe since it did not cross-hybridize with DNA of mosquito, *P. vivax, P. knowlesi, P. cynomolgi* and man. It did cross-hybridize with DNA of *P. chabaudi*, but since *P. chabaudi* is a parasite of thicket rat *(T. rutilans)*, found in only two localities in Africa, this should not pose any problem in the use of pUNK1-34 to detect *P. falciparum* infected mosquitoes.

The copy number of the insert of pUNK1-45 in *P. falciparum* genome can be estimated as fallows. The size of pUNK1-45 is about 8.3 kb (Table 3), and since the size of pUNK121 is 4.4 Kb, the insert size of pUNK1-45 is 4 Kb. Dot blot hybridization of pUNK1-45 probe with plasmid pUNK1-45 and extracted K1 genomic DNA showed that 12 pg of plasmid pUNK1-45 has the same intensity of 1ng of extracted K1 genomic DNA (figure 22). Assuming that *P. falciparum* genome contains 10^7 bp, the genomic complexity of *P. falciparum* compared to plasmid is $10^7/10^3$ or 10^4. The copy number of pUNK1-45 insert is equal to $12 \times 10^{-12} \times 10^4/10^{-9} = 120$ copy.

Figure 18 shows that intensity of autoradiogram of pUNK1-34 hybridized with 25 pg of plasmid pUNK1-34 was equal to 10 ng of extracted K1 genomic DNA. Thus the copy number of the insert of pUNK 1-34 in *P. falciparum* genome is about 25 copy.

The studies on the organization of pUNK1-34 and -45 in the genome of *P. falciparum* by Southern hybridization (figure 23-24) showed different results for the two plasmids. Figure 23 showed the insert of pUNK1-34 was contained only in Hind III-digested fragment of about 5 Kb. Smeared band of 6.6-50 Kb were seen with Southern hybridization of Bam HI-, EcoRI- and Sal I-digested fragments with pUNK1-34 as probe (figure 23 lane 10, 12, 13). This indicates that the insert of pUNK 1-34 is located in AT rich regions of the parasite genome as tandem repeats.

Southern hybridization patterns of restriction digested K1 genomic DNA with pUNK1-45 probe (figure 24) showed 3 hybridizable bands of Hind III-digested fragments. Bam HI, Sal I and EcoRI digestions showed a broad band of larger than 9.7 Kb, and there was also a light band of 7 Kb in the EcoRI digestion. These results indicate that insert of pUNK1-45 is located in AT-rich region of genome, probably in 3 sets of tandam repeats.

Recently , Fucharoen (1985) selected from *P. falciparum* library constructed by Tirawanchai, a strong hybridization clone pBRK1-30, and used it as probe to hybridize with EcoRI,-Bam HI-, AluI- and Hind III-digested K1 fragments. These enzyme-digested fragments also showed 1-2 bands on Southern hybridization. Using a less sensitive probe pBRK1-14 the number of Southern hybridization bands was increased to 7 or 8. Since hybridization bands revealed by repetitive DNA probes, eg. pBRK1-30, pUNK1-34 or pUNK1-45, were few in number it may be speculated that these repeat fragments are located on a limited number chromosome of the parasite.

The inserts of pUNK1-34 and pUNK1-45 were characterized by restriction mapping. The 2.1 kb insert of pUNK1-34 cantained a single site for AccI (5'...GT $\frac{AG}{CT}$ AC...3'), Cla I (5'...ATCGAT..,3') and Pvu II (5'... CAGCTG...3") and the 3.9 kb insert of pUNK1-45 had single sites for Nde I (5'...CATATG...3"), Kpn I (5'...GCTACC...3") and Cla I. Thus these two inserts do not contain homologous sequence. The different Southern hybridization patterns using the two probes support this suggestion.

It is surprising that sensitive probes such as Rep 2, constructed from cloning Tanzanian strain of *P. falciparum* with phage lambda (Franzen *et al*, (1984), Rep 20, constructed from Gambian isolate of *P. falciparum* with phage lambda vector (Oquendo, 1986), and clone number 26 selected from Tanzanian isolate of *P. falciparum* using M13 as vector, all contained the similar repeat sequence of 21 base pairs. This raises the question : is there a similar 21 base pair sequences in pUNK1-34 and -45. To answer this question it will be necessary to test whether the 21 base pair probe will hybridize with the plasmids. Alternatively, the insert of Rep. 20 could be used as a probe to determine whether homologous sequences exist in pUNK1-34 and pUNK1-45.

^{32}P-labelled DNA probes is useful in hybridization experiments but this technique is expensive and radioisotope is not stable . This make it less useful in field studies. Non-radioactive methods need to be developed. Biotinlabelled probes and photobiotin labelled probe have been used for nucleic acid hybridization (Langer *et al*, 1981 and Forster *et al*, 1985). Colorimetric detection of target nucleic acids on nitrocellulose filters with avidin alkaline phosphatase has been developed (Leary *et al* 1983). The substrate of this enzyme is 5-bromo-4-chloro-3-indolyl phosphate (BCIP). In enzymatic reaction BCI is the

product which has the ability to oxidize nitro-blue-tetrazolium and results in the deposition of a purple precipitation. In addition biotinlabelled probe can be selectively immunoprecipitated in the presence of antibiotin antibody(Länger *et al*, 1981). Non-radioactive techniques, such as biotin nucleic acid hybridization, can be used to detect viral, bacterial and parasites. Sethabutr *et al* (1985) developed biotinylated DNA probe to detect *Shigella* and enteroinvasive *Eschericha coli*. Oligonucleotides cross-linked to alkaline phosphatase have been developed to detect homologons sequence in herpes simplex virus and hepatitis B virus (Jablonski *et al*, 1986)